2021

Developmental changes in vibration sensing and vibration-cued hatching decisions in red–eyed treefrogs

BOSTON UNIVERSITY

GRADUATE SCHOOL OF ARTS AND SCIENCES

Dissertation

DEVELOPMENTAL CHANGES IN VIBRATION SENSING AND VIBRATION–CUED HATCHING DECISIONS IN RED–EYED TREEFROGS

by

JULIE JUNG

B.A., Williams College, 2015
M.A., Boston University, 2019

Submitted in partial fulfillment of the

requirements for the degree of

Doctor of Philosophy

2021

Approved by

First Reader _____

Karen M. Warkentin, Ph.D.
Professor of Biology
Professor of Women's, Gender & Sexuality Studies

Second Reader _____

Peter M. Buston, Ph.D.
Associate Professor of Biology

DEDICATION

I dedicate this dissertation to my family. I can credit everything good in my life to my loving umma and appa, who rededicated and rearranged their entire lives to facilitate the success of their children, and my oppa, who spent most of his childhood trying to shake his little sister (me) off his leg and then much of his adult life trying to make sure she is safe, supported, and happy.

ACKNOWLEDGMENTS

I would first and foremost like to thank my advisor, Dr. Karen Warkentin, for her incredible mentorship these last 6 years. During my interview with her at BU in 2015, Karen explained that hiring a Ph.D. student is a little like adopting an academic child – that even beyond those 5-7 years of study and direct supervision, this relationship will likely last a lifetime. I am infinitely grateful to Karen for making me feel welcome and supported from Day 1, and part of this team indefinitely. My partner Tom likes to joke that Karen is like a "cairn" for me. Cairns are small stacks of stones constructed along a (sometimes subtle) trail. As climbers, we use them as landmarks, to guide our way and keep us from getting lost. The analogy is apt, to say the least. Every time I have felt stuck or lost in my research, or even in life, I could count on Karen to prop me up and help me get back on track. Furthermore, Karen has pruned, polished, and progressed this dissertation to the n^{th} degree. Accepting her tracked changes on a manuscript draft is one of my greatest joys, like watching a time-lapse of my own work metamorphosing into something better and beautiful. Karen has devoted a lot of her time trying to open as many doors as possible for me, all the while giving me a shining example of what a brilliant and supportive scientist and mentor can look like. For this and more, I owe her a tremendous debt.

This dissertation has truly been a collective effort. Key input from my dissertation committee helped make this work possible. My committee members Drs. Pete Buston, James Gregory McDaniel, Fred Wasserman, James Traniello, and Jacqueline Webb have given their time and energy reviewing drafts, discussing vibration-cued hatching, and advising me about the process of pursuing a career in academia. Thank you as well to my

collaborators and co-authors: Jenni Serrano-Rojas, Ming Guo, Mark Crovella, and Beto Rueda Solano. It has been a pleasure to work with and learn from you all. I hope we can do it again soon.

I thank my lab family, past and present, for the hammock hangs, the birthday cakes, the aguardiente, the happy hugs (and the sad), the love, the friendship, the logistical and emotional support throughout the years: Javier Méndez Narváez, Brandon Güell, María José Salazar Nicholls, Kristina Cohen, Jesse Delia, Carolina Amorocho, and Alyssa Pereslete. I could never have made it here without you. And a special thank you is in order to Justin Touchon, for instilling and encouraging a love-hate-but-mostly-love of R and stats. Special thanks must also be given to the many team treefrog interns and visiting students throughout the years: Sonia Perez Arias, Su Jin Kim, Nora Moskowitz, Angelly Vasquez, Alina Chaiyasarikul, Karina Escobar, Crystal Tippett, Juana Rivera, Ana Ospina, Rachel Snyder, Adeline Almanzar, Caroline Guevara Molina, and Chloe Fouilloux. I couldn't have hoped for a greater group of gals to work with. I'll cherish the memories of us chasing frogs, poking eggs, playing bananagrams, desperately rubbing cofal on our mosquito bites, and sneaking up to the canopy tower or into the resort swimming pool for many years to come.

My research was supported by generous grants from the National Science Foundation (NSF), the Smithsonian Tropical Research Institute (STRI), and Boston University (BU). The exceptional support staff at STRI and BU also helped make this research possible. Finally, I want to thank my partner Tom and our pup Luna for always bringing me tea and keeping me sane.

DEVELOPMENTAL CHANGES IN VIBRATION SENSING AND VIBRATION-CUED HATCHING DECISIONS IN RED–EYED TREEFROGS

JULIE JUNG

Boston University Graduate School of Arts and Sciences, 2021

Major Professor: Karen M. Warkentin, Professor of Biology

ABSTRACT

Arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch prematurely to escape from egg predators, cued by vibrations in attacks. Young embryos modulate hatching based on multiple, non-redundant frequency and temporal properties of vibration cues, reducing the chance of false alarms that unnecessarily expose hatchlings to risk in the water. We used a variety of techniques, including vibration playbacks, to test two hypotheses concerning why hatching responses increase developmentally. First, we tested the hypothesis that sensory development improves cue detection. In ch.1, we assessed the role of vestibular mechanoreception in *A. callidryas* embryos. We found ontogenetic congruence of vestibular system function and MCH, suggesting that the developing ear plays a role in egg-motion sensing. In ch.2, we tested if lateral line mechanoreceptors contribute to MCH by blocking neuromast function with gentamicin then exposing embryos to vibration cues across ontogeny. We found that the lateral line mediates the earliest onset of MCH, and MCH continues to increase with increasing numbers of neuromasts. Second, we tested the hypothesis that, once sensory capabilities have developed, embryos are able to consider the cues available to them and weigh their costs and benefits in order to make an informed, optimal decision to hatch. Ch.3 explored the

ways in which embryo responses could be contextually modulated in complex ways. We showed that second-order temporal pattern elements such as prefixes and long gaps could be threatening in certain contexts and not in others, suggesting that embryos practice a very impressive, highly functioning decision-making process that incorporates multiple vibration properties to distinguish between threatening and non-threatening stimuli. Development had a drastic effect on this decision process because hatchlings face aquatic predators in ponds. Since older and bigger hatchlings were less likely to be killed by this new suite of predators, the costs of false alarms decreased as embryos approach spontaneous hatching and they should decide to hatch more readily compared to their younger and smaller counterparts. In ch.4, we showed that older embryos selectively accept more false alarms in response to ambiguous cues, providing evidence for ontogenetic adaptation in information use for escape-hatching decisions. Overall, this research will help us understand how animals facing different risk trade-offs use information to make crucial behavioral decisions.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ANOVA ..... Analysis of Variance

BU ..... Boston University

CI ..... Confidence Interval

FFT ..... Fast Fourier Transform

GFP ..... Green Fluorescent Protein

GLM ..... Generalized Linear Model

IQR ..... Interquartile Range

MCH ..... Mechanosensory Cued Hatching

MCI ..... Minishaker Clutch Interface

MTI ..... Minishaker Tine Interface

NSF ..... National Science Foundation

RMS ..... Root Mean Square

SE ..... Standard Error (of the Mean)

STRI ..... Smithsonian Tropical Research Institute

US ..... United States

VOR ..... Vestibulo-ocular Reflex

4-di-2-ASP ..... 4-(4-diethylaminostyryl)-1-methylpyridinium iodide

μ ..... micro (1×10^{-6})

CHAPTER 1. GENERAL INTRODUCTION

Developmental changes in behavior

Animal life consists of a series of decisions. Whether to fight or flee, hunt or hide, flirt or forget about it – every day organisms must make behavioral choices such as these in order to survive and reproduce. Classically, decisions are considered to comprise at least two steps (Castellano et al., 2012; Valone et al., 1996). First, sensory information about the environment is gathered, evaluated, and used to discriminate signals or cues. Second, a decision rule is engaged or applied to select the behavioral response. Both of these steps are affected by development, such that series of changes in ability contribute to changes in behavior. First, the development of bodies and brains enables and improves behavioral capabilities, changing what animals *can* do. These include the development of sensory systems that enable animals to perceive and respond to cues (Romagny et al., 2012) and the maturation of effectors and neural circuitry that enable and improve the performance of such responses (Bate, 1999). Moreover, because development changes animals' capabilities and needs, it also alters the costs and benefits of expressing a behavior in certain contexts (Wiedenmayer, 2009), thus changing what animals *should* do. The principle of adaptive ontogeny acknowledges that behavioral optimization requires balancing these changing costs and benefits (Hall and Oppenheim, 1987; Warkentin et al., 2019). Animal behavior changes with development for these two general reasons – changing capacities and changing optima (Warkentin et al., 2019; Wiedenmayer, 2009). However, we know relatively little about the development of relevant sensory receptors,

the ontogeny of risk assessment or decision strategies, or how they combine to shape behavior under changing cost-benefit trade-offs.

Ontogeny of defensive behaviors

Most animals face the risk of being eaten. Since predation imposes strong selective pressure, animals have evolved a remarkable diversity of defensive tactics to evade or escape predators (Lima and Dill, 1990). In addition to morphological defenses such as armor or camouflage, animals have a large behavioral repertoire encompassing avoidance, deception, intimidation, fighting, and fleeing that can allow them to avoid or survive encounters with predators. For example, many animals, including opossums and hog-nosed snakes, feign death to escape danger (Hartman, 1950; Humphreys and Ruxton, 2018); tiger moths defend against big brown bats using ultrasonic clicks that jam bat sonar (Corcoran et al., 2009); and golden spiny-tailed geckos deter predators by shooting sticky, foul-smelling secretions from glands on their tails (Rosenberg and Russell, 1980).

Defense strategies are often age-, stage- or size- dependent (Wiedenmayer, 2009). For instance, in some species juveniles rely on crypsis more than adults (Fitzgibbon, 1990; Martin et al., 2005), while in others older individuals are more likely to act aggressively (Shine et al., 2002; Stern, 1998) or escape to a part of their habitat where they are camouflaged or less accessible (Bateman and Fleming, 2014; Gish et al., 2012; Lang et al., 1977; Levri, 1998; Mateo, 1996; Schultz, 1981).

Many of the diverse defense strategies of prey, including plastically expressed behavioral defenses, depend on sensing informative cues from their environment, as well

as on specific motor skills. Thus, a threatened animal may fail to exhibit an appropriate adaptive behavior because either their sensory or motor abilities are immature and do not allow the full range of behaviors typical in older animals (Wiedenmayer, 2009). For instance, very young red deer calves freeze in response to an approaching predator, but after about a week they start fleeing (Espmark and Langvatn, 1985). The onset of the flight response depends on body weight and physical development, rather than just age, suggesting that the maturation of their motor systems limits their ability, and thus tendency, to flee. In rats, the maturation of the amygdala limits the processing of threat cues (Moriceau et al., 2004; Wiedenmayer and Barr, 2001). Thus, rat pups do not display defensive fear responses when exposed to a threat until 14 days of age, relatively late in development (Wiedenmayer and Barr, 1998). Sensory system development may often affect young animals' ability to display defensive behavioral responses to relevant stimuli.

The decision to flee from a perceived threat, or not, may yield either the correct response (i.e., fleeing in the presence of a predator, not fleeing in the absence of a predator) or two type of decision errors: false alarms (fleeing in the absence of a predator) and missed cues (failure to flee in the presence of a predator). The mortality costs of these decision errors can change across development. Prey that fail to flee or otherwise defend themselves from a predator attack are commonly eaten, no matter how developed they are. Thus, the risk of missing a cue may be relatively consistent across development. However, depending on the nature of the defense, false alarms may be more dangerous for less developed animals. For instance, fleeing may expose animals to additional predators, that had been unaware of their presence. In cases where the defensive behavior involves a habitat shift,

less developed animals may be less likely to survive in the new environment, which may host different predators or have different abiotic conditions. In such cases, the risk of fleeing unnecessarily from a predator attack decreases with development. For instance, the cost of dropping off a plant to escape predation by herbivores is higher for nymphs than for adult aphids, because nymphs are less likely to survive to find a new host plant (Roitberg et al., 1979; Tokunaga and Suzuki, 2008). Due to this high cost, young nymphs are less likely to respond to a predator attack by dropping off the plant (Gish et al., 2012; Losey and Denno, 1998; Montgomery and Nault, 1978).

Behavior depends critically on development. Evolutionarily, behavior is considered one of the most flexible or plastic elements of an animal's phenotype, and evolutionary change often comes about through the adjustment of developmental programs. Thus, it is essential that we examine developmental trajectories of behaviors and the causal mechanisms that underlie them. To this end, I examine how embryos sense vibrational cues and make behavioral decisions as they develop.

Study system

Red-eyed treefrogs, *Agalychnis callidryas,* are an excellent species in which to study how developmentally changing capabilities affect plastic defensive behaviors. As adults, these arboreal amphibians lay eggs on plants overhanging ponds. Usually within seven days, embryos hatch and fall into the water below, where they will develop as tadpoles. However, embryos can hatch in seconds and up to 30% prematurely to escape from terrestrial egg predators, such as snakes (Cohen et al., 2016; Warkentin, 1995) and

wasps (Warkentin, 2000). At least in the case of snakes, this escape-hatching behavior is cued by incidental vibrations in attacks (Warkentin 2005), and in both cases, escape-hatching success improves dramatically across embryonic development from oviposition to spontaneous hatching (Gomez-Mestre and Warkentin, 2007; Warkentin et al., 2006a). The earliest in development that these embryos have been induced to hatch is at 3 days post oviposition, in response to strong hypoxia cues from flooding. Escape-hatching responses to mechanosensory cues, however, do not appear until the following day, developmentally later than hatching responses to hypoxia (Warkentin et al., 2017). Thus, their onset is not constrained by the development of hatching ability. My dissertation research tests two other hypotheses about the factors that underlie developmental changes in *A. callidryas'* escape-hatching responses: sensory constraints and cognitive behavioral strategies.

Section I: changing sensory constraints

In order to test the hypothesis that sensory system development is the key factor enabling mechanosensory-cued hatching, we set out to determine the mechanism by which *A. callidryas* embryos sense egg motion. We examined two potential embryonic motion sensors, the vestibular system of the inner ear and the lateral line system. Both systems are well-known vertebrate motion detectors that depend critically on mechanosensory hair cells. To determine whether embryos use either system to perceive vibrations *in ovo*, we compared hatching responses of *A. callidryas* embryos in which each of these systems was functional vs non-functional.

In Chapter 2, we assessed the ontogenetic congruence of escape-hatching responses and a behavioral indicator of vestibular function known as the vestibulo-ocular reflex (VOR) in three ways. First, we determined that the ontogeny of VOR is congruent with the published ontogeny of escape success in attacks. Second, we compared VOR and hatching responses to vibration playbacks presented to whole clutches. Finally, we compared VOR and hatching responses of individual embryos to a simulated attack. The onset of VOR and hatching responses were largely concurrent at all three scales and latency to hatch in simulated attacks decreased with increasing VOR. Our results are consistent with a key role of the vestibular system in egg-motion sensing and mechanosensory-cued hatching (MCH) in *A. callidryas* embryos. However, low levels of hatching occurred earlier in development, before the vestibular system became functional, indicating that another sensor must also contribute to the hatching response.

In Chapter 3, we test if lateral line mechanoreceptors contribute to MCH. By blocking neuromast function with gentamicin then exposing embryos to physical disturbance cues across ontogeny, we found that the lateral line mediates the earliest onset of MCH, and MCH continues to increase with increasing numbers of neuromasts. Vestibular function onset also increased hatching, independent of neuromast function, indicating that young embryos use multiple mechanosensory systems. Moreover, even 5-day old embryos, relatively close to spontaneous hatching and with very well-developed ears, still use lateral line input to accelerate their hatching response. To learn how the lateral line system develops over the period of plastic hatching, we labelled hair cell bundles using a fluorophore, DiASP. Our ontogeny of neuromasts revealed that *A. callidryas* have many

more neuromasts than described for any other species at hatching, suggesting precocious sensory development may facilitate MCH. Collectively, our data support that the developing lateral line and vestibular system both contribute to MCH of red-eyed treefrog embryos, enabling escape from egg-predators.

Section II: cognitive behavioral strategies

We next tested two hypotheses addressing how, once mechanosensory capabilities have developed, *A. callidryas* embryos assess vibrational cues and apply behavioral decision rules in order to make a decision to hatch.

Chapter 4 explores the ways in which embryo responses can be contextually modulated in complex ways, focusing on temporal pattern cues. Egg predators such as snakes generate incidental vibrations that elicit early escape-hatching by embryos. Benign rainstorms also generate intense vibrations in egg clutches, with many properties that overlap those of snake-induced egg vibrations, but they pose no threat to embryos. Because of this, embryos face a discrimination challenge. To accurately assess risk and inform hatching, embryos use multiple vibration properties, including temporal pattern elements such as the durations of vibrations and lengths of intervening still intervals. However, measures of natural snake and rain vibration as simple duration-interval patterns are a poor match to embryo behavior. To assess a possible resolution to this mismatch, we use vibration playbacks to test if embryos use a second level of temporal pattern, long gaps within a rhythmic pattern, as indicators of risks. Our results show that identical vibration pulses, pulse groups, and periods of silence can be treated as risk cues in some contexts

and not in others and embryos employ a multi-faceted decision-making process to effectively distinguish between risk cues and benign stimuli.

In Chapter 5, we test if developmental changes in hatching in response to ambiguous cues follow adaptive predictions based on the developmentally changing costs of decision errors. Since hatchlings face aquatic predators in ponds (Touchon and Vonesh, 2016) and more developed hatchlings are less likely to be killed by this new suite of predators, the cost of false alarms decreases as embryos approach spontaneous hatching (Touchon et al., 2013; Warkentin, 1995; Warkentin, 1999; Willink et al., 2014). We found that older embryos selectively accept more false alarms in response to ambiguous cues presented in two distinct property domains, providing evidence for ontogenetic adaptation in information use for escape-hatching decisions. Thus, while development changes the relative costs and benefits that underlie behavioral decisions, ontogenetically changing decision rules allow embryos to effectively use ambiguous cues for defense.

Finally, Chapter 6 summarizes the key results of my dissertation research and considers their implications for future studies. Overall, this work contributes to the integrative biology of embryos as animals with rapidly changing abilities that face risk trade-offs and make important behavioral decisions using non-stereotyped incidental cues. It advances our understanding of information use, behavioral development, and biotremology and opens new opportunities for both mechanistic and comparative research.

CHAPTER 2. HOW DO RED-EYED TREEFROG EMBRYOS SENSE MOTION IN PREDATOR ATTACKS? ASSESSING THE ROLE OF VESTIBULAR MECHANORECEPTION

This chapter is published as:

Jung J, Kim S, Pérez Arias SM, McDaniel JG, Warkentin KM. 2019. How do red-eyed treefrog embryos sense motion in predator attacks? Assessing the role of vestibular mechanoreception. *Journal of Experimental Biology*. 222(21). doi:10.1243/jeb.206052

ABSTRACT

The widespread ability to alter hatching timing in response to environmental cues can serve as a defense against threats to eggs. Arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, hatch up to 30% prematurely to escape predation. This escape-hatching response is cued by physical disturbance of eggs during attacks, including vibrations or motion, and thus depends critically on mechanosensory ability. Predator-induced hatching appears later in development than flooding-induced, hypoxia-cued hatching; thus, its onset is not constrained by the development of hatching ability. It may, instead, reflect the development of mechanosensor function. We hypothesize that vestibular mechanoreception mediates escape-hatching in snake attacks, and that the developmental period when hatching-competent embryos fail to flee from snakes reflects a sensory constraint. We assessed the ontogenetic congruence of escape-hatching responses and an indicator of vestibular function, the vestibulo-ocular reflex (VOR), in

three ways. First, we measured VOR in two developmental series of embryos 3–7 days old to compare with the published ontogeny of escape success in attacks. Second, during the period of greatest variation in VOR and escape success, we compared hatching responses and VOR across sibships. Finally, in developmental series, we compared the response of individual embryos to a simulated attack cue with their VOR. The onset of VOR and hatching responses were largely concurrent at all three scales. Moreover, latency to hatch in simulated attacks decreased with increasing VOR. These results are consistent with a key role of the vestibular system in the escape-hatching response of *A. callidryas* embryos to attacks.

INTRODUCTION

Hatching is an essential embryo behavior that mediates the transition between two distinct stages of life, in the egg and post-hatching environments, when developing animals are exposed to different risks and opportunities. Variation in either environment can affect when is the best time to hatch. Environmentally cued hatching allows embryos to respond adaptively to their local environment by altering the timing of their hatching (Sih and Moore, 1993; Warkentin, 1995). Recent syntheses reveal that cued hatching responses are phylogenetically widespread (Warkentin, 2011a). Physical disturbance of eggs is particularly common as a cue for hatching, as observed in invertebrates (Endo et al., 2019; Mukai et al., 2014; Nishide and Tanaka, 2016; Oyarzun and Strathmann, 2011; Whittington and Kearn, 1988), fishes (Martin et al., 2011), amphibians (Buckley et al., 2005; Gomez-Mestre et al., 2008; Goyes Vallejos et al., 2018; Touchon et al., 2011; Warkentin, 1995;

Warkentin, 2000; Warkentin, 2011a), and reptiles (Doody, 2011; Doody and Paull, 2013; Doody et al., 2012). Physical disturbance cues can function in antipredator responses, conspecific-cued hatching, host-cued hatching of parasites, and embryo responses to physical conditions (Warkentin, 2011a; Warkentin, 2011b). Physical disturbance may be a particularly useful cue to impending predation of terrestrial eggs, since predators cannot eat eggs without touching and moving them, and terrestrial embryos appear to have less opportunity to receive chemical early warning cues than do aquatic embryos.

To our knowledge, the mechanosensory system mediating hatching responses to physical disturbance cues has not been assessed for any embryos. Indeed, we know relatively little about the developmental onset of mechanoreception, compared to its mature function, across taxa (Hill, 2008). In vertebrates, the predominant motion-detection system is the vestibular system of the inner ear. Otic mechanoreceptors appear during embryonic development in fishes (Becerra and Anadon, 1993; Bever and Fekete, 2002; Haddon and Lewis, 1996), amphibians (Fritzsch, 1996; Quick and Serrano, 2005), chicks (Alsina and Whitfield, 2017; Liang et al., 2010), mice (Fritzsch, 2003; Fritzsch et al., 2002), and humans (Fritzsch et al., 1998). Thus they could potentially mediate mechanosensory-cued hatching. In fishes and amphibians, the lateral line system also develops before hatching (Bever et al., 2003; Hill, 2008; Nieuwkoop and Faber, 1956; Stone, 1933; Thomas et al., 2015) and thus might play a role in mediating cued-hatching responses. Understanding the sensors that mediate cue perception is a key part of understanding any cued behavior and may be particularly crucial early in ontogeny, when both sensory abilities and behavior are changing rapidly. Unlike adults, with fully developed sensory systems, embryos' ability to

respond to particular cue types is constrained by the need for adequate prior development of the relevant sensors. It is essential to identify these sensors and assess their ontogeny in order to determine when developmental changes in behavior reflect the easing of sensory constraints and to understand the information available to embryos at different developmental stages. Understanding sensory ontogeny will also facilitate inquiry into other sources of developmental changes in behavior, such as ontogenetic adaptation of decision rules (Warkentin et al., 2019).

Red-eyed treefrog embryos, *Agalychnis callidryas* (Cope 1862), are tractable study organisms for research on predator-induced, mechanosensory-cued hatching of terrestrial eggs. Females lay gelatinous egg clutches upon leaves and other substrates overhanging ponds, such that hatching tadpoles generally fall into the water below as soon as they hatch (Gomez-Mestre et al., 2008; Pyburn, 1970). Thus hatching, for these embryos, entails a change from arboreal to aquatic habitats with concomitant changes in selection pressures and potential predators (Warkentin, 1995). Predation is the largest cause of mortality for *A. callidryas* embryos monitored at ponds in Costa Rica and Panama, and attacks during the last third of the typical undisturbed embryonic period induce rapid escape-hatching responses of embryos (Gomez-Mestre and Warkentin, 2007; Warkentin, 1995; Warkentin, 2000). Embryos hatch in response to physical disturbance by predators and, at least for snake attacks, playback of recorded attack-vibrations is sufficient to elicit premature hatching (Hughey et al., 2015; Warkentin, 2005). Embryos can hatch within seconds by rapidly releasing hatching enzymes to digest a small hole in the membrane, then squeezing through it (Cohen et al., 2016). Embryos also hatch prematurely in response to flooding,

cued by hypoxia (Warkentin, 2002), and drying, based on unknown cues (Salica et al., 2017).

We recently discovered that the developmental onset of hatching responses to hypoxia and mechanosensory cues differs in *A. callidryas* (Warkentin et al., 2017). Specifically, there is a period of development when embryos are competent to hatch, as demonstrated by their consistent hatching response to strong hypoxia and the presence of hatching gland cells, yet still unresponsive to mechanosensory disturbance cues or natural predators (Cohen et al., 2019; Warkentin et al., 2017). Up to 10% of eggs laid can be consumed during this period (Gomez-Mestre and Warkentin, 2007; Warkentin, 1995; Warkentin, 2000; Warkentin et al., 2017), suggesting that an earlier onset of escape-hatching responses to predators could be beneficial. The existence of this hatching-competent but unresponsive-to-predators period indicates that something beyond hatching ability limits the onset of the anti-predator response (Warkentin et al., 2017). The survival cost of early hatching decreases gradually, over days, not hours, of development (Warkentin, 1995; Warkentin, 1999; Willink et al., 2014); this suggests that changes in adaptive embryo decisions are unlikely to impose a narrow developmental limit on the onset of the anti-predator response. Instead, the rapid developmental increase in response to a simulated attack cue, from 0–100% hatching over a few hours (Warkentin et al., 2017), suggests a sensory constraint may limit embryos ability to detect attacks. Thus, as an initial step to assess what sensory system mediates vibration perception in attacks, we looked for ontogenetic congruence of sensor development and the onset of the escape-hatching behavior.

We hypothesize that *A. callidryas* embryos use inner ear mechanoreceptors to sense motion cues. If the vestibular system is the primary mechanism by which red-eyed treefrog embryos sense the physical disturbance of their egg clutches, and is required to perceive predator attacks, its development may limit the onset of escape-hatching responses in this context. To assess this, we looked for developmental correlations between escape hatching behavior and a marker of vestibular function, the vestibulo-ocular reflex (VOR). This reflex generates eye movements that compensate for head movement, producing a more stable image in the retina (Straka, 2010). VOR can be used as a convenient behavioral indicator of vestibular function, as it depends critically on vestibular system development (Jen, 2009). The development of the gravitational, roll-induced VOR has been extensively studied in the frog *Xenopus laevis* (Horn, 2006; Horn and Gabriel, 2011; Horn et al., 1986a; Horn et al., 1986b; Rayer and Horn, 1986), the fish *Oreochromis mossambicus* (Sebastian and Horn, 1999; Sebastian et al., 2001), and the salamander *Pleurodeles waltl* (Gabriel et al., 2012), demonstrating that VOR amplitude is a sensitive indicator of vestibular function. In these aquatic species, modifications of vestibular input either by vestibular lesions (Horn et al., 1986a; Rayer and Horn, 1986; Schaefer and Meyer, 1974) or by altered gravitational conditions during critical periods of vestibular system development (Gabriel et al., 2012; Horn, 2006; Horn and Gabriel, 2011; Sebastian et al., 2001) reliably lead to significant reductions in VOR.

In adults, the vestibulo-ocular reflexes include responses to angular acceleration, mediated by the semicircular canals, and to gravitoinertial acceleration, mediated by the otoconial organs (Angelaki and Cullen, 2008; Straka, 2010). However, gravitoinertial

acceleration sensing begins developmentally earlier than angular acceleration sensing in small vertebrates (Straka, 2010), about 10 days earlier in *X. laevis,* in much less developed animals (Lambert et al., 2008). Semicircular canal dimensions limit the onset of angular acceleration detection, with a minimum lumen radius of 60 μm required for endolymph displacement sufficient for sensor function in *X. laevis* (Lambert et al., 2008; Straka, 2010). In *A. callidryas*, at the onset of hatching responses to physical disturbance, the semicircular canals are not yet formed (Jung et al., 2018), and even when embryos are hatching spontaneously their semicircular canal lumen radius is only about 40 μm (J. Jung, unpublished data), below the threshold for sensor function in *Xenopus.* The gravitoinertial signals from otoconial organs are inherently ambiguous and must be integrated with input from semicircular canals to distinguish translational linear acceleration from rotation relative to gravity (Angelaki, 2004; Angelaki and Cullen, 2008). Thus, before semicircular canals reach a minimum functional size, animals cannot distinguish between linear and rotational acceleration. Given the requirement for post-hatching growth before semicircular canals reach a functional size, in *A. callidryas* as in *X. laevis*, vestibular sensing in embryos is limited to gravitoinertial sensing by otoconial organs.

To test our hypothesis that *A. callidryas* embryos use inner ear mechanoreceptors to sense motion cues, we began by quantifying the basic ontogeny of VOR in *A. callidryas* embryos. We predicted that the developmental onset of VOR (increase in magnitude from absent to consistently strong) would align with the previously documented ontogeny of *A. callidryas'* escape-hatching success in predator attacks. In attacks by egg-predatory snakes and wasps, we have never observed hatching before about 2.5 days prematurely. In our

Panamanian study population, escape-hatching success is zero at age 3 d, present but relatively low and variable at 4 d, and consistently high thereafter, with spontaneous hatching in the evening at 6 d (Hite et al., 2018; Warkentin, 2000; Warkentin et al., 2006a). In Warkentin's 1990s research on the Osa Peninsula, Costa Rica, with *A. callidryas* developing more slowly under cooler conditions, predator-induced hatching began at age 5 d, with spontaneous hatching in the evening at 7 d (Almanzar and Warkentin, 2018; Gomez-Mestre and Warkentin, 2007; Gomez-Mestre et al., 2008; Warkentin, 1995). Accordingly, if a strong VOR were present at the onset of hatching competence at 3 d, otoconial organ development would be implausible as a constraint underlying the later onset of attack-induced hatching. Moreover, assuming that VOR is a reliable marker for vestibular function, if VOR onset were clearly later than the onset of escape responses to attacks (e.g., not present until 5 d), it would reject a key role for the vestibular system in sensing attacks.

Next, focusing on the developmental period of greatest variation in VOR and hatching response, we directly compared the hatching responses of egg clutches to vibration playback with the VOR of a subset of hatched and unhatched individuals from each clutch. During this period of high variation, and potentially rapid developmental change, we predicted a positive relationship between magnitude of VOR and hatching response, with some threshold value below which otoconial organ function is insufficient to cue hatching.

Finally, we applied a simulated predator attack cue to individual eggs to compare hatching responses of embryos to their VOR, as clutches developed. Here as well, we

predicted that the onset of vestibular function would match the onset of hatching responses. A lack of correlation of VOR magnitude and hatching response across either clutches or individuals, during the period of high variation and response onsets, would suggest that these are developmentally independent events that simply happen to occur during the same general period. The presence of correlated VOR and hatching responses both among clutches and among individuals would be consistent with a functional linkage.

METHODS

Egg clutch collection and care

We collected 0–3 d old *A. callidryas* egg clutches and the leaves on which they were laid from the Experimental Pond in Gamboa, Panama (9.120894 N, 79.704015 W). Clutches were brought to a nearby ambient temperature and humidity laboratory at the Smithsonian Tropical Research Institute, mounted on plastic cards for support, positioned over aged tap water in plastic cups, and misted with rainwater frequently to maintain hydration. Tests of hatching responses were conducted in the ambient-conditions laboratory, and individual hatchlings were tested for VOR in an adjacent air-conditioned laboratory. All embryos used were morphologically normal, in developmental synchrony with siblings in their clutch, and in intact, turgid eggs at the start of the experiment. Most clutches are laid between 10 pm and 2 am, so we assign embryos to daily age-classes and report developmental timing starting from midnight of their oviposition night (Warkentin, 2002; Warkentin et al., 2005). Across the onset of hatching competence, tested individuals

were staged based on morphological markers described in Warkentin et al. (2017). Some specimens were preserved for morphological studies (to be presented elsewhere) and all other hatchlings were returned to their pond. This research was conducted under permits from the Panamanian Environmental Ministry (SC/A-15-14, SE/A-46-15) and approved by the Institutional Animal Care and Use Committees of Boston University (14-008) and the Smithsonian Tropical Research Institute (2014-0601-2017).

Measurement of the vestibulo-ocular reflex (VOR)

We measured roll-induced VOR of newly hatched tadpoles or manually decapsulated embryos (henceforth, collectively 'hatchlings') using a custom-built, Arduino-based, portable tadpole rotator (Supplementary Figure 2.1, J. G. McDaniel, Adrian Tanner, and K. M. Warkentin; Boston University Engineering Products Innovation Center). The rotator smoothly turns a shaft at the push of a 'clockwise' or 'counterclockwise' button and was programmed for 15° rotational increments. A conditioning mass and rubber plate mounted on the shaft limit vibration transfer from the motor to the test animal, and a printed plastic cup glued to the rubber plate enables field replacement of the animal interface. To hold hatchlings, we mounted a section of plastic pipette in the center of the cup, in line with the rotator shaft, using silicone seal. The hatchling chamber was 13.5 mm long and 3 mm in internal diameter, with a slight widening at the mouth so as not to restrict eye motion, and horizontally leveled in relation to gravity. To test a hatchling, the chamber was filled with aged tap water and the animal was backed into it using a transfer pipette or length of tubing on a syringe, positioning its snout just

within the tube. No anesthesia was necessary and individuals could be tested within minutes of hatching.

The chamber was surrounded by a light diffuser and illuminated on both sides by LED lights (Panasonic 9W, 100-127V, 90mA), providing a uniform white visual field. It faced a horizontally leveled MPE-65 mm macro lens on a digital camera (Canon D70) with cable shutter release, mounted on a focusing rail on a tripod. Following Horn (Horn et al., 1986b; Horn and Sebastian, 1996), we rolled hatchlings about their body axis 180° in each direction, photographing them in frontal view each 15° (Supplementary Movie 2.1). We continuously observed hatchlings on the camera view-screen, manually applied rotation increments, and took each photograph as soon as body and eye rotation had stopped, to minimize testing time. Most animals remained immobile through each 180° roll sequence; for those that moved more than their eyes, we restarted the sequence from 0° to obtain a continuous series of measurements. From each photograph, we measured right and left eye angle and body axis angle using ImageJ (Schneider et al., 2012). From each angular measurement series, we constructed an individual VOR curve using a sine-fitting function in Python (Version 2.7.9, Build 1, Python Software Foundation). We assessed the curve fit and calculated the VOR amplitude from the sine function. The peak-to-peak amplitude of the curve corresponds to the hatchling's VOR magnitude (Figure 2.1).

We visually checked each sine curve fit and rejected those that did not meet the following criteria: 1) curve fits of the two eyes show similar wavelengths, are horizontally aligned, and have parallel or near-parallel waveforms, 2) the wavelength is plausible for VOR, with zero crossing at or near the zero body angle, and 3) eye rotation is opposite to

body rotation (i.e., curve is not upside-down). Individuals whose curve fits failed one or more of these VOR criteria (Supplementary Figure 2.2) were considered to have a VOR of zero. Of 406 hatchlings tested, 92 failed the VOR curve fit criteria (N = 4 of 36 in series **Ia** below, N = 38 of 89 in series **Ib**, N = 19 of 169 in series **II**, N = 31 of 112 in series **III**).

(I) Ontogeny of vestibular function

First, to determine the basic ontogenetic timing of the onset of vestibular sensory function in *A. callidryas,* we measured the VOR of embryos at different ages (series **Ia)**. From 19–25 June 2014, we tested VOR daily across the plastic hatching period, in the afternoon of each day (13:27–17:09 h), using a set of non-sibling embryos at each age (N = 7, 10, 10, and 9 hatchlings, at ages 3–6 d respectively; total N = 36 hatchlings from 14 clutches). The mean daily temperature in Gamboa ± SE across incubation and testing days was 27.1 ± 0.2°C (measured at a Smithsonian weather station about 400 m from the ambient lab). Second, to assess how VOR varied among and within egg clutches across development, we tested developmental series of five clutches, from 10–20 August 2014 (series **Ib**, incubated and tested at mean daily temperatures of 26.6 ± 0.2°C**)**. We concentrated our sampling in the period of greatest change, testing ~ three siblings per age at 6 h intervals from 3.75–4.75 d, with a final sample at 5.75 d (total N = 88 hatchlings; N = 15, 16, 15, 15, 15, 12 individuals per age group).

We removed each individual egg from its clutch just prior to VOR testing, placed it in a small dish, and gently rolled and jiggled it with a blunt probe to induce hatching. The youngest embryos were unresponsive to this stimulus and, instead, manually

decapsulated with fine forceps under a dissecting microscope. Hatchlings were tested for VOR within 3 minutes of leaving their egg capsule.

(II) VOR and hatching response in vibration playback to whole clutches

To assess if variation in VOR and the hatching response are related, across the period of high variation in both traits, we paired vibration playbacks to 36 clutches with VOR measurements on a subset of embryos from each clutch, from 26 June to 21 July 2015 (series **II**, incubated and tested at mean daily temperatures of 27.6 ± 0.2°C). We tested clutches at ages 3.7–4.9 d and stages 2–7 (Warkentin et al., 2017), from before any hatching response to vibration until responses became fairly strong. To focus on fine-scale developmental changes and avoid age-imprecision due to variation in oviposition timing, we report results based on stage. Compared to 2014, development tended to be accelerated under the warm El Niño conditions in 2015 (Warkentin et al., 2017).

We played a synthetic low-frequency vibration stimulus (Figure 2.2C) designed to elicit very high hatching, based on prior playbacks to 5-d-old clutches (Caldwell et al., 2009; Warkentin et al., 2006b). We generated noise in Matlab and filtered it using a custom script (available upon request), to compensate for nonlinearities in the shaker transfer function and generate a frequency distribution resembling that of snake attacks (Caldwell et al., 2009), with high energy below 60 Hz and intensity dropping off above that (Figure 2.2C). To test our match to the desired frequency distribution, we recorded playbacks of the stimulus embedding a small (0.14 g) AP19 accelerometer (AP Technology International B.V., Oosterhout, The Netherlands) within a clutch. Accelerometers added

~5% to the mass of each clutch, such that test clutches remained within the natural range of interclutch mass variation (Warkentin, 2005). Transduced vibrational signals were powered/amplified by an APC7 signal conditioner and digitized with an external sound card (MSE-U33HB; Onkyo USA, Saddle River, NJ, USA). The output was recorded using Raven Pro 1.3 bioacoustics software (Cornell University Laboratory of Ornithology, Ithaca, NY, USA) on a Macbook Pro computer. The RMS amplitude of the playback stimulus, measured from recorded periods of vibration only, excluding silent periods, was 10 m/s^2. The intensity of frequencies below 20 Hz was limited by shaker capabilities. The base temporal pattern consisted of 0.5 s pulses of vibration, with roughly rectangular amplitude envelopes, separated by 1.5 s intervals of silence (Figure 2.2B). This was divided into pulse-groups consisting of 10 pulses separated by 30-s gaps of silence (Figure 2.2B). We included a three-pulse "primer" plus 30-s gap before the repeating10-pulse pattern began, since this element also increases hatching response (Figure 2.2B, Jung, Guo, McDaniel and Warkentin, unpublished data).

Playback methods followed Caldwell et al. (Caldwell et al., 2009; Caldwell et al., 2010). Stimuli (Figure 2.2A) were presented through an array of blunt metal tines inserted among eggs (Figure 2.S3, Movie 2.S2) attached via a rigid post to an electrodynamic minishaker (Model 4810; Brüel & Kjær, Nærum, Denmark). Shaker output was controlled by Audacity 2.1.0 (Free Software Foundation, Boston, MA) on a 2014 MacbookAir, via a custom-made amplifier designed to have a flat frequency response from DC to 5 kHz (E. Hazen, Boston University Electronic Design Facility). Playback clutches on their plastic cards were mounted on a flat-sided plastic stand (~1.5 kg), then carefully slid forward so

the tines entered the clutch between eggs. We used only healthy clutches that fit within the tine field, and tines were rinsed with rainwater between trials. We watched for any hatching induced by the set-up procedure (only 3 individuals, from 3 clutches), then allowed five hatching-free minutes for acclimation before starting the playback. For playback, the shaker moved the tines up and down, so eggs were shaken vertically, and hatched tadpoles fell into a tray of water below the clutch.

For each trial, we counted the embryos that hatched during the playback period and 5-min of post-playback observation. We then immediately (within 5–10 minutes) measured VOR of a subset of 3 hatchlings per clutch that had hatched in response to playback, unless fewer had hatched. To check for hatching competence of the remaining eggs, after post-playback observation, we manually stimulated eggs, rubbing and jiggling them with a blunt metal probe, for about two minutes, then submerged any unhatched eggs in hypoxic water. Any embryos that failed to hatch under manual stimulation and hypoxia were considered not competent to hatch and excluded from the count of test individuals in calculations of proportion hatched per clutch (proportion excluded = 0.078 ± 0.022, mean ± SE across clutches). We measured VOR of 3 additional hatchlings that hatched in response to either manual stimulation or hypoxia, but not vibration playback; numbers of manually-stimulated and hypoxia-cued hatchings tested for VOR varied among clutches (total of 3, unless fewer remained after playback). We staged all VOR-tested hatchlings (N = 143 hatchlings total) from their frontal photos following a staging system based on Warkentin et al. (2017).

(III) VOR and hatching response to simulated attack on individual embryos

To examine the correlation between hatching responses to physical disturbance cues and vestibular function on an individual level, we assessed both traits in developmental series of embryos across the onset of mechanosensory-cued hatching (series **III**). To assess hatching responses of embryos to a simulated attack, we removed individual eggs from their clutch, placed each in a petri dish with a drop of water, and manually jiggled them with a moistened blunt metal probe, alternating 15 s of stimulation and 15 s of rest for 5 min or until the egg hatched (Warkentin et al., 2017) (Movie S3). We tested two embryos per clutch from 11 clutches every 3 hours, on August 11–13, 2015. The mean daily temperature across incubation and testing days was 27.6 ± 0.3°C. As with vibration playbacks, we observed embryos for 5 min before, during, and after stimulation (15 minutes total), and considered any hatching during and after stimulation (10 minutes) to be a response to the stimulus. All sibships were initially tested for their hatching response to hypoxia and, in most cases, we began testing responses to the egg-jiggling stimulus only after siblings had demonstrated an ability to hatch; the data on developmental timing of onset of the response to each cue are reported elsewhere (Warkentin et al., 2017). We continued testing each clutch every 3 h until both test embryos had hatched at two time points, thus capturing a range of developmental ages (3.25–4.625 d) and stages (2–7) from those unresponsive to the jiggling cue, through the onset of response, to strongly responsive (total N=112 individuals, 6–18 per clutch). For each hatchling we recorded latency to hatch, from stimulus onset, or failure to hatch after 5 min of post-stimulus observation. We manually decapsulated unhatched embryos, and photographed all animals in frontal view

to assess development (from stages 2–8) following a staging system based on Warkentin et al. (2017). We then immediately measured their roll-induced VOR.

Statistics

When data met parametric assumptions, we used ANOVAs and Tukey post-hoc tests to find effects and comparisons. Otherwise, we used the Wilcoxon Rank Sum and Wilcoxon Each Pair methods for non-parametric tests of effects and comparisons. In the first developmental assay that examined the hatching response of multiple siblings per clutch (series **Ib**), we fit a 4-parameter logistic model, grouped by clutch, and performed an analysis of means for inflection point estimates. In the following assays where we considered multiple siblings per clutch, we analyzed our results using mixed models with clutch as a random effect. To analyze predictors of hatching, we used binomial GLMMs with clutch as a random effect and performed likelihood ratio tests to compare nested models. All statistical tests were carried out in JMP Pro 13 (version 13.2.0, SAS Institute Inc. 2016) or the R statistical environment (version 3.3.3, R Development Core Team 2014, http://www.r-project.org) in RStudio (version 1.1.383, RStudio Team 2015).

RESULTS

(I) Ontogeny of vestibular function

Across embryos tested at daily intervals (series **Ia**), VOR amplitude increased with age (Wilcoxon Rank Sum: χ^2=16.2797, df=3, P=0.0010, Figure 2.3A). VOR did not change

significantly from age 4–6 d (Wilcoxon Each Pair, all P>0.4274) but hatchlings tested at age 3 d showed lower VOR than those aged 4–6 d (Wilcoxon Each Pair, all P<0.0014, Figure 2.3A). In the second developmental series, with replication within clutches (series **Ib**), VOR increased with age in a sigmoidal fashion (R^2=0.91, Figure 2.4B), and clutches varied in inflection point estimates (Analysis of means; upper limit exceeded in clutch 101 and 102, P<0.01; Figure 2.4B).

(II) VOR and hatching response in vibration playback to whole clutches

Based on post-playback hypoxia testing, all individuals included in VOR analyses (N=169) were able to hatch, but only 63 of them hatched in response to vibration playbacks (series **II**). VOR amplitude increased significantly across developmental stages (one-way ANOVA, $f_{5,162}$=79.2953, P<0.0001). Across the first four stages we tested (stages 2–5, Warkentin et al. 2017), no embryos in any clutches hatched in response to vibration playbacks and VOR was consistently low (5.3 ± 1.0°; N=22 hatchlings, 8 clutches; Figure 2.5A). Compared to VOR at stages 2–5, VOR was higher at stage 6 (N=63 hatchlings, Tukey test from one-way ANOVA, P<0.0001) and stage 7 (N=84 hatchlings, P<0.0001, Figure 2.5A). Up until stage 5, no individuals hatched. At stage 6, vibration-cued hatching began, but the low hatching response rates within clutches were not significantly higher than zero at earlier stages (Tukey test from one-way ANOVA, P>0.3809); clutch hatching rates at stage 7 were significantly higher than at all prior stages (P<0.0001).

Considering all tested individuals, those that hatched in playback had a significantly higher VOR than individuals that did not hatch in playback, but hatched in response to manual stimulation or hypoxia (Mixed Model, VOR Amplitude ~ Hatching with Clutch as a random effect: χ^2=4.8028, P=0.02841). For the subset of 23 clutches where hatching occurred, individuals that hatched in playbacks tended to have higher VOR than siblings that did not hatch, but the difference was not significant (Mixed Model, χ^2=3.0898, P=0.07878). However, individuals that hatched only in response to hypoxia had significantly lower VORs than those that hatched in response to playbacks (Mixed Model, χ^2=6.2563, P=0.0438). For clutches with hatching, we compared mean VOR of each clutch (from 5.61 ± 0.14 individuals per clutch, range 4–6) with proportion hatched. Proportion hatched per clutch increased with mean VOR (Figure 2.5B, linear regression, $F_{1,21}$=8.0252, P=0.0100); no individuals hatched with VOR less than 21.38° and mean VOR of those that hatched in response to playback was 35.92° ± 0.95°.

For individual embryos (N=112) subjected to a simulated attack (series **III**), VOR increased in magnitude across embryonic developmental stages (Mixed Model, χ^2=96.215, P<2.2e-16, Figure 2.6A) and varied among clutches (Mixed Model, χ^2=8.3355, P=0.003888). No stage 2 embryos hatched. Hatching in response to individual egg-jiggling began at stage 3 with a hatching rate of 20.8% (Figure 2.6B), which is when some embryos started showing a measurable VOR (Figure 2.6A). By stage 4, almost half the embryos hatched (47.4%) and by stage 7, all embryos tested hatched in response to the jiggling cue (Figure 2.6B).

(III) VOR and hatching response to simulated attack on individual embryos

Both developmental stage and VOR amplitude were significant and strong predictors of hatching (Figure 2.7A-B, binomial GLMM), and the model incorporating both variables was better than models with either one alone (AIC values 120 vs. 122 and 125). More developed embryos with greater VOR were more likely to hatch, with hatching response increasing 14% for every stage (χ^2=13.285, P=0.02085) and 24% for every 10 degrees of VOR amplitude (χ^2=11.951, P=0.0005461, Figure 2.7A-B). However, the 61 embryos that hatched in response to egg-jiggling included 7 individuals with no detectable VOR, ranging from stage 3 to 5 (Figure 2.8).

Considering the subset of animals that hatched in response to egg jiggling, their latency to hatch decreased with VOR amplitude (Figure 2.8, Latency ~ VOR with Clutch as a random effect, χ^2=16.55, P=4.738e-5). If we add stage and the interaction between stage and VOR into the model, there is a main effect of stage (χ^2=13.3925, P=0.009509), and an interaction effect (χ^2=12.0126, P=0.017258), but no main effect of VOR (χ^2=1.3143, P=0.251613). Closer examination of the interaction indicates a significant VOR effect only at stage 6 (χ^2=5.0734, P=0.0243), but note that sample sizes were lower at other stages (in order from stage 3, N=5, 9, 18, 26, 3).

DISCUSSION

Embryos use physical disturbance (egg motion) as a cue to hatch among fishes (Martin et al., 2011), amphibians (Buckley et al., 2005; Gomez-Mestre et al., 2008; Goyes Vallejos et al., 2018; Touchon et al., 2011; Warkentin, 1995; Warkentin, 2000; Warkentin,

2011b), and reptiles (Doody, 2011; Doody and Paull, 2013; Doody et al., 2012), as well as many invertebrates (Endo et al., 2019; Mukai et al., 2014; Oyarzun and Strathmann, 2011; Tanaka et al., 2016; Whittington and Kearn, 1988). However, the specific sensors mediating the environmentally cued hatching responses of embryos are entirely unknown. We examined the role of the vestibular system – specifically the developing otoconial organs – in the escape-hatching response of red-eyed treefrogs. In four experiments, at population, clutch, and individual levels, we found developmental congruence between the onset of the VOR and the escape-hatching response to real and simulated predator attack and vibration playbacks, consistent with our hypothesis that these gravitoinertial sensors play a key role in mediating mechanosensory-cued hatching.

VOR as an indicator of vestibular system function

Our tests for ontogenetic congruence of vestibular system function and escape-hatching behavior are based on the vestibulo-ocular reflex (VOR), or eye movements induced by roll and tilt of the body (Horn et al., 2013), which we could measure within minutes of hatching using a tadpole-in-tube rotation protocol. Since input from the vestibular system controls the muscles responsible for VOR, it is well-established that the VOR is not expressed without vestibular system function (Cohen, 1974; Precht, 1976). Moreover, the onset of VOR appears not to be limited by eye muscle development. Extraocular motoneurons develop and establish axonal connections with target eye muscles very early in embryogenesis (Gilland and Baker, 2005; Glover, 2003). In 96 of 406 hatchlings tested, we observed non-VOR-related eye movements (criteria listed in

methods) with a measurable magnitude greater than that of individuals with a small but clear VOR (Figure 2.S2). This indicates that hatchlings, prior to developing a working VOR, can change their eye angle – just in a way that does not match up with their body rotation. The data from these individuals supports that the onset of VOR is not limited by when embryos become physically capable of moving their eyes. Moreover, the presence of non-VOR-related eye movements motivate our criterion rejecting individuals with non-parallel curve fits. The eye muscles that enable the VOR receive their information from both vestibular organs (Precht, 1976). In *Xenopus*, complete unilateral vestibular lesions and selective lesions of each utricular organ reduce the VOR of both eyes (Horn et al., 1986b). Thus, we considered non-parallel curves for the two eyes to indicate non-VOR-related eye movements (Figure 2.S2A).

Ontogenetic congruence of VOR and mechanosensory-cued hatching

When we began this work, we knew that hatching ability does not limit the onset of hatching responses to predator cues, because younger embryos demonstrate hatching competence in response to strong hypoxia (Cohen et al., 2019; Warkentin et al., 2017). Moreover, the rapid developmental increase in hatching response to egg-jiggling – contrasted with the much slower developmental decrease in the costs of early hatching – suggests that some sensory constraint imposes a developmental limit on the onset of the anti-predator response (Warkentin et al., 2017). We performed four experiments to examine the role of vestibular mechanoreception in embryos' risk assessment by comparing the ontogeny of responses at a population level, at a clutch level, and at an

individual level.

First, at a population level, we found that the developmental onset of the gravitational, roll-induced vestibulo-ocular reflex in red-eyed treefrog embryos (individually in series **Ia** and across clutches in series **Ib**) is congruent with the documented onset of escape-hatching responses to predator attacks in the Gamboa population of *A. callidryas* (Almanzar and Warkentin, 2018; Hite et al., 2018; Warkentin, 2000; Warkentin et al., 2006a). If the onset of VOR were clearly before or after the developmental period when predator-induced hatching begins in this population of *A. callidryas*, it would have rejected the hypothesized key role of vestibular system development in enabling the antipredator response. Moreover, clutches appeared to vary slightly in their onset of VOR (**Ib**), congruent with the greater variation in escape-hatching success of clutches attacked when the response first appears, and decreased variation later in development (Gomez-Mestre et al., 2008; Warkentin et al., 2006a).

Next, we examined the ontogeny of VOR in more detail through the period of greatest change (series **II**) and tested its relationship to the hatching response using vibration playbacks to entire clutches (Movie 2.S2). Across the onset of ear function, embryos below a VOR threshold of 21° did not hatch during vibration playbacks, even though they could hatch if flooded. Moreover, clutch hatching response increased with clutch mean VOR at supra-threshold levels. These data are also consistent with a key role of vestibular mechanosensing in mediating vibration-cued hatching.

In our last series (**III**), we compared VOR and the hatching response to simulated attacks on individual embryos (Movie 2.S3), rather than whole clutches, and saw that they

were still highly correlated. In addition, embryos with greater VOR hatched more rapidly in response to egg jiggling. Hatching occurred developmentally earlier in response to targeted jiggling cues (**III**) than in response to whole-clutch vibration playback (**II**), at stage 3 vs. stage 6. Moreover, embryos started showing a measurable VOR at earlier developmental stages in the egg jiggling series (**III**), relative to the clutch vibration series (**II**) (compare Figure 2.5A vs. 2.6A). VOR development was correlated with stage, but not perfectly. For instance, some stage 3 animals showed VOR but most did not, and one stage 5 animal lacked VOR, but most showed it. VOR amplitude predicted hatching more strongly than did developmental stage although, controlling for VOR, stage explains some additional variation and vice-versa. This individual-level correlation between vestibular function and hatching is consistent with a role of the developing embryonic vestibular system in mechanosensory-cued hatching.

Across successively finer levels of developmental precision, our results reveal a substantial increase in mechanosensory-cued hatching responses with the development of vestibular function, consistent with a role for otoconial organs in mediating the response. In general, the timing of onset of vestibular function is consistent with the onset of escape success in predator attacks (Almanzar and Warkentin, 2018; Hite et al., 2018; Warkentin, 2000; Warkentin et al., 2006a). In our vibration playbacks to clutches, no embryos lacking VOR hatched. In our egg-jiggling developmental series, we found a strong correlation of VOR with increased hatching response and decreased hatching latency. However, some evidence suggests that additional mechanoreceptor systems can also play a role in escape-hatching (Figure 2.7B).

Mechanosensory-cued hatching before vestibular function

Of the 61 embryos that hatched in response to our individual egg-jiggling cue, seven individuals (11%) had no detectable VOR; they hatched an average of 4.85 h before their siblings showed VOR. Hatching of embryos lacking VOR in response to jiggling cues is relatively rare and does not reject a key role of the otoconial organs in risk assessment by embryos, given the strength of the relationship between VOR and hatching. However, the occurrence of any mechanosensory-cued hatching prior to vestibular function indicates that vestibular mechanoreceptors are not the only sensors that can mediate hatching when eggs are physically disturbed, at least under some types of disturbance. *A. callidryas* embryos clearly use cues in multiple sensory modalities, including hypoxia (Rogge and Warkentin, 2008) and light level (Güell and Warkentin, 2018), to inform hatching. These embryos might also use multiple mechanosensors, either to perceive different cue components available in attacks and egg-jiggling or as potentially redundant or synergistic sensors of the same cue element. Two other candidate sensor types—lateral line neuromasts and cutaneous mechanoreceptors—may also be relevant to mechanosensory-cued hatching in the egg-jiggling context.

Other mechanosensory systems

The lateral line is a system of mechanoreceptors that detect movement, pressure gradients, and vibration in fishes and aquatic amphibians (Mogdans and Bleckmann, 2012). The effective stimulus to lateral line is low frequency particle motion of the surrounding fluid, relative to neuromasts distributed on the animal's surface (Strelioff and Honrubia,

1978; Weeg and Bass, 2002). *A. callidryas* embryos develop a lateral line system on their head, body, and tail by 3 d, well before mechanosensory-cued hatching begins at 4 d (Cohen et al., 2019; Warkentin et al., 2017). However, the number of superficial neuromasts, visualized with the fluorescent vital dye 4-di-2-ASP (Sigma D-3418), continues to increase through the onset of mechanosensory-cued hatching (Jung and Warkentin, unpublished data). The constant ciliary circulation of the perivitelline fluid within *A. callidryas* eggs (Rogge and Warkentin, 2008; Warkentin et al., 2005) presumably stimulates the lateral line, and any change in this circulation pattern would therefore be perceptible to embryos.

The sensation of touch in adult frogs and tadpoles depends on cutaneous mechanoreceptors that are diverse and highly specialized (Catton, 1976; Fromy et al., 2008; Spray, 1976; Weston, 1970). A single mechanoreceptive afferent can encode more than one type of stimulus, for example temperature and texture (Hunt and McIntyre, 1960), as well as mechanical stimuli such as pressure and vibration (Ribot-Ciscar et al., 1989). Since all somatosensory neurons arise from precursor neural crest cells early in embryonic development, much prior to the development of the vestibular system (Jenkins and Lumpkin, 2017; Weston, 1970), pre-VOR *A. callidryas* embryos are likely to already have cutaneous mechanoreceptors. These could enable embryos to sense contact cues, through the membrane, as a probe or a predator touches the egg capsule. Moreover, if the inertia of embryos is higher than their surroundings, moving an egg might also change how strongly the embryos' skin presses against the adjacent membrane, altering contact cues.

Multiple mechanosensory cues in attacks and multiple mechanosensory systems

Several types of mechanosensory cues could occur in egg-predator attacks—and in egg-jiggling—including whole-egg motion, passive embryo motion within the capsule, and tactile contact that may deform egg-capsules or contact embryos through their perivitelline membranes (Figure 2.9). Whole-egg motion occurs in vibration-playbacks, egg-jiggling, and predator attacks. As the embryo is accelerated along with its surrounding capsule, this will activate gravitoinertial sensors in the vestibular system—i.e., in the developing utricular and perhaps also saccular and/or lagenar maculae (Fritzsch and Straka, 2014; Straka and Dieringer, 2004), which differentiate as the otic vesicles divide into compartments (Quick and Serrano, 2005). If the embryo remains in the same position relative to its capsule, whole-egg motion alone would likely not alter perivitelline fluid flow and seems unlikely to stimulate the lateral line or cutaneous touch receptors.

Passive embryo motion within the capsule occurs when embryos are displaced in their perivitelline chamber as the capsule is moved. The inertia of the embryo likely differs from the surrounding fluid and capsule, such that the embryo may lag a bit behind as the egg accelerates around it. For instance, if the egg were accelerated up the embryo could be pressed against the bottom of the chamber, and if the egg were accelerated down the embryo could be lifted off the bottom. This could change both cutaneous stimulation and perivitelline fluid flow if the embryo's body were sufficiently displaced within the capsule. Tactile contact occurs for a subset of eggs in predator attacks on, and tine-based vibration playbacks to, whole egg clutches, and for all eggs exposed to individual egg-jiggling stimulation. If the contact deforms egg capsules (e.g., dents or squashes them), even

without contacting the embryo inside, it may change perivitelline fluid flow and lateral line input (Figure 2.9). Contact with the embryos through the membrane would also directly stimulate cutaneous touch receptors. Of course, active embryo movements within the egg capsule will also stimulate all three mechanoreceptor systems.

Our egg-jiggling (Movie 2.S3) and vibration-playback (Movie 2.S2) experiments differed in several important ways. First, the jiggling stimulus (Movie 2.S3) represents a targeted attack on individual eggs rather than a generalized stimulus to whole clutches (Movie 2.S2). Second, it was a more complex multimodal stimulus that combined whole egg motion with tactile elements and included both lateral and rolling movements. Since predators must touch eggs to eat them, risk of mortality in attacks is presumably higher for eggs receiving motion and contact cues than for those receiving motion cues alone. Both targeted jiggling of and predator attacks on individual eggs likely stimulate the otoconial organs, the lateral line, and touch receptors in the skin. But other eggs in attacks and in vibration playbacks likely experience only whole-egg motion and vestibular stimulation. This variation in the cues available to embryos may contribute to the variation in individual responses and the different responses to vibration-playback and egg-jiggling stimuli. Moreover, in the jiggling series, embryos began showing VOR (thus, developing vestibular function) at earlier developmental stages compared to in the tine playback series (Figure 2.6A vs. 2.5A), which may also have contributed to their earlier mechanosensory-cued hatching.

Ontogenetic changes in embryo use of multimodal mechanosensory cues

Whatever sensory system mediates hatching in egg jiggling for animals lacking VOR would presumably add to the stimulation experienced by older animals that have developed functional otoconial organs. Moreover, at a given stage of development, cues indicating greater risk should be more likely to elicit hatching. Thus, at the same stage, we expect an individually targeted "attack" stimulus to more strongly elicit hatching than a stimulus transmitted through the clutch. Consistent with this, stage 6 or 7 animals with strong VOR show a stronger hatching response to egg jiggling than to vibration playbacks to clutches. Nonetheless, if an animal has less-developed mechanoreceptors and cannot sense components of a stimulus, it will be limited in its risk-assessment ability. Stage 3 animals lacking VOR, and vestibular function, presumably receive just cutaneous and perhaps lateral line input in attacks and our mechanosensory stimuli. In contrast, stage 3–5 animals with low VOR likely also receive weak vestibular input; combining this with cutaneous and/or lateral line input may generate sufficient total stimulation to elicit hatching. Without additional input from another mechanosensory system, weak vestibular input may be insufficient to elicit hatching. Different types of mechanosensory cues likely stimulate different mechanoreceptor types, or combinations thereof, providing different and potentially synergistic or complementary information about risk. Thus, *A. callidryas* embryos may use multimodal mechanosensory cues to inform escape-hatching decisions, particularly at the onset of vibration-cued hatching when their mechanosensory systems are less developed.

We recently developed a new vibration playback system to generate whole-egg

motion without tactile contact cues or egg-shape deformation, demonstrating that egg-motion alone is sufficient to induce hatching (Warkentin et al., 2019). A second playback-system component adds a tactile contact cue, which appears to synergize with motion to increase hatching of 4-day embryos (Fouilloux, Jung, Ospina, Snyder, & Warkentin unpublished). Lateral line blocking and/or vestibular system ablation experiments, in conjunction with vibration playbacks, would be useful to assess the individual and potentially interacting roles of these mechanosensory systems in the hatching decisions of *A. callidryas* embryos.

CONCLUSION

Hatching is a developmentally critical behavior that immediately impacts survival in multiple ecological contexts. Environmentally cued hatching is widespread and well-documented in all three major clades of bilateria and, in many species, embryos respond to multiple different factors or contexts (Warkentin, 2011a). Physical disturbance of eggs is a particularly salient and common cue to hatch among embryos of fishes (Martin et al., 2011), amphibians (Buckley et al., 2005; Gomez-Mestre et al., 2008; Goyes Vallejos et al., 2018; Touchon et al., 2011; Warkentin, 1995; Warkentin, 2000; Warkentin, 2011b), and reptiles (Doody, 2011; Doody and Paull, 2013; Doody et al., 2012), as well as many invertebrates (Endo et al., 2019; Mukai et al., 2014; Oyarzun and Strathmann, 2011; Tanaka et al., 2016; Whittington and Kearn, 1988). Presumably, all the vertebrates that use physical disturbance as a hatching cue have vestibular systems and cutaneous mechanoreceptors, but only the fish and amphibians have lateral lines. Moreover, some of

the contexts that induce hatching in vertebrates seem likely to provide only whole-egg motion cues. For instance, grunion embryos are tightly coiled within their eggs and pressed against the capsule wall at a stage when tumbling in waves elicits hatching (Martin et al., 2011; Speer-Blank and Martin, 2004). This embryo size and position seem likely to prevent passive displacement within the perivitelline chamber as eggs are moved. Pig-nosed turtle embryos hatch in response to a whole-egg motion stimulus presented via an electronic shaker in the laboratory (Doody et al., 2012). Neither the lateral line nor cutaneous sensing seem likely to play a role in these instances, suggesting the vestibular system could mediate motion-cued hatching responses in multiple— perhaps many—vertebrate embryos. The mechanisms that enable, regulate, and inform hatching change developmentally, altering embryos' capacities for behavioral responses to cues. Thus, information on embryos' sensory development will clarify how and why development changes behavior. This research elucidates how changing sensory and behavioral abilities can affect an essential early behavior and reveals a fundamental mechanism underlying phenotypic plasticity at a critical life history switch point.

FIGURE LEGEND

Figure 2.1 – Example vestibulo-ocular reflex (VOR) curves for *Agalychnis callidryas* hatchlings. (A, B) Data points show eye angle (left in blue, right in red) plotted against body angle and lines are best fit curves for each eye. The peak-to-peak amplitude (*) of the curve measures the magnitude of VOR. (C, D) Frontal view of hatchlings tested, showing left (blue) and right (red) eye angles relative to the body axis (white). (A) Low VOR for a hatchling of age 3.6 d (C). (B) Clear VOR in a hatchling of age 4.5 d (D).

Figure 2.2 – Vibration playback stimulus. (A) The entire stimulus included 7 pulse groups divided by 30 s gaps plus a 3-pulse primer before the first 30 s gap. (B) The first minute of the stimulus, including the primer and one pulse group, comprised of ten 0.5 s pulses of vibration, separated by 1.5 s intervals. (C) Frequency spectrum of acceleration, normalized from peak power. This stimulus induces near 100% hatching in 5-day-old clutches.

Figure 2.3 – Ontogeny of the vestibulo-ocular reflex (VOR) across embryonic development of *Agalychnis callidryas* (series Ia). Hatchings were tested at 24 h intervals from age 3–6 d, at approximately 3 pm. Red numbers show sample size of non-sibling individuals per age (total N = 36 hatchlings tested from 14 different clutches). Individual hatchlings were tested for VOR immediately after hatching or decapsulation and data points are color-coded by developmental stage. Box plots show medians, interquartile range (IQR), and extent of data to ± 1.5×IQR. Different letters indicate significant

differences in VOR amplitudes between ages.

Figure 2.4 – Ontogeny of the vestibulo-ocular reflex (VOR) across embryonic development in five sibships of *Agalychnis callidryas* (series Ib). Individual hatchlings were tested from developmental series of five clutches (shown in different colors and symbols), with ~3 siblings per test point (total N=88 hatchlings; N=15, 16, 15, 15, 15, 12 per age). Lines show 4-parameter logistic curve fit for each clutch.

Figure 2.5 – Relationships among vestibulo-ocular reflex (VOR) amplitude, development, and hatching response of *Agalychnis callidryas* embryos to vibration playbacks (series II). (A) Filled and unfilled box plots indicate VOR of individuals (N=169) that did not hatch or hatched, respectively, in response to vibration playbacks to whole clutches across development. Red numbers show N of individuals tested per response, per stage. Box plots show medians, interquartile range (IQR) and extent of data to ± 1.5×IQR, and outliers as points. Different letters indicate significant differences between stages. (B) Total proportion hatched, for the 23 clutches from which at least one embryo hatched, plotted against clutch mean VOR amplitude measured from 169 hatchlings (4–6 individuals per clutch). Color indicates the modal developmental stage of individuals in each clutch. Dashed line indicates the linear regression fit, shading indicates 95% confidence interval.

Figure 2.6 – Ontogeny of vestibulo-ocular reflex (VOR) and hatching responses in *Agalychnis callidryas* (series III). (A) Ontogeny of VOR across stages, from developmental series of 11 egg clutches, with two siblings tested per time point (N = 112

individuals). Red numbers show N of individuals per response, per stage. Different letters indicate significant differences between stages. Box plots show medians, interquartile range (IQR) and extent of data to ± 1.5×IQR, and points show outliers. Filled and unfilled box plots indicate embryos that did not hatch and hatched, respectively, in response to manual egg jiggling. (B) Ontogeny of hatching response to manual jiggling of individual eggs. Proportion hatched (colored boxes) is of individual embryos tested per stage. Boxes are colored by developmental stage.

Figure 2.7 – Effect of (A) developmental stage and (B) vestibulo-ocular reflex (VOR) amplitude on hatching response of *Agalychnis callidryas* embryos to egg jiggling (series III). Values of 0 (unhatched, black circles) and 1 (hatched, colored circles) are jittered vertically to show data points. Hatched embryos are colored by developmental stage. Integer values (developmental stages) are jittered, while continuous individual measurements (VOR amplitude) are not. Dashed lines are predicted fits from binomial generalized linear mixed models; shading indicates the 95% confidence interval.

Figure 2.8 – Latency of Agalychnis callidryas embryos to hatch in response to egg jiggling, in relation to vestibulo-ocular reflex (VOR) amplitude (series III). Data are for N=61 embryos tested individually. Developmental stages are indicated by color. Dashed line indicates linear regression fit; shading shows the 95% confidence interval.

Figure 2.9 – Types of mechanosensory cues in egg-predator attacks. Attacked embryos may experience whole-egg motion (solid black), passive embryo motion within the perivitelline chamber (white), and direct contact with eggs (dashed) that may a. deform egg

capsules or b. touch embryos through their capsule.

Figure 2.1

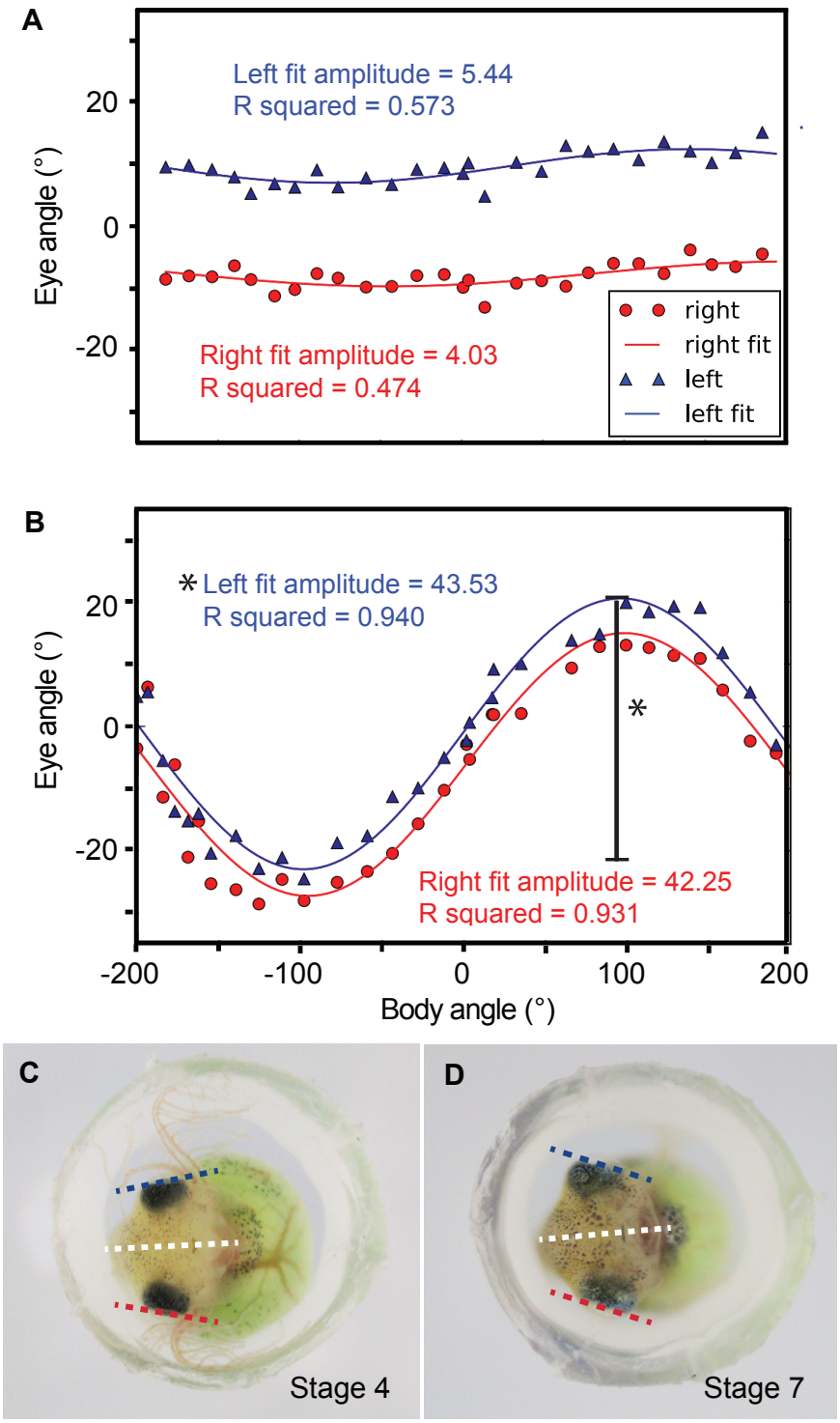

A
Left fit amplitude = 5.44
R squared = 0.573
Right fit amplitude = 4.03
R squared = 0.474
right
right fit
left
left fit
Eye angle (°)
20
0
-20
B
* Left fit amplitude = 43.53
R squared = 0.940
Right fit amplitude = 42.25
R squared = 0.931
*
Eye angle (°)
20
0
-20
-200
-100
0
100
200
Body angle (°)
C
Stage 4
D
Stage 7

Figure 2.2

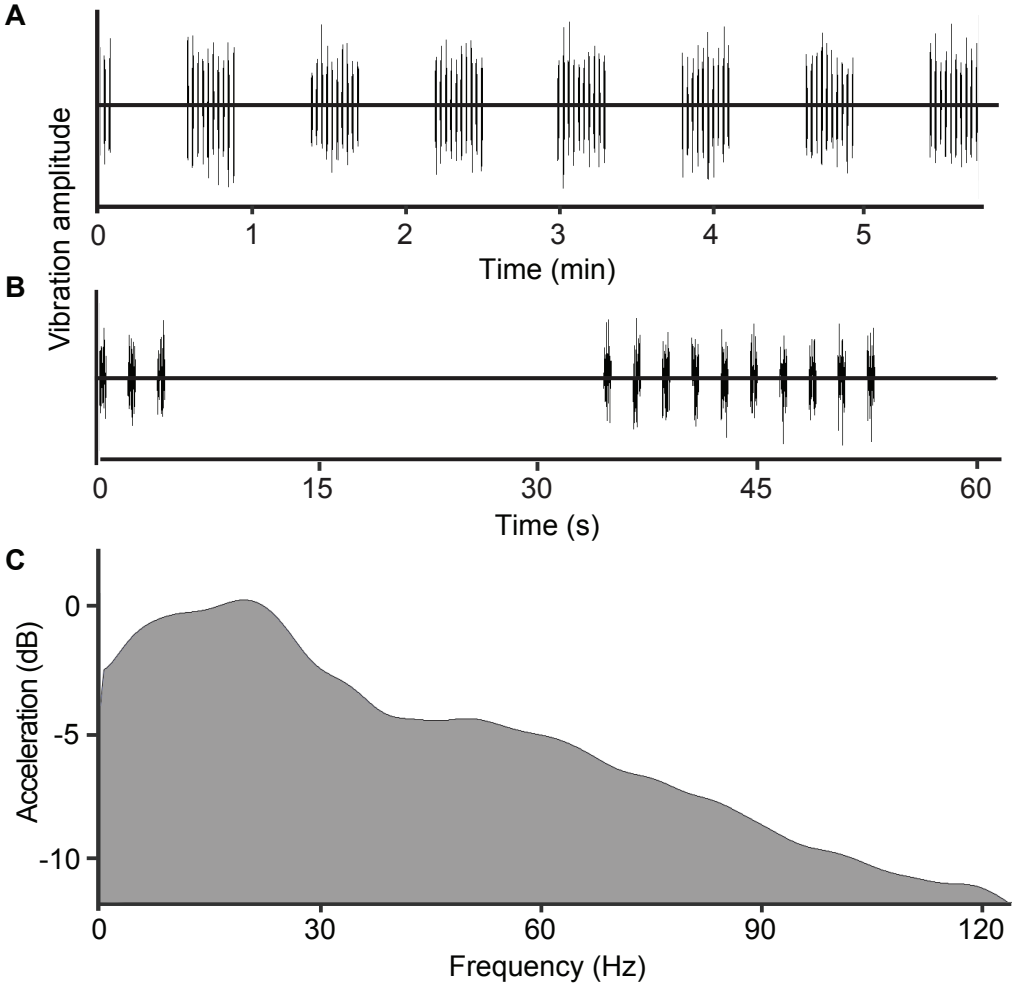

A
Vibration amplitude
0
1
2
3
4
5
Time (min)
B
0
15
30
45
60
Time (s)
C
Acceleration (dB)
0
-5
-10
0
30
60
90
120
Frequency (Hz)

Figure 2.3

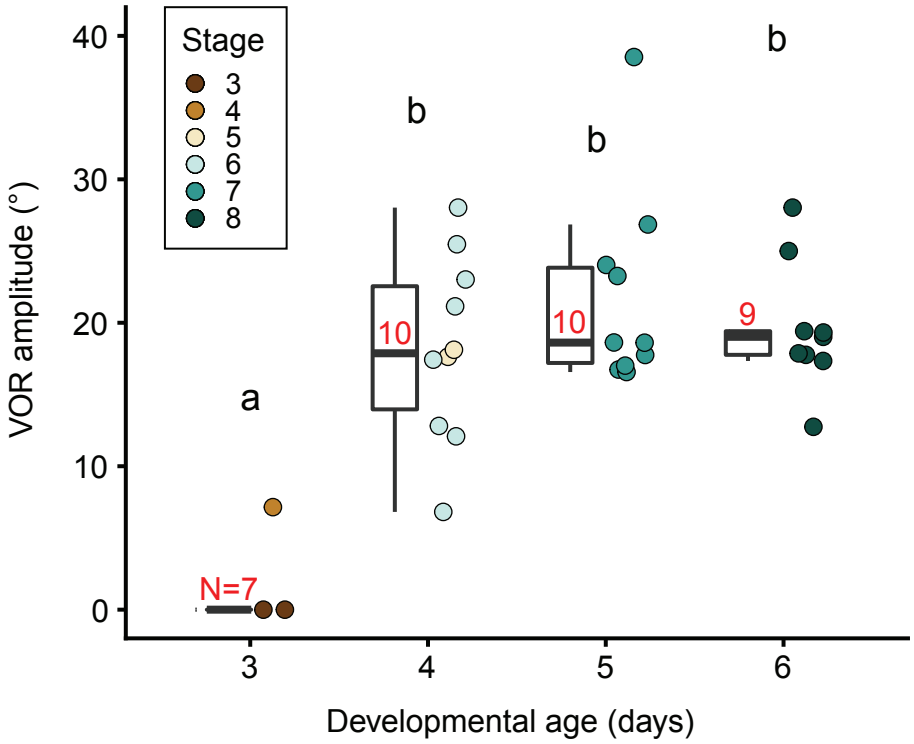

Stage
3
4
5
6
7
8
VOR amplitude (°)
40
30
20
10
0
a
b
b
b
N=7
10
10
9
3
4
5
6
Developmental age (days)

Figure 2.4

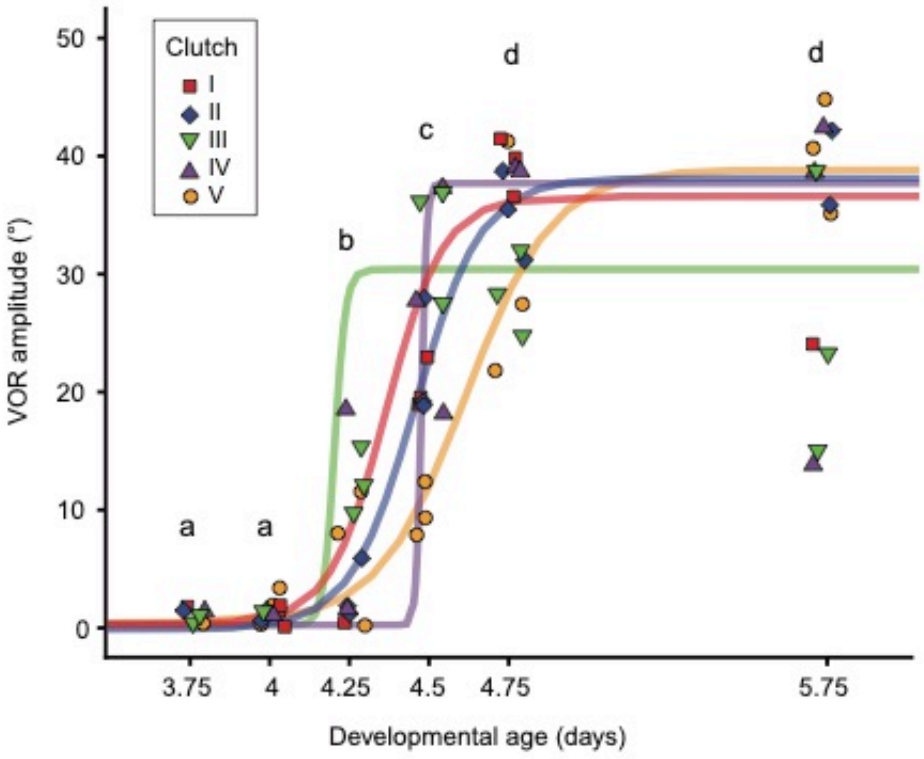

Clutch
I
II
III
IV
V
VOR amplitude (°)
50
40
30
20
10
0
a
a
b
c
d
d
3.75
4
4.25
4.5
4.75
5.75
Developmental age (days)

Figure 2.5

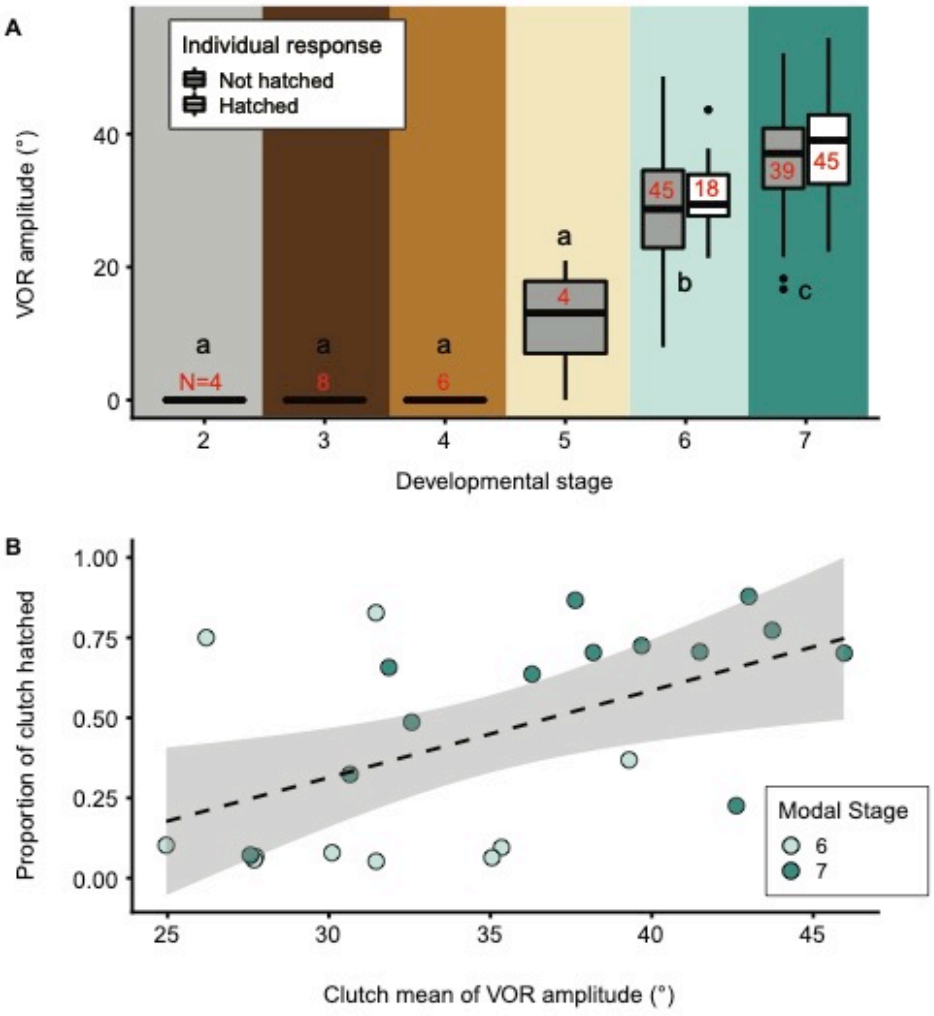

A
Individual response
Not hatched
Hatched
VOR amplitude (°)
40
20
0
a
a
a
a
b
c
N=4
8
6
4
45
18
39
45
2
3
4
5
6
7
Developmental stage
B
Proportion of clutch hatched
1.00
0.75
0.50
0.25
0.00
Modal Stage
6
7
25
30
35
40
45
Clutch mean of VOR amplitude (°)

Figure 2.6

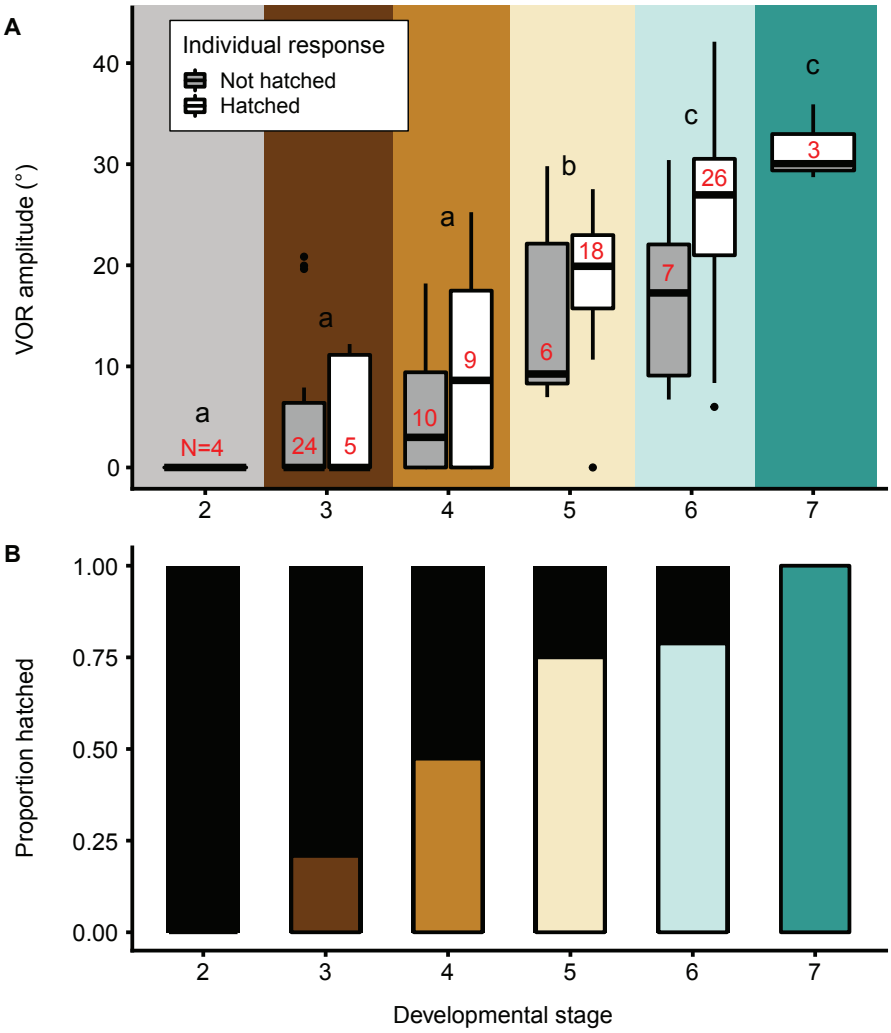

A
Individual response
Not hatched
Hatched
VOR amplitude (°)
40
30
20
10
0
a
N=4
a
24
5
a
10
9
b
6
18
c
7
26
c
3
2
3
4
5
6
7
B
Proportion hatched
1.00
0.75
0.50
0.25
0.00
Developmental stage

Figure 2.7

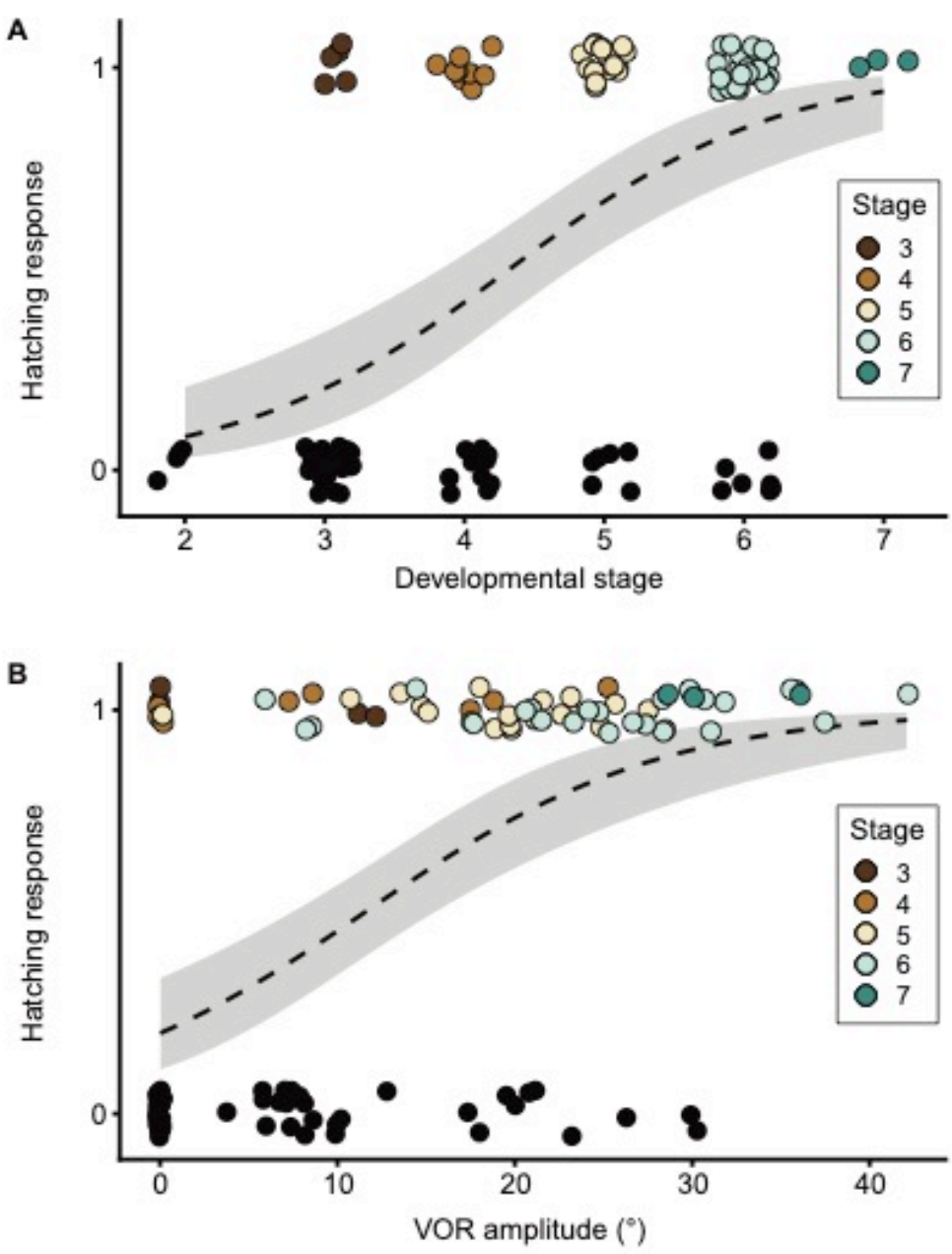

A
1
0
Hatching response
Stage
3
4
5
6
7
2
3
4
5
6
7
Developmental stage
B
1
0
Hatching response
Stage
3
4
5
6
7
0
10
20
30
40
VOR amplitude (°)

Figure 2.8

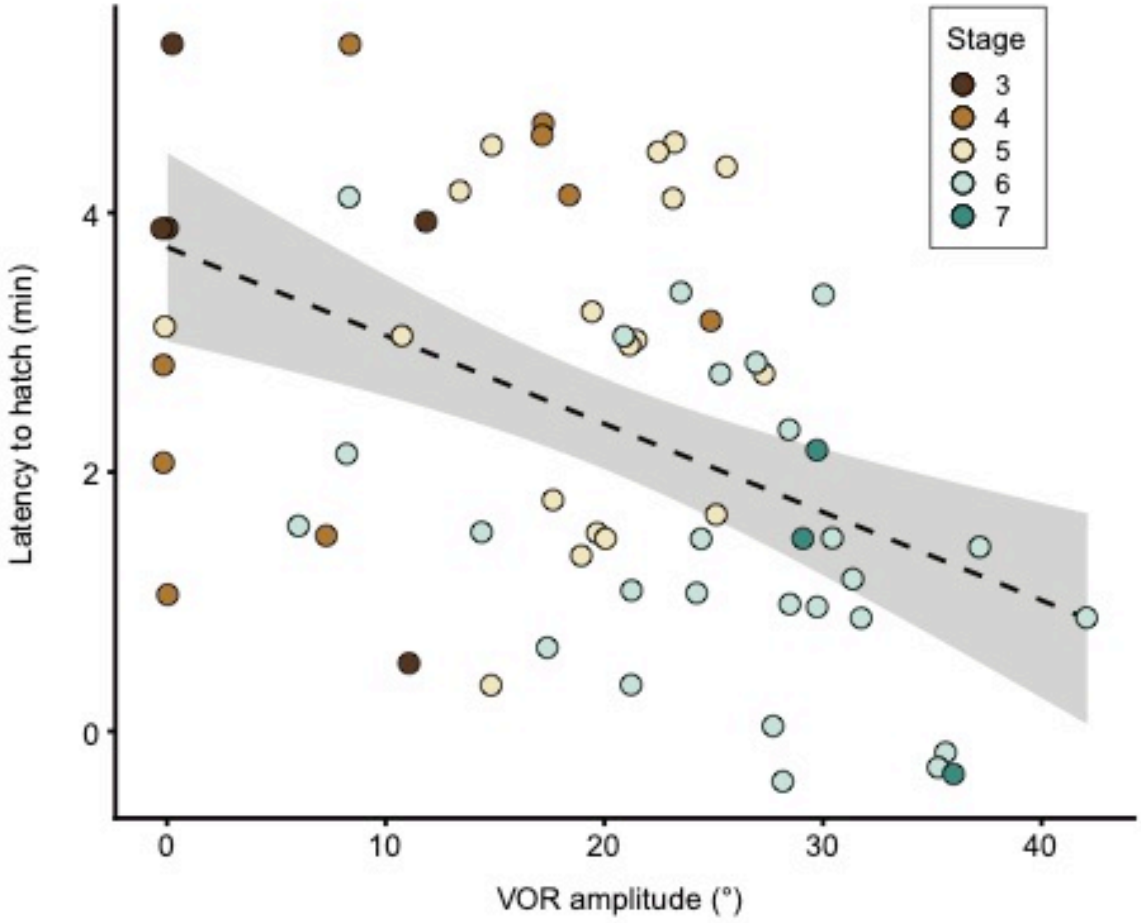
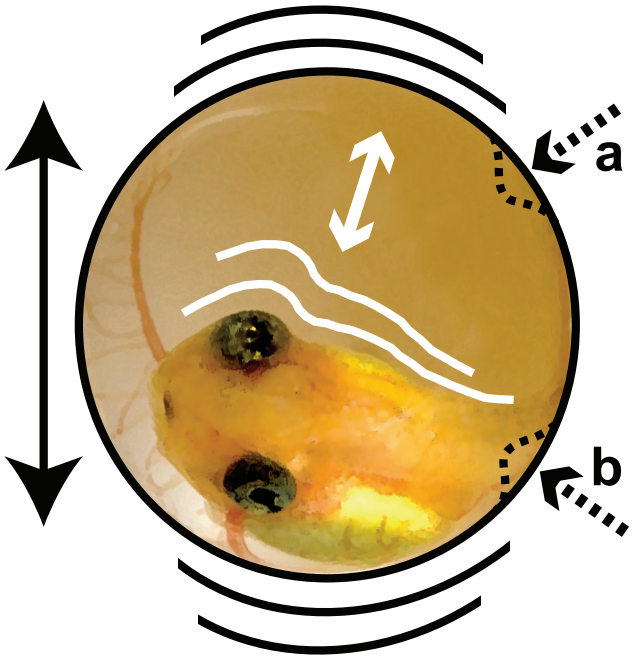

Stage
3
4
5
6
7
Latency to hatch (min)
0
2
4
0
10
20
30
40
VOR amplitude (°)

Figure 2.9



SUPPLEMENTARY INFORMATION

Supplementary Movies are available online at:

http://jeb.biologists.org/lookup/doi/10.1242/jeb.206052.supplemental

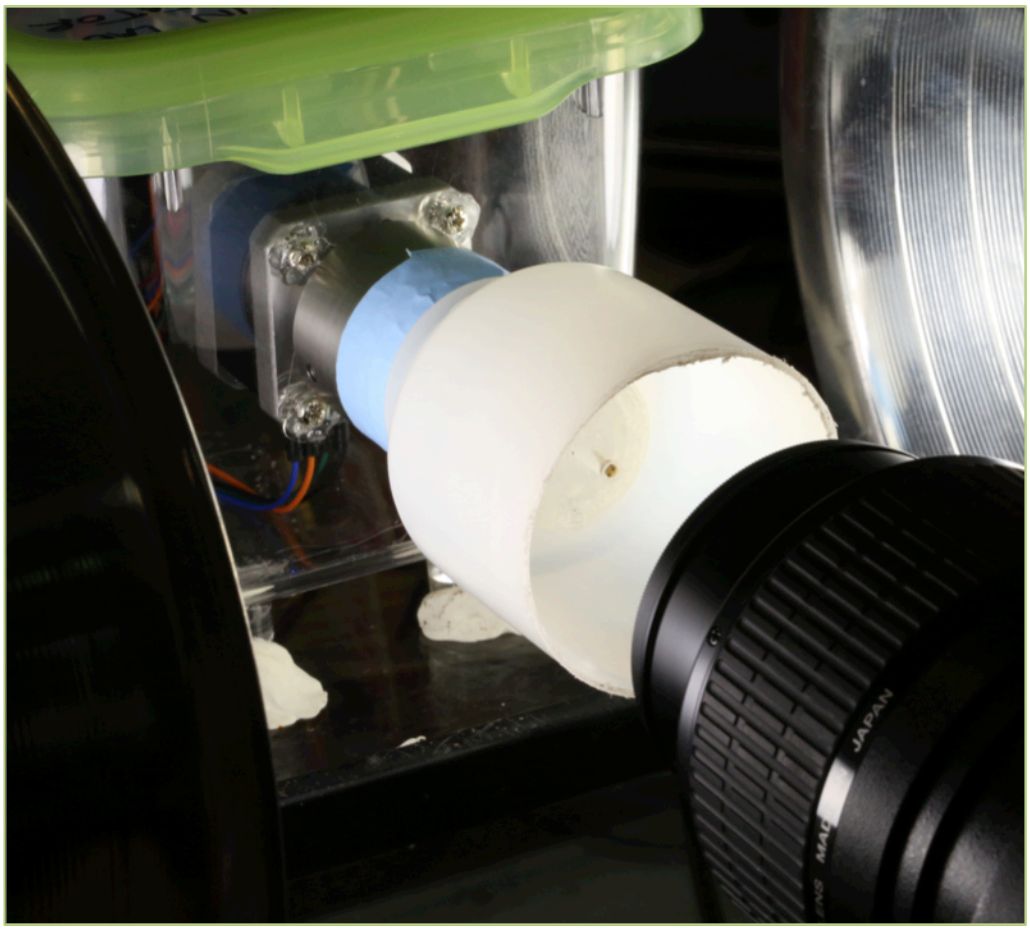
Supplementary Movie 2.1 – Measuring the vestibulo-ocular reflex (VOR). The clip shows a frontal view of an *Agalychnis callidryas* hatchling within the tadpole rotator, as it is rotated through a series of 15° rotational increments. This individual shows eye rotation opposite to body rotation, demonstrating a VOR. The video was recorded using an MPE-65 mm macro lens.

Supplementary Movie 2.2 – Vibration playback to a 4-d old *Agalychnis callidryas* egg clutch. The vibrational stimulus was presented through an array of blunt metal times inserted among eggs. The clip shows a series of ten 0.5 s vibration pulses, separated by 1.5 s silent intervals, and then the beginning of a longer silent gap during which two embryos hatch from the left side of the clutch.

Supplementary Movie 2.3 – Manual egg-jiggling stimulus. Individual *Agalychnis callidryas* eggs were jiggled with a blunt metal probe, alternating 15 s of stimulation and 15 s of rest for 5 min or until the egg hatched. The clip shows 5 s of stimulation, after which the embryo begins shaking motions – indicating the start of the hatching process – and the experimenter ceases stimulation. Then the embryo hatches.

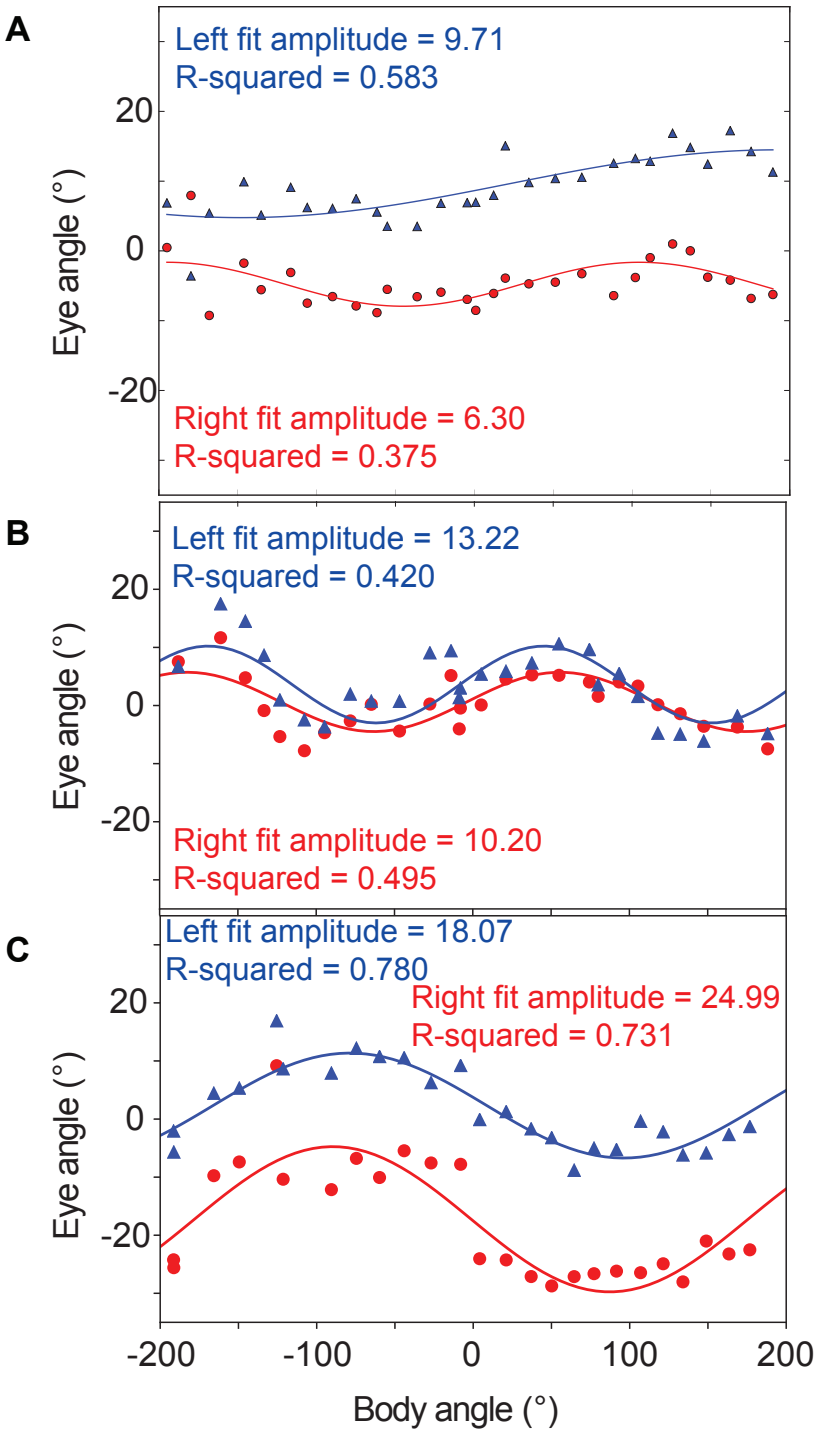
Supplementary Figure 2.1 – Measuring the vestibulo-ocular reflex (VOR) with a custom-made tadpole rotator. The hatchling was placed in a tube, within a light diffusor, attached to the rotator shaft and facing a MPE-65 mm macro lens and camera.

Supplementary Figure 2.1



Supplementary Figure 2.2 – *Agalychnis callidryas* hatchlings' eye movements unrelated to the vestibulo-ocular reflex (VOR). Example measurement series of eye and body angles demonstrate non-VOR eye movements with a measurable amplitude exceeding that of small but clear VOR. We considered VOR amplitude to be zero when a hatchlings' curve fit showed (A) different wavelengths or offset waveforms causing non-parallel lines for each eye, (B) wavelengths too short or too long for VOR curves, or (C) an upside down sine curve, relative to VOR curves, indicating eye rotation that magnified rather than reduced the effect of body rotation.

Supplementary Figure 2.2



Supplementary Figure 2.3 – *Agalychnis callidryas* egg clutch set up for vibration playback. Clutches were mounted vertically, then a set of blunt metal tines connected to a shaker were inserted among the eggs to deliver the vibrational stimulus.

Supplementary Figure 2.3

CHAPTER 3. MULTIMODAL MECHANOSENSING ENABLES TREEFROG EMBRYOS TO ESCAPE EGG-PREDATORS

This chapter is published as:

Jung J, Serrano-Rojas SJ, Warkentin KM. 2020. Multimodal mechanosensing enables treefrog embryos to escape egg-predators. *Journal of Experimental Biology*. 223(24). doi:10.1242/jeb.236141

ABSTRACT

Mechanosensory-cued hatching (MCH) is widespread, diverse, and improves survival in many animals. From flatworms and insects to frogs and turtles, embryos use mechanosensory cues and signals to inform hatching timing, yet mechanisms mediating mechanosensing *in ovo* are largely unknown. The arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas,* hatch prematurely to escape predation, cued by physical disturbance in snake attacks. When otoconial organs in the developing vestibular system become functional, this response strengthens, but its earlier occurrence indicates another sensor must contribute. Post-hatching, tadpoles use lateral line neuromasts to detect water motion. We ablated neuromast function with gentamicin to assess their role in *A. callidryas*' hatching response to disturbance. Prior to vestibular function, this nearly eliminated the hatching response to a complex simulated attack cue, egg-jiggling, revealing that neuromasts mediate early MCH. Vestibular function onset increased hatching, independent of neuromast function, indicating young embryos use multiple mechanosensory systems. MCH increased developmentally. All older embryos hatched in response to egg-jiggling,

but neuromast function reduced response latency. In contrast, neuromast ablation had no effect on timing or level of hatching in motion-only vibration playbacks. It appears only a subset of egg-disturbance cues stimulate neuromasts; thus embryos in attacked clutches may receive uni- or multimodal stimuli. *A. callidryas* embryos have more neuromasts than described for any other species at hatching, suggesting precocious sensory development may facilitate MCH. Our findings provide insight into the behavioral roles of two mechanosensory systems *in ovo* and open possibilities for exploring sensory perception across taxa in early life stages.

INTRODUCTION

Animals ubiquitously use vibration to inform their behavior (Cocroft et al., 2014; Hill et al., 2019; Hill, 2009). Most research in this area focuses on vibrational communication signals in the contexts of mate choice, competition, and parental care (Cocroft et al., 2014). These signals are intentionally produced and generally operate between conspecifics. In addition, passively produced, unintentional vibrational cues can inform behavior. A smaller subset of research examines how animals use such incidental vibrations and other physical disturbance cues, generated either abiotically or biotically, to express context-appropriate behaviors. Conspecifics can generate disturbance cues (Doody et al., 2012), but other sources abound, such as weather (Marquez et al. 2016) and, more commonly, predators and prey (Bacher et al., 1997; Brownell and Leo van Hemmen, 2001; Castellanos and Barbosa, 2006; Oberst et al., 2017; Pfannenstiel et al., 1995; Roberts, 2018). Physical disturbances generated incidentally as animals move might be especially

useful as cues because they can be difficult to conceal in a predator-prey context. Even embryos can sample incidental disturbance cues from their environments (Warkentin 2005; Warkentin, 2011a; Warkentin et al., In press). Like later life stages, embryos use physical disturbance cues from abiotic sources such as rainfall (Roberts, 2001) and wave action (Griem and Martin, 2000; Martin et al., 2011), or biotic sources such as conspecifics (Endo et al., 2019; Noguera and Velando, 2019), hosts (Wang et al., 2012; Whittington and Kearn, 1988; Whittington and Kearn, 2011), and predators (Doody and Paull, 2013; Warkentin, 2005). From flatworms and insects to frogs and turtles, embryos of all sorts use disturbance cues to inform their hatching timing, yet the mechanisms mediating vibration and other mechanosensing *in ovo* are largely unknown.

The red-eyed treefrog, *Agalychnis callidryas*, is an excellent species in which to study how embryos detect and respond to physical disturbance or vibrations. As adults, these arboreal amphibians lay eggs on plants overhanging rainforest ponds. Usually within seven days, embryos hatch and fall into the water below, where they will develop as tadpoles, but they can hatch prematurely to escape from threats to eggs, including pathogens, flooding, and terrestrial egg predators (Warkentin and Caldwell, 2009). This escape-hatching response changes dramatically over development. The earliest observed hatching occurs at 3 days post-oviposition in response to strong hypoxia cues, but the onset of predator-induced, mechanosensory-cued hatching (MCH) does not occur until the following day (Warkentin et al., 2017). During the period of development between the onset of hatching responses to hypoxia and to mechanosensory cues, embryos are clearly able to hatch to escape threats. Nonetheless, they do not use this ability to flee from

predators. Hatching ability is, therefore, not the sole constraint limiting the onset of escape-hatching responses to predator attacks.

The embryos of *Agalychnis callidryas* clearly use cues in multiple sensory modalities, including vibration (Warkentin 2005, Warkentin et al. 2019), hypoxia (Rogge and Warkentin, 2008) and light level (Güell and Warkentin, 2018), to inform hatching. In sensing physical disturbances, these embryos might use one or multiple mechanosensory systems, either to perceive different cue components available in attacks or as potentially redundant or synergistic sensors of the same cue component. As a first step toward determining the role of mechanosensory system development in the ontogeny of predator-induced hatching ability, we examined a general vertebrate motion sensor, the vestibular system of the inner ear (Jung et al., 2019). When otoconial organs in the developing vestibular system become functional, MCH increases substantially, but its earlier occurrence at a low level indicates that vestibular mechanoreceptors cannot be the only sensors that enable hatching when eggs are physically disturbed (Jung et al., 2019).

The mechanosensory lateral line system is found in all fishes and aquatic life stages of amphibians and serves to detect movement, vibrations, and pressure gradients in the surrounding water. It is comprised of mechanoreceptive neuromast sensory organs with hair cells that are sensitive to local water displacements (Lannoo, 1999) and are similar in morphology and function to hair cells in the auditory and vestibular system of vertebrates (Mogdans, 2019; Roberts et al., 1988). The lateral line system and vestibular system are responsive to many of the same stimulus fields (Braun and Coombs, 2000) and the hair cells of the lateral line and inner ear even have comparable thresholds of pressure detection

(Van Netten, 2006). When something moves in the water, it creates water motion that deflects the ciliary bundles of the hair cells of the neuromasts, which opens mechanically gated ion channels (Harris et al., 1970; Sand et al., 1975). Upon deflection by water flow, hair cells within neuromasts depolarize and release neurotransmitter onto afferent neuron terminals, which then convey this information to the hindbrain (Liao, 2010; Raible and Kruse, 2000). This stimulus information helps fishes and aquatic amphibians orient in currents (Elepfandt, 1982; Montgomery et al., 1997), maintain position within a school (Partridge and Pitcher, 1980), find prey (Bleckmann, 1980; Hoekstra and Janssen, 1985; Montgomery and Macdonald, 1987; Pohlmann et al., 2004), and detect predators (Montgomery, 1989; Schwalbe et al., 2012). Fishes and aquatic amphibians are able to detect low-level water motion with both the lateral line system and the inner ear, but the relative roles of these sensory systems is unclear (Karlsen and Sand, 1987).

Here we investigate the potential contribution of the embryonic lateral line system to sensing physical disturbance cues that inform escape-hatching decisions of *A. callidryas* embryos during snake attacks. When snakes bite, bump, and pull at eggs, embryos may receive complex mechanosensory stimuli, including motion, pressure, and tactile elements (Jung et al., 2019). The flow of perivitelline fluid, which constantly circulates around embryos, may also be altered as snakes stretch or squash egg capsules, thereby altering input to the lateral line system in the egg. However, not all embryos in an attacked clutch receive these complex cues. Embryos more distant from the snake may receive only whole-egg motion cues, transmitted through the clutch, but it is unclear if or how such whole-egg motion could stimulate the lateral line system (Jung et al., 2019). To assess the role of the

lateral line and vestibular systems in sensing complex mechanosensory stimuli, we used a simulated attack cue (jiggling eggs in a standardized fashion with a blunt probe; Warkentin et al., 2017). We tested embryos with functional and inactivated lateral line systems, before and after the onset of vestibular function. To assess if egg motion alone stimulates the lateral line system, we used a custom-made vibration playback system to shake eggs in trays without providing concurrent tactile or egg deformation cues (Warkentin et al., 2019; Warkentin et al., In press) and tested embryos with functional and inactivated lateral line systems. Thus, we assessed the role of two mechanosensory systems, in two disturbance cue contexts, in a critical anti-predator behavior demonstrated by developing embryos.

METHODS

Egg clutch collection and care

We collected young (0–1 d old) *A. callidryas* egg clutches and the leaves on which they were laid from the Experimental Pond in Gamboa, Panama (9.120894 N, 79.704015 W) and brought them to a nearby laboratory (at ambient temperature and humidity) at the Smithsonian Tropical Research Institute. We mounted clutches on plastic cards for support, positioned them over aged tap water in plastic cups, then placed them inside plastic bins with screen lids connected to an automatic misting system (Mist King, Jungle Hobbies, www.mistking.com) set to mist clutches with rainwater at regular intervals to maintain hydration. Most clutches are laid between 10 pm and 2 am, so we assigned embryos to daily age-classes and reported developmental timing starting from midnight of their

oviposition night (Warkentin, 2002; Warkentin, 2005). All tested individuals were staged based on external morphological markers (Warkentin, 2017) and were morphologically normal, in developmental synchrony with siblings in their clutch, and in intact, turgid eggs at the start of experiments. Hatchlings from gentamicin-treated eggs were reared in the laboratory under ambient conditions until hair cells regenerated (Hernández et al., 2007; Ma et al., 2008; Thomas et al., 2015; Williams and Holder, 2000), as confirmed with 4-di-2-ASP staining (details below), within 3 d of hatching. All hatchlings were returned to the pond from which they were collected. This research was conducted under permits from the Panamanian Environmental Ministry (SC/A-10-18 and SE/A-42-19) and approved by the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (2017-0601-2020-2).

Vestibulo-ocular reflex (VOR) measurement

To assess vestibular function, we measured roll-induced VOR of newly hatched tadpoles or manually decapsulated embryos using previously described methods (Horn and Sebastian, 1996; Horn et al., 1986a; Jung et al., 2019). We placed hatchlings in a close-fitting tube of water and used a custom-made tadpole rotator to roll them about their body axis 180° in each direction, photographing them in frontal view each 15° (Jung et al., 2019). This method allowed us to measure vestibular function rapidly and without anesthesia. From each photograph, we measured right and left eye angle and body axis angle using ImageJ (Schneider et al., 2012). From each angular measurement series, we constructed an individual VOR curve using a sine-fitting function in Python (Version 2.7.9, Build 1,

Python Software Foundation). The peak-to-peak amplitude of the curve fit is the measured VOR magnitude.

Lateral line system visualization with 4-di-2-ASP

To visualize hair cells in neuromasts, newly hatched or decapsulated tadpoles were immersed in a 500 µM solution of the fluorophore 4-(4-diethylaminostyryl)-1-methylpyridinium iodide (4-di-2-ASP, Sigma D-3418, sigmaaldrich.com) for five minutes at room temperature in the dark. Tadpoles were then rinsed in aged tap water to remove excess fluorophore and placed in a shallow petri dish or snout-up in a modified pipette mount. This method allowed us to label neuromast hair cells and photograph them in live embryos without anesthesia or treatment with a paralytic. We used an Olympus stereoscope equipped with epifluorescence (GFP filter set) to photograph each individual. We considered neuromasts showing fluorescence to be functional and a complete lack of fluorescence to indicate neuromast inactivation. Fluorescence gradually faded within hours and disappeared within a day of treatment. Treated individuals developed normally compared to untreated controls.

Lateral line system knockout with gentamicin

To test the role of the lateral line system in predator-induced, mechanosensory-cued escape-hatching behavior, we used the ototoxic aminoglycoside antibiotic gentamicin to temporarily ablate neuromast function (Kroese and van den Bercken, 1982; Montgomery et al., 1997; Van Trump et al., 2010). At embryonic age 3 d, we removed individual eggs

from clutches and placed groups of eight siblings into hexagonal polystyrene weigh boats (4.5 cm across x 1 cm depth). We added 1 mL of rainwater to each boat, partially submerging eggs, and waited 1 h for water absorption to increase and standardize turgidity before treatment. To begin treatment, we removed the remaining water and added a fresh 1mL of rainwater into each boat. Control eggs remained in rainwater, while treatment eggs received a timed dosage series of gentamicin sulphate solution-10 (MP Biomedicals™, fishersci.com) mixed into the rainwater to gradually increase the concentration (starting dose 2 µL, + 2 µL at 3 h, + 2 µL at 6 h, + 0.5 µL at 9 h, + 0.5 µL at 10.5 h). The final concentration was the minimum dosage that effectively blocked lateral line system function, determined based on pilot experiments. The gradual increase in concentration was necessary to maintain egg turgor and membrane integrity, as needed for hatching assays, also based on pilot experiments. At 13 h, we extracted 0.5 mL of solution from each boat to increase air exposure of eggs and ensure sufficient oxygen availability for these terrestrial eggs as they continued developing in the boats until hatching-response tests were carried out. Only eggs that maintained normal turgidity until testing were used to assess embryo responses to mechanosensory cues. We confirmed lateral line system blocking with 4-di-2-ASP staining in each experiment (Figure 3.1A-B). To assess if gentamicin damaged hair cells in embryonic ears, as suggested previously (Bagger-Sjoback, 1997; Simmons et al., 2004; Van Trump et al., 2010; Yan et al., 1991), we compared VOR of stage-matched (within one developmental stage of each other) control and gentamicin-treated siblings, across all experiments described below (Figure 3.1C).

Hatching-response test: manual egg jiggling

To assess the MCH response of gentamicin-treated (Figure 3.1A) and control (Figure 3.1B) embryos across ontogeny, we performed a standard egg-jiggling assay (simulated attack), using a blunt probe to manually jiggle eggs in an intermittent pattern for 5 min (Jung et al., 2019; Warkentin et al., 2017). This simulated attack includes complex motion and tactile elements, and transient deformation of egg membranes. We tested 1202 individual embryos from 60 clutches (at least 5 individuals per treatment per clutch) from July 2 to August 5, 2019. We moved sibling pairs of eggs (treatment and control individuals) into their own weigh boats with a drop of water and manually jiggled them with a blunt metal probe, alternating 15 s of stimulation and 15 s of rest (i.e., stimulating eggs in a pair alternately) for 5 minutes or until the embryo hatched.

We exposed embryos to jiggling cues during three developmental periods (Figure 3.2A): (1) across the onset of vestibular function (N=544 individuals later subdivided based on VOR measurements; range, mean ± SEM age: 4.03–4.36 d, 4.19 ± 0.003 d; stage 27–28, 27.19 ± 0.017), (2) after vestibular function was well-established (N=360 individuals; 4.54–5.03 d, 4.74 ± 0.007 d; stage: 27–30, 28.99 ± 0.010), and (3) closer to spontaneous hatching (N=298 individuals, age: 5.29–5.56 d, 5.39 ± 0.003 d, stage: 29–32, 30.44 ± 0.046). For the youngest age group, we measured the VOR of all individuals that hatched (N=67 individuals) and checked for lateral line system blocking of all hatched, gentamicin-treated individuals (N=3). We used VOR measurements to determine vestibular function onset times, dividing siblings into subsets tested before and after VOR onset. For the two older groups, we measured VOR in at least one gentamicin-treated and

one control individual per clutch (N=111 individuals). We used 4-di-2-ASP staining to check for lateral line system blocking in at least one individual per gentamicin-treatment boat with hatching and visualized neuromasts in one untreated control per testing session (i.e., the period of hours over which a group of trays were tested; N=31 individuals).

For the youngest developmental period, we determined the start of testing for each sibship based on external developmental markers (Warkentin, 2017). We began testing early in stage 27, near the mean first appearance of MCH across clutches (Warkentin et al., 2017) and shortly before the onset of vestibular function (Jung et al., 2019), then stimulated pairs of siblings sequentially until VOR was visible in tested hatchlings. After analysis of VOR tests, we estimated the onset of vestibular function for each clutch as the first time point at which any individual from the clutch showed a VOR > 10°. Applying this criterion generated two groups: "no VOR" (N=286 individuals; age 4.03–4.36, 4.17 ± 0.005 d; all stage 27) and "with VOR" (N=258 individuals, age: 4.07–4.36, 4.20 ± 0.005; stage 27–28, 27.23 ± 0.027). These two groups differ significantly in age (Wilcoxon Rank Sum: z=3.8, P=0.00007). In prior work using manual egg-jiggling at stages 26–29 and measuring the VOR of every individual, the hatching response of individuals "without vestibular function" was similarly low (23%) using criteria of <1° or <10°, and it increased substantially as VOR increased to ca. 30–40° (Jung et al., 2019). Here, using the VOR of measured individuals to estimate the presence/absence of vestibular function in their unmeasured siblings allowed us to test for MCH in many more individuals than we could measure for VOR, but entails some classification errors. Applying our criterion to a prior VOR dataset (Jung et al., 2019) we estimated 14% false positives (individuals lacking VOR

but classified as “with VOR”) and 22% false negatives (individuals with VOR but classified as “no VOR”) from among the untested siblings; such classification errors would reduce the chance of detecting an effect of vestibular function on hatching in the current data.

For each individual, we recorded hatching response (Figure 3.2B) and latency (seconds from stimulus onset) or failure to hatch after 5 min of post-stimulus observation (Figure 3.2C). Proportion hatched was calculated per treatment per clutch.

Hatching-response test: vibration playbacks

To provide a motion-only stimulus, without concurrent tactile cues, we performed vibration playbacks to groups of embryos held in custom-made egg-trays (Warkentin et al., 2019; Warkentin et al., In press). Trays held up to 15 eggs (≥8, 11.79 ± 0.25 eggs per tray) in individual funnel-shaped spaces, allowing hatched tadpoles to slide through the tray to water below. We tested 1132 individuals in 96 trays from 63 clutches from July 25 to August 7, 2019 (age: 5.40–5.75, 5.56 ± 0.009 d, stage: 30–33.67, 31.27 ± 0.080). We aimed to test embryos of the same age as the 5 d individuals tested with jiggling cues, but due to logistic constraints playback embryos were, on average, 4 hours older than jiggled embryos. Embryos were treated with gentamicin or as rainwater controls at age 3 d, as described above, and moved to individual spaces in egg-trays early at embryonic age 4 d, while eggs could be easily handled without inducing hatching. Full trays were maintained

on racks over aged tap water in humidors until testing, with regular misting until age 5 d (Warkentin et al., 2019; Warkentin et al., In press).

We designed a synthetic low-frequency vibration stimulus to elicit high hatching rates, based on prior playbacks to 5 d embryos (Caldwell et al., 2009; Jung et al., 2019; Warkentin et al., 2006b; Warkentin et al., 2017; Warkentin et al., 2019; Warkentin et al., In press). We generated white noise in MATLAB and filtered it using a custom script (available upon request) to compensate for nonlinearities in the shaker transfer function and generate a frequency distribution similar to snake-attack vibrations (Caldwell et al., 2009), with high energy below 50 Hz and intensity dropping off above that (Figure 3.3A). The temporal pulse pattern included a 3-pulse 'primer' and a series of seven 10-pulse groups separated by 30 s intervals of silence (Figure 3.3B). Within each pulse group, the base temporal pattern had a cycle length of 2 s, consisting of 0.5 s pulses of vibration with roughly rectangular amplitude envelopes separated by 1.5 s intervals of silence (Figure 3.3C). To record playback vibrations, we attached a small (0.14 g) AP19 accelerometer (AP Technology International B.V., Oosterhout, The Netherlands) to an egg-tray using dental wax (Warkentin et al., In press). Accelerometer output was routed through an AP5000/10 charge-to-voltage converter to a B&K 1704 signal conditioner (Brüel & Kjær, Nærum, Denmark), digitized with a Focusrite Scarlett 2i2 external sound card (focusriteplc.com), and recorded using Raven Pro 1.3 bioacoustics software (Cornell University Laboratory of Ornithology, Ithaca, NY, USA) on a Macbook Air computer. The RMS amplitude of the playback vibrations, excluding intervals of silence, was 6.5 ms^{-2}.

Playback methods followed published work detailing vibration presentation via egg-trays (Warkentin et al., 2019; Warkentin et al., In press). For testing, we clamped egg-trays holding embryos to a custom-made interface on a rigid post attached to an electrodynamic minishaker (Model 4810; Brüel & Kjær, Nærum, Denmark). The shaker, post, and tray were horizontally leveled, with foam supports under the post and tray edge and water under the embryos. Thus, embryos were moved horizontally and hatchlings fell into the water below. Shaker output was controlled by Audacity 2.1.0 on a 2014 Macbook Air, via a custom-made amplifier (E. Hazen, Boston University Electronic Design Facility). We recorded any hatching induced by the set-up procedure (N=3 individuals), then allowed five minutes for acclimation before starting the playback (N=38 individuals hatched during acclimation). Individuals that hatched before the stimulus started were not considered part of the test. We noted if and when (to the nearest second) each embryo hatched during stimulus playback and 3 minutes of post-playback observation (Figure 3.3D-F). We then immediately (within 20 min) measured VOR of a subset of individuals, including a pair of gentamicin-treated and control individuals from 32 clutches (plus 14 clutches with unpaired data). We confirmed hatching competence of embryos remaining unhatched by manually stimulating them with a blunt metal probe (N=251 individuals). We also used 4-di-2-ASP staining to confirm lateral line blocking in at least one individual per treated tray with hatching and to visualize neuromasts in one control per testing session (N=43 treated, 5 untreated individuals). We staged 3 haphazardly selected hatchlings from each tray (Warkentin, 2017).

Lateral line system ontogeny

To determine at what point during lateral line development the hatching response to jiggling cues begins, we compared the ontogeny of the lateral line system and of MCH, using a developmental series of 79 embryos from 25 clutches from August 6–11, 2018. For each individual, we first tested for MCH, using the same manual egg-jiggling stimulus as in the first hatching response test (Warkentin et al., 2017). Test clutches were transported just prior to hatching competence and tested in an air-conditioned laboratory of the Smithsonian Tropical Research Institute in Gamboa. We recorded latency to hatch and embryos that remained unhatched after 5 min were manually decapsulated using sharp forceps. We staged embryos under a stereoscope (Warkentin, 2017). Neuromasts were then stained using 4-di-2-ASP as described above and photographed in in frontal and dorsal views. The frontal view showed neuromasts in the ventral, oral, infraorbital, nasal, and supraorbital lines (Figure 3.4A), while the dorsal view showed neuromasts in the nasal, supraorbital, middle, and dorsal lines (Figure 3.4B). All neuromasts within each line were labeled and counted using ImageJ software (Schneider et al., 2012). We took several photos in each view, in different focal planes, framing, and magnification, then counted neuromasts in each line from the photo(s) that showed them best. Counts from supraorbital and middle neuromast lines were extrapolated from one side, assuming bilateral symmetry within individuals. When lines extended across multiple photos, we used landmarks to avoid double-counting or missing neuromasts. We averaged counts per line from two independent counters and summed them across lines to estimate the total number of

neuromasts per individual (Figure 3.4C, Table 3.1). We also measured tadpole total length from dorsal images (Figure 3.4D).

Statistics

All statistical tests were carried out in the R statistical environment (version 3.6.2, R Development Core Team 2019, http://www.r-project.org) in RStudio (version 1.2.5033, RStudio Team 2019). We used generalized linear mixed models within the 'lme4' package (Bates et al., 2015) with clutch as a random effect and likelihood ratio tests to compare nested models for fixed effects and interaction effects on the number of neuromasts (error distribution: negative binomial), hatching responses (binomial), and first hatching latency (gamma). We used the fitconds function within the 'fitplc' package to plot curve fits for all hatching latencies (weibull) within trays (Duursma and Choat, 2017).

RESULTS

Lateral line system knockout with gentamicin

Our gentamicin treatment completely inactivated all neuromasts with no evidence of differences in the VOR of stage-matched gentamicin treated and non-treated siblings (χ^2_1=2.3, P=0.1302, Figure 3.1).

Hatching-response test: egg jiggling

Across the onset of VOR (mean age 4.17 vs. 4.2 d), both lateral line and vestibular function strongly and independently increased the likelihood of hatching in response to egg-jiggling (gentamicin: χ^2_1=88.2, P<2.2e-16; VOR: χ^2_1=30.4, P=3.5e-8; interaction: χ^2_1=0.7, P=0.4, Figure 3.2A-B). In embryos lacking vestibular function (mean age 4.17 d), gentamicin reduced the hatching response significantly from 10% to 1% (χ^2_1=14.4, P=0.0001, Figure 3.2B). Just after the onset of VOR, gentamicin still reduced hatching significantly (41% vs. 2%; χ^2_1=73.8, P<2.2e-16, Figure 3.2B). While both age and neuromast function increased hatching, the gentamicin effect on MCH decreased with age (age: χ^2_3=200.1, P<2.2e-16; gentamicin: χ^2_1=105.7, P<2.2e-16; interaction: χ^2_3=19.9, P=0.0002; Figure 3.2B). At 4.7 d, gentamicin still significantly reduced hatching (70% vs. 41%; χ^2_1=35.4, P=2.7e-09) but at 5.4 d, close to spontaneous hatching, all jiggled eggs hatched, with or without functional neuromasts (Figure 3.2B).

Latency to hatch decreased with age and lateral line function (age: χ^2_3=79.2, P<2.2e-16; gentamicin: χ^2_1=36.9, P=1.266e-09; interaction: χ^2_3=3.8, P=0.29; Figure 3.2C). The low hatching response of gentamicin-treated embryos before and just after the onset of VOR limited our sample of latency, reducing statistical power for comparisons (Figure 3.2C). However, gentamicin treatment increased latency to hatch at both 4.7 d (χ^2_1=17.1, P=3.5e-05) and 5.4 d (χ^2_1=21.2, P=4.1e-06) with no indication that the effect decreased with age (interaction: χ^2_1=2.84, P=0.09; Figure 3.2C).

Hatching-response test: vibration playbacks

To assess if egg motion alone can stimulate the lateral line system, we used vibration playbacks to shake gentamicin-treated and untreated embryos held in custom-made egg trays. In these playbacks, lateral line function had no effect on the proportion of embryos hatched (χ^2_1=1.8, P=0.1787, Figure 3D) or the timing of hatching (χ^2_1=1.1, P=0.2986, Figure 3.3E-F).

Lateral line system ontogeny

The number of neuromasts in all seven lines increased with developmental stage (Figure 3.4C, Table 3.1). Total number of neuromasts increased with size (Figure 3.4D), age (Figure 3.4E) and, most strongly, developmental stage (Figure 3.4F). From stage 27 to 29, the number of neuromasts more than doubled (Figure 3.4F). Both developmental stage (χ^2_1=50.9, P=9.5e-13) and the total number of neuromasts (χ^2_1=15.7, P=7.5e-5) were significant predictors of hatching in egg-jiggling experiments (Figure 3.4G). When embryos had fewer than a threshold number of neuromasts (247), none hatched, whereas embryos with more neuromasts often hatched.

DISCUSSION

We demonstrate that the developing lateral line and vestibular systems both contribute to escape-hatching of red-eyed treefrog embryos, and that the roles of these sensors change during development and vary with disturbance cue type.

Lateral line system knockout with gentamicin

Using gentamicin, we achieved complete temporary lateral line ablation in *A. callidryas* embryos with no evidence of vestibular system damage (Figure 3.1). Others found evidence that gentamicin can cause vestibular system damage when administered intramuscularly (Bagger-Sjoback, 1997; Yan et al., 1991) or by direct immersion post-hatching (Simmons et al., 2004; Song et al., 1995; Van Trump et al., 2010). Our study is the first to investigate the effects of gentamicin administered incrementally to embryos developing *in ovo*, which was necessary to avoid water loss from eggs and, later, test for hatching responses. The gradual passage of gentamicin across the vitelline membrane into the perivitelline space while eggs were in the treatment bath, and the potential loss of gentamicin from older eggs maintained in egg-trays before vibration playbacks, means we do not know the precise concentrations embryos were exposed to over time. However, our longest exposure durations and peak exposure concentrations exceeded those in previous studies (Simmons et al., 2004; Song et al., 1995; Van Trump et al., 2010), supporting that for some animals under some exposure conditions gentamicin can selectively damage hair cells in the lateral line without impairing the function of hair cells in the vestibular system. Lateral line neuromasts are directly exposed to the fluid bathing an embryo, but hair cells of the inner ear are not, and the barriers protecting these internal cells likely change with development.

Notably, we found no evidence for ear damage in early stages, at the onset of vestibular function, and also in more developed 5 day old embryos. Immature and recently regenerated hair cells are resistant to aminoglycoside antibiotics (Dai et al., 2006; Hashino

and Salvi, 1996; Murakami et al., 2003; Van Trump et al., 2010). Thus, in the youngest tested embryos, vestibular hair cells may have had only brief gentamicin exposure after maturing to a point of vulnerability. In the lateral line system, neuromasts began to regain fluorescence, when stained with 4-di-2-ASP, within hours of hatching and cessation of gentamicin exposure. We suspect this was due to maturation of gentamicin-resistant developing hair cells. Embryos tested at 5 days would have had functional hair cells in their ears for over 24 h (29.8 h from mean onset of VOR to mean testing age) under gentamicin treatment, yet we also found no evidence for vestibular damage in these older embryos. This suggests that, compared to more mature tadpoles (Bagger-Sjoback, 1997; Simmons et al., 2004; Van Trump et al., 2010; Yan et al., 1991), embryonic anatomy may better protect otic hair cells against externally administered gentamicin.

Hatching-response test: manual egg jiggling

In embryos lacking vestibular function, a significant effect of gentamicin treatment on hatching response reveals that the onset of lateral line function precedes the onset of vestibular function and plays a key role in very early MCH (Figure 3.2B). Immediately following the onset of VOR, the lateral line system continues to contribute strongly to risk assessment and hatching (Figure 3.2B). The overall higher hatching rate in VOR-positive animals, across both treated and control individuals (Figure 3.2B), is consistent with a key role of otoconial organs in embryonic vibration sensing (Jung et al., 2019). However, at the onset of MCH neither lateral line nor vestibular function alone enabled a strong response; the multimodal combination of input from both senses greatly increased the

likelihood of hatching in a simulated attack (Figure 3.2B).

Soon after *A. callidryas* gain hatching competence, the lateral line system plays a critical role in sensing and responding to predator cues. We tested the effect of gentamicin on jiggling-induced hatching in two later periods to determine if the dependence of MCH on lateral line function changes developmentally. In *A. callidryas*, embryos hatch spontaneously from 5–7 d, while younger embryos almost never hatch if undisturbed (Güell and Warkentin, 2018; Hite et al., 2018; Warkentin, 2000; Warkentin et al., 2001). From age 4 to 5 d, embryos become more likely to escape during snake and wasp attacks (Gomez-Mestre and Warkentin, 2007; Warkentin, 1995; Warkentin, 2000) and to hatch in vibration playbacks (Jung et al., 2019; Warkentin et al., 2019; Warkentin et al., In press). Over this same developmental period, our results show that MCH responses become less dependent on multimodal input from the lateral line plus vestibular system. This might reflect increasing strength of vestibular input as embryo ears develop (Jung et al., 2018).

When animals use multimodal sensory integration to inform behavior, a sensor may contribute to a response without being required for its occurrence (Angelaki and Cullen, 2008). We examined latency from stimulus onset to hatching response (Figure 3.2C) as a potentially more sensitive indicator of lateral line system contributions to embryo decisions, since latency affects escape success during predator attacks (Almanzar and Warkentin, 2018; Chaiyasarikul and Warkentin, 2017). We found that even embryos near the stage of spontaneous hatching, when they have a very strong vestibular-mediated hatching response, still use lateral line input to accelerate their response to simulated attack cues (Figure 3.2C).

Sensory integration of lateral line and inner ear inputs

Across development, embryos use both lateral line and inner ear mechanoreceptors to mediate their hatching response. Moreover, input from these two mechanosensory systems is non-redundant, motivating consideration of possible neural integration mechanisms. At least initially, the lateral line circuit and the vestibular circuit operate on parallel but separate tracks (Fay and Edds-Walton, 2008; McCormick, 1999). There are reports of both sensory systems sending fibers from their peripheral receptors to common first-order nuclei, but the convergence is minor compared to their distinct termination areas in the midbrain (McCormick et al., 2016). One shared synaptic target in the hindbrain is the Mauthner (M-) cell, a paired sensorimotor structure that receives input from the inner ear (Szabo et al., 2007) and the mechanosensory lateral line (Mirjany et al., 2011) and sends output to relay and motoneurons that activate axial musculature (Eaton et al., 2001). These cells mediate fast C-start escape responses in fishes and amphibians (Eaton et al., 2001; Korn and Faber, 1975; Korn and Faber, 2005; Korn et al., 1974), and have been suggested to play a role in hatching to escape from egg predators in zebrafish embryos (Eaton and Nissanov, 1985). However, hatching in fishes and amphibians requires enzymatic digestion of the egg envelope as a first step, with muscular activity secondarily playing a role in final membrane rupture and exit from the egg (Cohen et al., 2019; Korwin-Kossakowski, 2012). In the hatching process of *A. callidryas*, enzyme release and membrane rupture typically occur before the strong S-shaped contractions of axial musculature (swimming motions) that propel the embryo from its capsule (Cohen et al., 2019). Moreover, M-cells mediate very fast responses, on the order of milliseconds (Eaton and Nissanov, 1985), while

mechanosensory-cued hatching in *A. callidryas* occurs many seconds to several minutes after the onset of stimulation (Warkentin et al., 2007; Warkentin et al., 2017), suggesting that embryos integrate information over time before deciding to hatch. Indeed, embryos propensity to hatch increases even during periods of vibrational silence following certain vibration patterns (Jung et al. in prep). Thus, it seems likely that hatching decisions are made, and input from sensory modalities may be integrated, at higher levels in the brain (McCormick et al., 2016). It would be particularly interesting to identify the mechanism controlling the rapid temporally and spatially regulated release of hatching enzyme (Cohen et al., 2019; Salazar-Nicholls et al., 2020) and link it via neural integration mechanisms to the sensory inputs that trigger the hatching process.

Hatching-response test: vibration playbacks

The fact that lateral line function had no effect on the proportion of embryos hatching (Figure 3.3D) or their latency to hatch (Figure 3.3E) in response to motion-only vibration playbacks suggests that only a subset of more complex physical disturbances stimulate the lateral line system, while potentially any egg motion may stimulate the vestibular system. Thus, some embryos in attacked egg clutches receive unimodal vestibular stimulation while others receive multimodal vestibular and lateral line system input. Some may also receive input from cutaneous touch receptors (Blaxter, 1987; O'Brien et al., 2012). Given the role of lateral line system input in reducing hatching latency, as well as increasing hatching likelihood in younger embryos, this variation in the type(s) of sensory input an embryo receives under different predation contexts likely

contributes to the variation in the likelihood and timing of hatching during attacks (Almanzar and Warkentin, 2018; Warkentin, 2000; Warkentin et al., 2006a). The variation in received mechanosensory stimuli within a clutch may also be a mechanism contributing to threat-sensitive embryo behavior (Ferrari and Chivers, 2009; Hughey et al., 2015; Mathis et al., 2008; Van Buskirk, 2016); since predators must touch eggs to eat them, the risk of mortality during attacks is likely higher for eggs receiving multimodal cues than for those receiving motion cues alone.

Lateral line system ontogeny

In a recent study, we found hatching in response to egg-jiggling to begin, on average, at stage 27.3 and considered hatching "consistent" at stage 28.8 (Figure 3.4C), the second time both of two tested siblings hatched (Jung et al., 2019; Warkentin et al., 2017). This known onset of MCH aligns ontogenetically with a rapid increase of neuromast number in developing embryos (Figure 3.4C). The pattern of a neuromast-number threshold for hatching (Figure 3.4G) suggests that lateral line system development may limit the onset of disturbance cue sensing and associated hatching behavior.

Lateral line morphology in comparative context

The number of neuromasts at hatching and their proliferation after hatching varies greatly across taxa. Some fishes hatch with only two functional neuromasts, and early larvae simply float in the water column [e.g. flounder (Kawamura and Ishida, 1985), tuna (Kawamura et al., 2003), grouper (Mukai et al., 2006), catfish (Mukai et al., 2010)].

Subsequent rapid lateral line system development is correlated with behavioral changes (avoiding obstacles, feeding, migrating, settling, and surviving seasonal floods) and parallels rapid development of other sense organs such as eyes, ears, taste buds, olfactory epithelium (Kawamura and Ishida, 1985; Kawamura et al., 2003; Mukai et al., 2006; Mukai et al., 2010). Neuromast proliferation can also occur slowly. For instance, cod larvae hatch with five lateral body neuromasts, and only add one more by feeding onset, 2–3 weeks later (Blaxter, 1984). The developmental stage at which neuromast function begins differs among species, and appears related to hatchlings' habitat and habits (Otsuka and Nagai, 1997). For instance, ayu hatch with 20 pairs of well-developed neuromasts and migrate downstream immediately upon hatching (Kawamura et al., 1983). In contrast, pale chub hatch without a single neuromast and remain in the spawning bed for 4 d, during which neuromasts develop rapidly; at emergence into the river, larvae are responsive to water flow and have nearly caught up to ayu in neuromast number (Kawamura et al., 1983). Across species, developmental increases in the number of neuromasts are closely linked to the ontogeny of mechanosensory-guided behavior (Blaxter and Fuiman, 1989; Kawamura and Ishida, 1985; Llanos-Rivera et al., 2014). However, neuromast size, shape, and hair cell polarity can also affect mechanosensory sensory function (Becker et al., 2016; Webb and Shirey, 2003).

Anatomically and physiologically, amphibian neuromasts resemble superficial neuromasts of teleost fishes (Metcalfe, 1985; Simmons et al., 2004). However, we know relatively little about their early development, except in a few species. At hatching, the salamander *Ambystoma mexicanum* has all 60 neuromasts, while in the frog *Lithobates*

(Rana) pipiens most neuromasts have not yet formed; however, by the onset of feeding, the lateral line appears fully formed and functional in both species (Smith et al., 1988). Among amphibians, early lateral line system development has been characterized in detail in *Xenopus laevis* (Roberts et al., 2009; Shelton, 1970; Simmons et al., 2004; Winklbauer, 1989), but studies rarely distinguish hatching timing. Reported hatching stage varies such that *X. laevis* have between 0 (Carroll and Hedrick, 1974) and 14 neuromasts at hatching, but embryos with just 6–8 neuromasts swim into water currents and responsiveness increases over the next 10 h with an increase in neuromast number (Roberts et al., 2009). In our study system, the hatching response of *A. callidryas* embryos to disturbance begins only when they have hundreds of neuromasts (Figure 3.4G). Despite the large increase in neuromast number, there appears to be little change in neuromast size (personal observation from confocal images by María José Salazar Nicholls and Julie Jung) or shape (Cohen et al., 2019) from 3 to 5 days post oviposition. It is not yet clear how physical disturbance of eggs stimulates the neuromasts of embryos inside them; however, induced perturbations of perivitelline fluid motion within the egg capsule may provide a weaker or less clear stimulus to the lateral line system than currents in open water do after hatching. Additionally, the cost of unnecessarily swimming into a current may be lower than that of hatching prematurely.

The number of neuromasts *A. callidryas* have at hatching is high, even compared to other anurans in late larval development (Lannoo, 1987). *A. callidryas* that hatched in response to egg-jiggling, at 7.7–11.0 mm total length, had neuromast counts of 323.6 ± 11.9 (range 247–434). For comparison, in a study of 36 other anuran species examined in

late larval development, at their expected peak of neuromast numbers (Lannoo, 1987; Winklbauer, 1989), the most neuromasts reported was 332 in *Rana aurora*, at 25–27.5 mm snout-vent length (Lannoo, 1987). Since lateral line development depends on the size of the animal, and tadpoles tend to add neuromasts during post-hatching development (Fabrezi et al., 2012; Winklbauer, 1989), these other species presumably have fewer neuromasts at hatching. This suggests that *A. callidryas* have precocious lateral line development, as well as high neuromast numbers. However, we know nothing about lateral line ontogeny in other anurans with documented or suspected mechanosensory-cued escape-hatching behavior (Brown and Iskandar, 2000; Brown et al., 2010; Buckley et al., 2005; Chivers et al., 2001; Gomez-Mestre et al., 2008; Poo and Bickford, 2014; Sih and Moore, 1993; Smith and Fortune, 2009; Touchon et al., 2011). Egg predation might be a selective force favoring earlier or greater development of mechanosensory systems. A comparative analysis of lateral line and vestibular system development in relation to the distribution of MCH in anurans (Warkentin, 2011a; Warkentin, 2011b) would be informative. Assessing links between sensory morphology and embryo behavior could reveal patterns of functional variation and adaptive evolution and provide further insight into how embryos use sensory information.

Embryos across diverse taxa respond to physical disturbance cues from many ecologically relevant sources (Warkentin, 2011a; Warkentin, 2011b; Warkentin et al., In press). In contexts such as antipredator defense (Doody and Paull, 2013; Warkentin, 2005) and sibling hatching synchronization (Endo et al., 2019; Noguera and Velando, 2019), embryos' ability to sense these cues is often essential for their survival, yet we know very

little about the sensory systems that mediate MCH or even, in many cases, which elements of physical disturbance cues are most relevant. Herein, we show that to detect egg-disturbance cues *A. callidryas* embryos use their vestibular system, a sensory mechanism likely to be relevant across vertebrates, and their lateral line system, a mechanism which may contribute to MCH in other frogs and fishes. Our study serves as proof-of-concept for the feasibility of combining neuromast ablation *in ovo* with hatching-response tests, and it highlights the value of latency as a sensitive response variable in the context of multimodal mechanosensing. The methods we present open opportunities for comparative research into embryo sensory ecology with other species that hatch in response to mechanosensory cues. Our findings provide insight into the functional roles of two different mechanosensory systems prior to hatching and reveal new possibilities for exploring embryonic sensory perception across taxa.

TABLE AND FIGURE LEGENDS

Figure 3.1 – Gentamicin ablates lateral line system function in *Agalychnis callidryas* embryos, with no effect on vestibular system. (A) A representative gentamicin-treated individual, showing complete ablation of functional neuromasts, and (B) an age- and stage-matched control sibling, showing functional neuromasts (orange). (C) Vestibulo-ocular reflex (VOR) amplitude of gentamicin-treated and control *A. callidryas*. Gray lines connect points representing stage-matched siblings, in the same or adjacent developmental stages, across treatments. ns, not significant ($P > 0.05$).

Figure 3.2 – Ontogeny of the hatching response to egg jiggling by gentamicin-treated and control *A. callidryas* embryos, across and after the onset of vestibular function. Vestibular function onset was indicated by the VOR. (A) Developmental timeline of testing periods, with mean ages (points) and ranges (rectangles) color coded. (B) Hatching response. (C) Hatching latency. Data points are clutch-means and boxplots show means, quantiles, and 1.5x the interquartile range (IQR) across clutches, within treatment × age category, ns, $P>0.05$; *** $P<0.001$.

Figure 3.3 – Hatching response to motion-only vibration playback by gentamicin-treated and control *A. callidryas* embryos. Embryos were tested at a mean age of 5.56 d, stage 31.3. (A) Frequency spectrum from recording of playback stimulus. (B) Waveform of entire stimulus. (C) Waveform of a single pulse group as indicated by the red box in B,

showing the base temporal pattern (0.5 s duration, 1.5 s interval). (D) Proportion of embryos hatched and (E) latency for the first individual to hatch after stimulus began. Points are values per tray, boxplots show means, quantiles, and 1.5xIQR across trays. (F) All individual hatching latencies (points) in playbacks, with Weibull curve fits and 95% confidence interval (CI; shading). Vertical solid lines indicate the mean latency by which 50% of individuals within treatments had hatched (estimated from bootstrap); dashed lines indicate the 95% CI. ns, P>0.01.

Figure 3.4 – Lateral line ontogeny and development of the hatching response to egg-jiggling of *A. callidryas* embryos. Representative (A) frontal and (B) dorsal views of hatchlings stained with 4-di-2-ASP to show neuromasts, with lines color-labeled. (C) Mean (± SE) number of neuromasts in each line (bilaterally) across development. Inset shows previously reported (Jung et al., 2019; Warkentin et al., 2017) stages when mechanosensory-cued hatching (MCH) is first and consistently expressed; diamonds represent means and 95% confidence intervals, box and whiskers show IQR and extent of data to ± 1.5×IQR. Total number of neuromasts increased with (D) total length (χ^2=5.2, df=1, P=0.02319), (E) age (χ^2=62.4, df=1, P<2.9e-15), and (F) developmental stage (χ^2=6263.8, df=1, P<2.2e-16; linear regression and 95% CI indicated). (G) The hatching response increased with neuromast number; the predicted curve fit (dashed line) and 95% CI (shading) from a binomial generalized linear mixed model are indicated. For F and G, categorical variables are jittered to show data points and stages are color coded. Dotted lines in D, E, and G show the threshold number of neuromasts at which hatching began.

Table 3.1 – Lateral line system ontogeny. Mean (±SE) number of neuromasts in each line (bilaterally) of the lateral line system in *A. callidryas* embryos across the onset of mechanosensory-cued hatching.

Figure 3.1

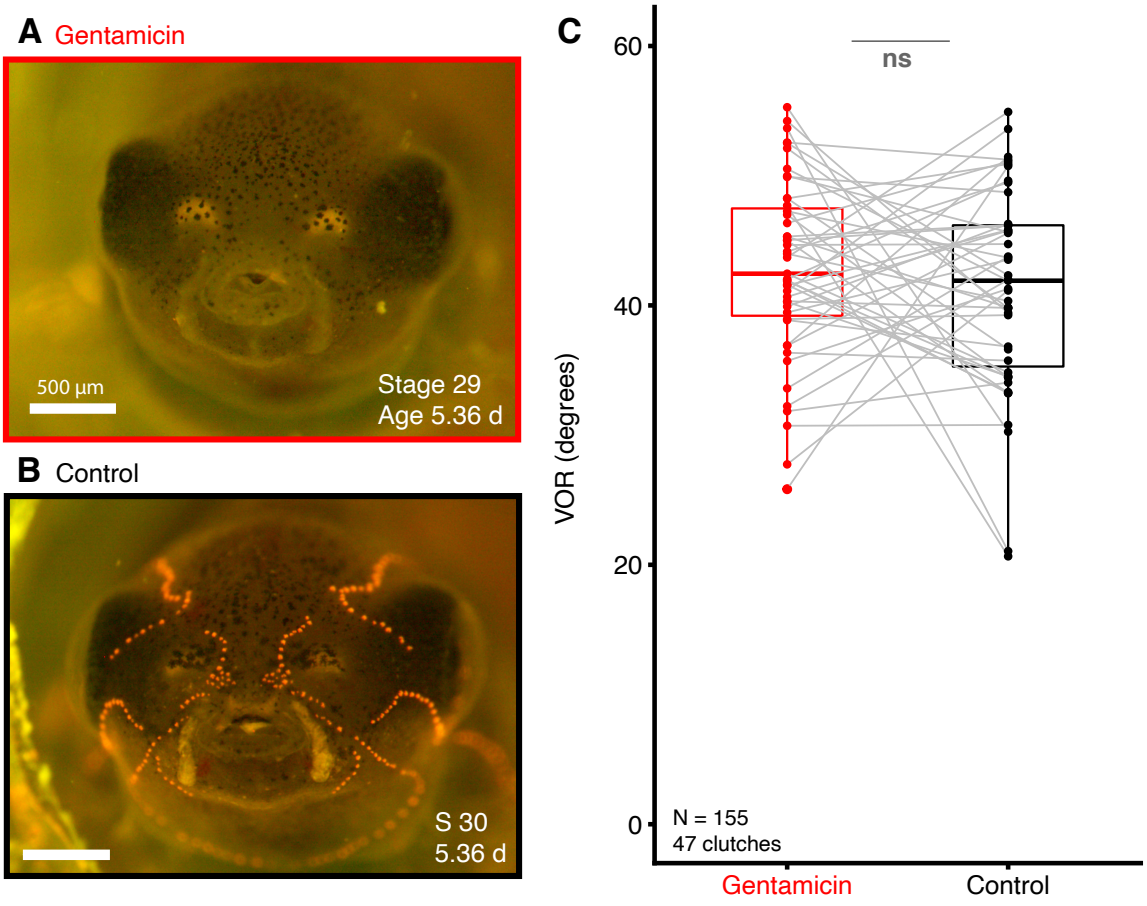

A Gentamicin
500 µm
Stage 29
Age 5.36 d
B Control
S 30
5.36 d
C
60
40
20
0
VOR (degrees)
ns
N = 155
47 clutches
Gentamicin
Control

Figure 3.2

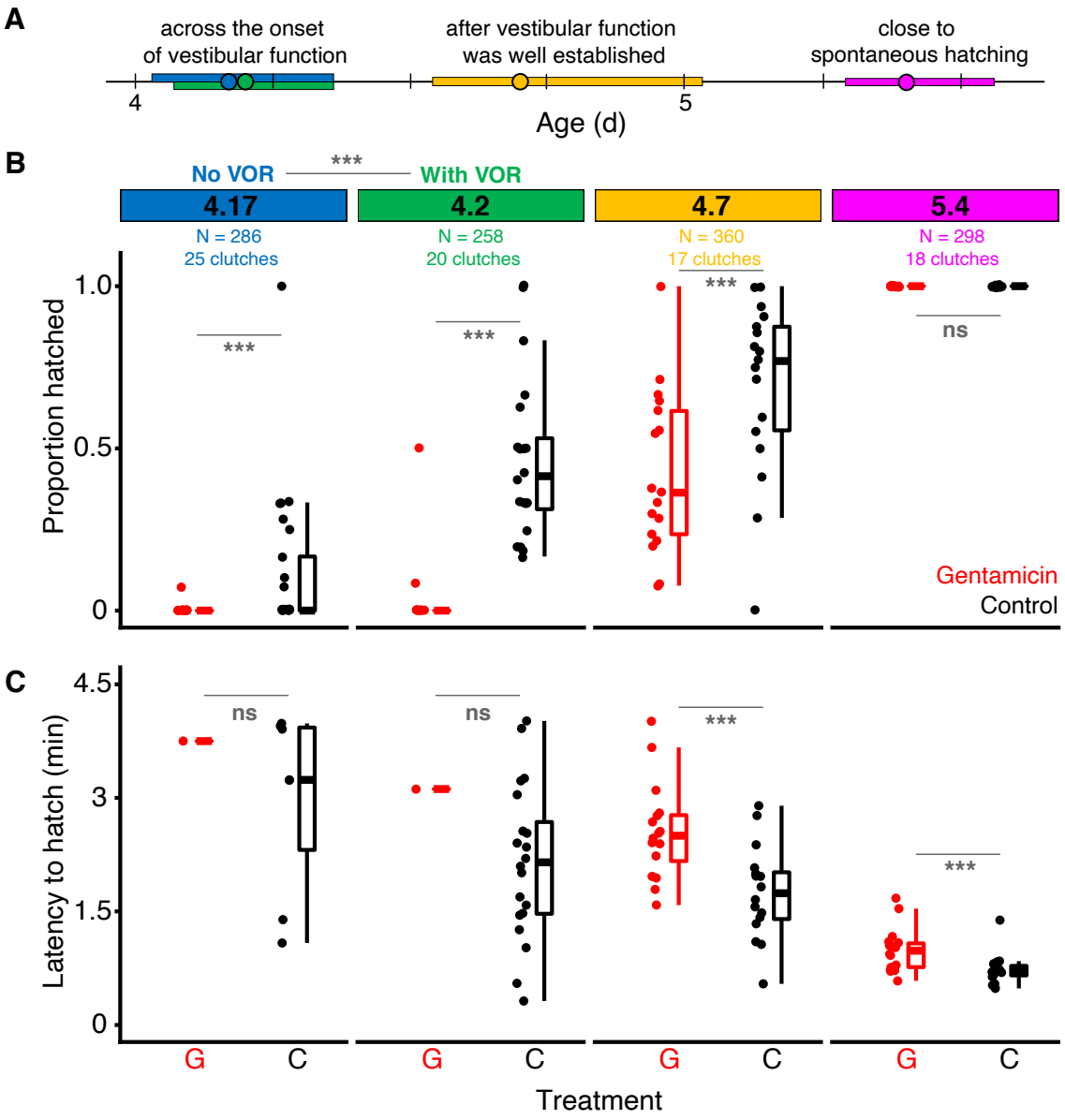

A
across the onset
of vestibular function
after vestibular function
was well established
close to
spontaneous hatching
4
5
Age (d)
B
No VOR
***
With VOR
4.17
4.2
4.7
5.4
N = 286
25 clutches
N = 258
20 clutches
N = 360
17 clutches
N = 298
18 clutches
***
***
***
ns
Proportion hatched
1.0
0.5
0
Gentamicin
Control
C
Latency to hatch (min)
4.5
3
1.5
0
ns
ns
***
***
G
C
G
C
G
C
G
C
Treatment

Figure 3.3

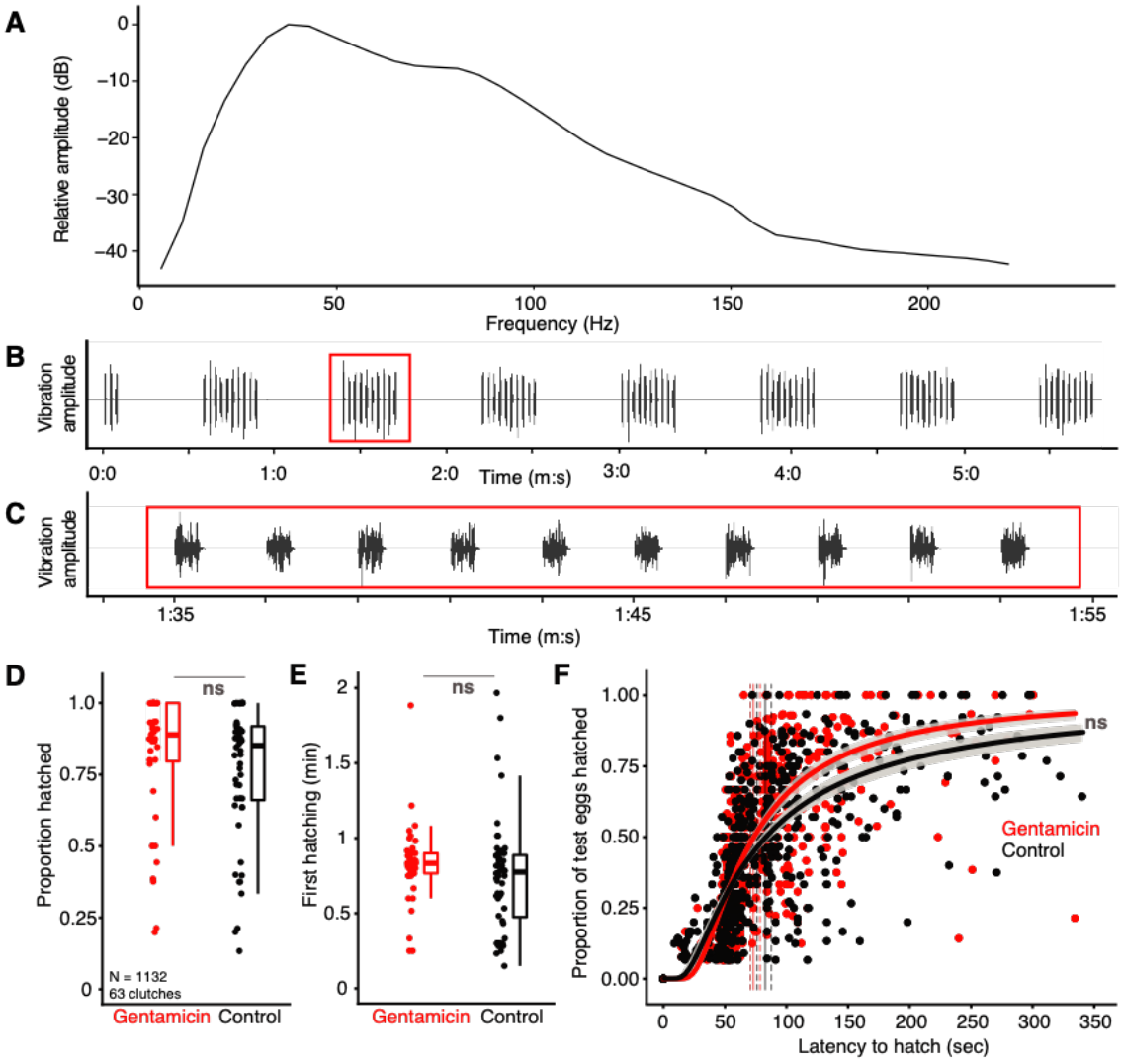

A
Relative amplitude (dB)
0
−10
−20
−30
−40
0
50
100
150
200
Frequency (Hz)
B
Vibration amplitude
0:0
1:0
2:0
Time (m:s)
3:0
4:0
5:0
C
Vibration amplitude
1:35
1:45
1:55
Time (m:s)
D
Proportion hatched
1.0
0.75
0.5
0.25
0
ns
N = 1132
63 clutches
Gentamicin
Control
E
First hatching (min)
2
1.5
1
0.5
0
ns
Gentamicin
Control
F
Proportion of test eggs hatched
1.00
0.75
0.50
0.25
0.00
ns
Gentamicin
Control
0
50
100
150
200
250
300
350
Latency to hatch (sec)

Figure 3.4

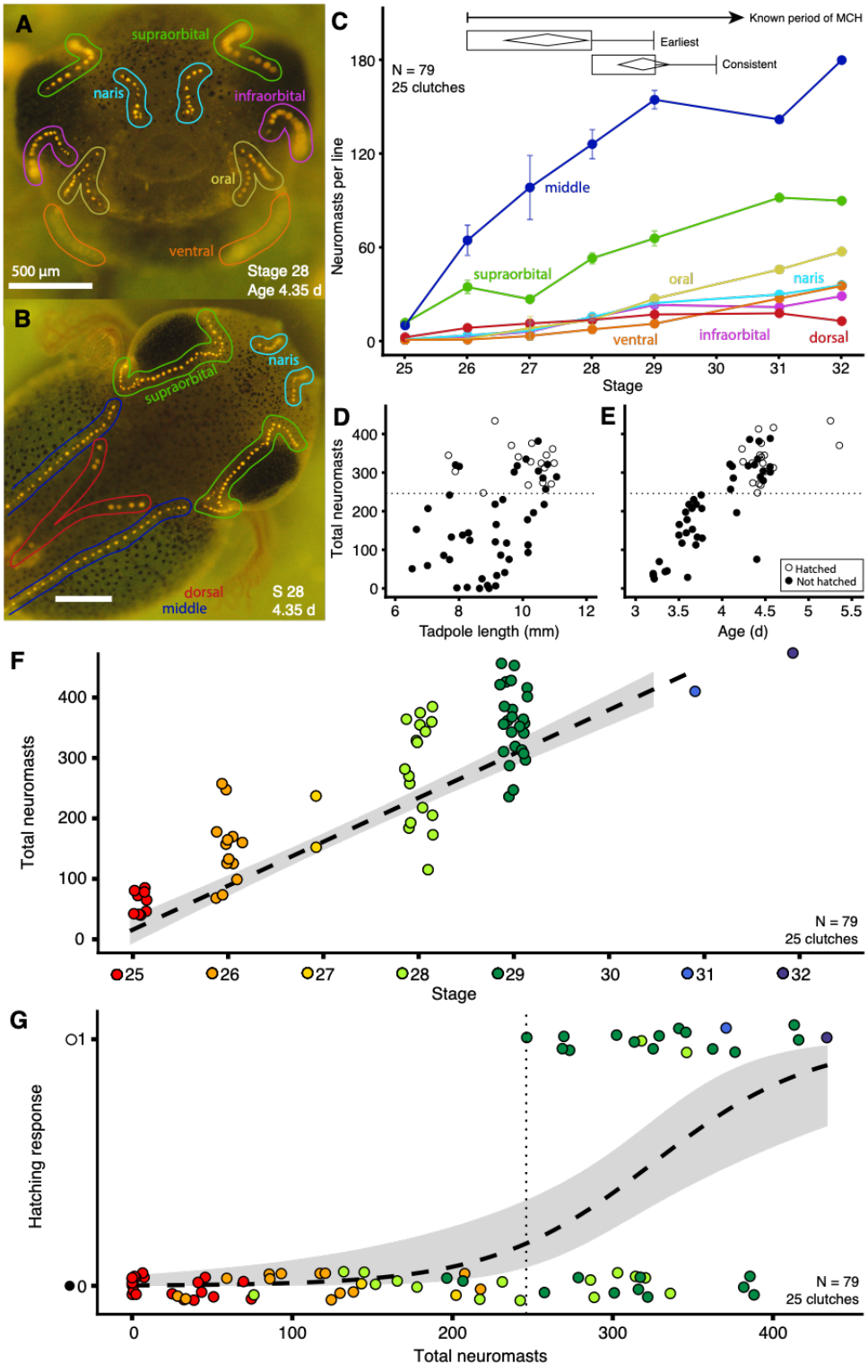

A
supraorbital
naris
infraorbital
oral
ventral
500 µm
Stage 28
Age 4.35 d
B
naris
supraorbital
dorsal
middle
S 28
4.35 d
C
Known period of MCH
Earliest
Consistent
N = 79
25 clutches
Neuromasts per line
middle
supraorbital
oral
naris
ventral
infraorbital
dorsal
Stage
D
Total neuromasts
Tadpole length (mm)
E
Hatched
Not hatched
Age (d)
F
Total neuromasts
N = 79
25 clutches
Stage
G
Hatching response
N = 79
25 clutches
Total neuromasts

Table 3.1

Stage	*n*	*Infraorbital*	*Supraorbital**	*Naris*	*Oral*	*Ventral*	*Middle**	*Dorsal*	*Bilateral Total*
25	20	0.05	10.90	0.33	0.00	0.03	9.20	1.62	22.12 ± 5.7
26	13	2.08	33.81	2.85	0.65	0.12	63.69	7.65	110.85 ± 15.9
27	2	5.25	26.00	5.75	7.50	2.50	97.50	10.50	155.00 ± 42.5
28	17	14.24	52.24	14.76	12.74	6.71	125.24	12.76	238.68 ± 20.4
29	25	22.36	64.96	23.52	26.24	10.30	153.64	16.24	317.26 ± 11.8
31	1	21.00	91.00	29.00	45.00	26.50	141.00	17.00	370.50
32	1	28.00	89.00	35.00	56.50	34.50	179.00	12.00	434.00

* one side counted, values presented assume symmetry

CHAPTER 4. FROG EMBRYOS USE MULTIPLE LEVELS OF TEMPORAL PATTERN IN RISK ASSESSMENT FOR VIBRATION-CUED ESCAPE HATCHING

ABSTRACT

Stereotyped signals can be a fast, effective means of communicating danger, but animals assessing predation risk must often use more variable incidental cues. *Agalychnis callidryas* embryos hatch prematurely to escape from egg predators, cued by vibrations in attacks, but benign rain generates vibrations with overlapping properties. Facing high false alarm costs, embryos use multiple vibration properties to inform hatching, including temporal pattern elements such as pulse durations and inter-pulse intervals. However, measures of snake and rain vibration as simple pulse-interval patterns are a poor match to embryo behavior. We used vibration playbacks to assess if embryos use a second level of temporal pattern, long gaps within a rhythmic pattern, as indicators of risks. Long vibration-free periods are common during snake attacks but absent from hard rain. Long gaps after a few initial vibrations increase the hatching response to a subsequent vibration series. Moreover, vibration patterns as short as three pulses, separated by long periods of silence, can induce as much hatching as rhythmic pulse series with five times more vibration. Embryos can retain information that increases hatching over at least 45 s of silence. This work highlights that embryo behavior is contextually modulated in complex ways. Identical vibration pulses, pulse groups, and periods of silence can be treated as risk cues in some contexts and not in others. Embryos employ a multi-faceted decision-making process to effectively distinguish between risk cues and benign stimuli.

INTRODUCTION

The importance of temporal pattern processing

Recent years have seen growing interest in understanding how receivers extract, interpret, and integrate different types of environmental information to make behavioral decisions (Hebets and Papaj, 2005; Higham and Hebets, 2013). Critical to the analysis of environmental information is the ability to perceive and process changes in stimulus intensity. Many signals and cues are characterized by rapid changes in intensity over time. The temporal structure of signals is well-documented to be important in foraging and communication contexts of diverse animal taxa (Buck and Buck, 1968; Edwards et al., 2005; Emlen, 1972; Ghazanfar et al., 2001a; Kay, 1982; Langner, 1992; Liégeois-Chauvel et al., 1999; Loftus-Hills and Littlejohn, 1971; Pollack, 2001; Remez et al., 1981; Siniscalchi et al., 2012; Straughan, 1975). For instance, temporal-pattern perception and processing have been studied extensively in echolocating bats, which hunt flying insect prey by emitting high-frequency sounds and analyzing the returning echoes (Covey and Casseday, 1999; Melendez et al., 2006). They have also been a strong focus in research on communicating frogs and insects, which use the temporal properties of songs and calls to identify and select mates (Gerhardt, 1991; Pollack, 2000; Tunstall and Pollack, 2005).

Anurans are particularly well suited for investigating questions of how temporal information is perceived and processed, since acoustic signals, which are inherently intermittent or amplitude-modulated over time, play an important role in their reproductive biology, enabling females to identify and choose between conspecific males (Gerhardt,

1982; Gerhardt, 1991; Gerhardt, 2001; Gerhardt and Schul, 1999). In many anuran species different call types, such as advertisement and aggressive calls, are spectrally identical (i.e., contain the same component carrier frequencies), but differ significantly in how signal amplitude is modulated over time (Brenowitz and Rose, 1994; Burmeister et al., 1999; Gerhardt, 1982; Loftus-Hills and Littlejohn, 1971; Rose and Brenowitz, 1997; Straughan, 1975); and many anurans actively discriminate between call types that differ only in temporal structure (Diekamp and Gerhardt, 1995; Gerhardt, 2001; Rose et al., 1988; Ryan, 1983).

Most research on temporal pattern processing to date has focused on complex signals in the context of communication. Evolved signals, such as acoustic alarm or advertisement calls or aposematic visual patterns, represent traits that have been selected for their function in communication, specifically their effect on receivers. In contrast, cues, such as the visual looming rate of an approaching animal or the sound of rain, are sensory stimuli that are produced incidentally by biotic and abiotic sources; they have not been selected for their effect on receivers. Nonetheless, cues provide information that animals use to make behavioral decisions; this can enhance fitness and is sometimes critical for survival (Maynard-Smith and Harper, 2003). Receivers are, therefore, under selection to detect and assess cues as well as signals, and both can be studied by examining their effects on the behavior of receivers (Hasson, 1994; Hasson, 1997; Smith and Harper, 1995). However, we know much less about how animals process information from cues to make decisions, compared to the better-studied context of communication signals.

Many acoustically communicating animals attend to multiple levels of temporal

patterns in signals. For instance, they may assess overall call repetition rate as well as the finer-scale pattern of pulse repetition rate within calls (Gerhardt et al., 2007; Ryan, 1983). Such discretely multi-level temporal structure is often clear and highly salient in signals (Burmeister et al., 1999; Gerhardt and Schul, 1999; von Helversen, 1993) but may be less obvious in incidental cues, which can present relatively broad and continuous distributions of temporal property values (Warkentin, 2005; Warkentin et al., 2006b). This does not mean that complexities of temporal structure are irrelevant to receivers assessing cues, but it can create challenges for studying them.

For instance, an animal assessing risk based on an amplitude-modulated incidental cue (e.g. sound or vibration) may experience periods when cue amplitude exceeds its detection threshold and periods when it does not, generating a temporal pattern of perceived events and intervals (Figure. 4.1). An investigator recording the cue, however, might perceive shorter or longer events, if their recording equipment had a higher or lower threshold (e.g. noise floor). Moreover, the animal might temporally group the events it perceives, parsing sub-threshold intervals into "within-series" and "between-series" categories (as in inter-pulse and inter-call intervals). However, to an investigator perceiving a continuous distribution of intervals, this distinction may be unclear.

Although potentially challenging to study, such complex temporal patterns in incidental cues might be important sources of information for receivers. As a starting point to assess this, we posit that the behavior and biomechanics of foraging might generate multi-level temporal patterns in the resulting incidental vibrations that other animals, such as predators or prey, could exploit to distinguish them from abiotic background noise

(Appel and Cocroft, 2014; Meyhöfer et al., 1994). We use vibration-cued hatching as a test case to determine whether prey assessing risk can use multiple levels of temporal pattern, a type of information processing that is common for signals but largely unexamined for incidental cues.

Incidental vibrations as hatching cues

Hatching is an important event in animal lives that marks the transition between two distinct life stages, exposed to different environments associated with different risks and opportunities. Across taxa, many embryos adjust their timing of hatching in response to environmental cues (Warkentin, 2011b). Vibration-cued hatching is well-documented in a diverse array of vertebrates and invertebrates, presenting opportunities to use playback experiments to examine their information-assessment processes (reviewed in Warkentin, Jung & McDaniel, in press).

We worked with embryos of the red-eyed treefrog, *Agalychnis callidryas,* a tractable and well-studied species for investigating risk assessment based on incidental cues. These embryos hatch prematurely to escape the threat of predation by egg-eating snakes, cued by the vibrations of their egg clutches during attacks (Warkentin, 1995; Warkentin, 2005). However, snakes are not the only force that shakes these jiggly, gelatinous egg clutches as they hang from plants over rainforest ponds. Tropical rainstorms generate intense egg clutch vibrations with amplitudes, frequencies, and temporal properties that overlap those that occur in predator attacks, but they pose no threat to eggs and do not induce premature hatching (Warkentin, 2005). Thus, these embryos must solve

a discrimination challenge when making the decision to hatch in response to a physical disturbance cue.

To distinguish threats from benign disturbances and make appropriate decisions about whether to hatch in response, *A. callidryas* embryos extract and combine information from multiple vibration properties (Warkentin and Caldwell, 2009). Playbacks of vibrations recorded from egg clutches during snake attacks can elicit hatching, with no additional predator cues (Warkentin, 2005), and so can simple synthetic stimuli constructed from bouts of white noise (Caldwell et al., 2009; Caldwell et al., 2010; Jung et al., 2019; Jung et al., 2020; Warkentin et al., 2006b). These embryos use information from frequency spectra to modulate hatching, hatching most in response to vibrations dominated by low frequencies and less as vibration frequency increases (Caldwell et al., 2009). The frequency sensitivity of hatching matches well with the spectra of vibrations in predator attacks, which are dominated by low frequencies; it contrasts with rainstorm spectra, which contain both low, snake-like frequencies and a broader range of high frequencies (Caldwell et al., 2009). These high frequencies, absent from predator vibrations, are relatively clear indicators of low risk and their presence decreases hatching responses (Caldwell et al., 2009). However, since low frequencies present in snake attacks overlap with low frequencies present in rainstorms, they are a fundamentally ambiguous cue. To avoid false alarms, embryos receiving low frequency vibrations must use other cue properties.

Temporal properties also affect *A. callidryas'* hatching decisions (Warkentin et al., 2006b). In playbacks of simple rhythmic stimuli, constructed from pulses of low-frequency noise, varying pulse duration and inter-pulse intervals revealed a single strong peak in

hatching, with the response decreasing as either property departed from this peak (Figure 4.2). We might expect that, as with frequency properties, the temporal properties of natural vibrations would match embryo responses to playbacks of synthetic stimuli. Indeed, the average durations and intervals measured from rainstorm vibrations recorded from egg clutches fall outside the expected area of high hatching (Figure 4.2)(Warkentin, 2005; Warkentin et al., 2006b). However, the temporal pattern that elicits peak hatching, measured using synthetic stimuli, is far from the average temporal patterns measured from real snake attacks (Figure 4.2) (Warkentin, 2005; Warkentin et al., 2006b).

Based on embryo responses to these synthetic stimuli, it seems neither attacks by parrot snakes, *Leptophis ahaetulla*, nor cat-eyed snakes, *Leptodeira annulata*, would induce hatching, yet these embryos do hatch in both real snake attacks (Warkentin 1995, Gomez-Mestre & Warkentin 2007) and playbacks of recorded snake vibrations (Warkentin, 2005). Warkentin (2005) used an arbitrary threshold, based on the noise floor of recordings, to measure vibration durations and intervals from recordings. Thus, a simple solution could be that embryos use a different threshold, thus perceiving different temporal patterns (Figure 4.1). However, re-analysis of recorded snake vibrations systematically varying the threshold amplitude used to distinguish vibration pulses and intervals failed to resolve the mismatch (Guo, McDaniel, Crovella and Warkentin, unpublished). Thus, our understanding of how embryos use temporal pattern information to inform hatching decisions is clearly incomplete. Another potential cause of the mismatch could be that embryos assess temporal pattern at multiple levels. If so, measured mean property values may not reflect how embryos parse temporal patterns.

The potential importance of multi-level temporal patterns

Snakes biting and pulling at eggs, attempting to detach and swallow them, generate intermittent, amplitude-modulated vibrations with relatively short intervals between movements. These may be challenging to distinguish from temporal patterns of hard rain, where vibrations from individual drops merge together to produce extended, amplitude-modulated periods of vibration. However, snake attacks are often punctuated by periods when the snake swallows eggs disconnected from the clutch, generating longer gaps between series of closely spaced vibrations. Such gaps are unlikely to occur during hard rain, thus might help to distinguish it from snake attacks (Warkentin, 2005). Moreover, both at the onset of attacks and when returning to the clutch after swallowing eggs, snakes often tongue-flick the eggs and then pause to assess their chemical cues before biting. Tongue-flicking is an important sensory behavior unique to squamates in which chemical stimuli gathered by the tongue are delivered to the vomeronasal organ situated in the roof of the mouth (Daghfous et al., 2012; Schulterbrandt et al., 2008). In playbacks of recorded rain, embryos that experienced less intense vibrations from the start of storms hatched less in response to subsequent more intense vibrations, suggesting a response-damping mechanism such as habituation occurs (Caldwell et al., 2010). It is possible that an opposing, sensitizing effect occurs in response to vibrations from tongue flicks followed by pauses, which could contribute to increased hatching in response to snake attacks. If either longer gaps within more closely spaced vibration series or brief vibrational "prefixes" ahead of vibration series enhance hatching responses, this would mean that the effect of periods of silence depends on their surrounding vibrational context and indicate

that embryos, like adults, can parse temporal patterns at multiple levels. We used playback experiments to test this possibility.

We composed a series of synthetic vibration stimuli from three core elements, designed based on the results of prior playbacks to *A. callidryas* embryos. These included short, low frequency vibration pulses and short silent intervals that, in combination, elicited high hatching in playbacks of simple rhythms (Figure 4.2). In addition, in some stimuli, we included longer silent periods ("gaps") of a length that substantially reduces the hatching response to the short pulses when combined in a simple rhythm (Figure 4.2). If the effect of these long gaps is independent of vibrational context, they should consistently decrease hatching, compared to stimuli without them, or with fewer of them. If, however, embryos parse temporal pattern on more than one level, we hypothesize that in some vibrational contexts long gaps will not reduce and may even increase the hatching response. Evidence supporting this would motivate and inform new kinds of analyses of natural vibration patterns.

METHODS

Egg collection and care

We collected healthy *A. callidryas* egg clutches, between the ages of 0–3 d, from the Experimental Pond in Gamboa, Panama (9.120894 N, 79.704015 W) and brought them and the leaves on which they were laid to a nearby open-air laboratory at the Smithsonian Tropical Research Institute. We mounted leaf sections with clutches adhered to them on

plastic cards for support, put the cards in plastic cups containing small amounts of aged tap water to house any hatched tadpoles, and misted the clutches with rainwater multiple times a day to prevent dehydration. Across experiments, embryos were tested at 5 d of age (mean±sem 5.68±0.008 d) to reduce variation in hatching responses due to embryo development. Sets of stimuli were presented in random order within temporal blocks over several nights of testing to accumulate at least ten replicates per stimulus. If a stimulus set (one replicate of each stimulus) was incomplete when testable clutches ran out for a night, we began the next playback day with a partial set to balance sample sizes across stimuli. Each egg clutch or tray of eggs was used for only one trial. All tadpoles were returned to the Experimental Pond soon after hatching. This research was conducted under permits from the Panamanian Environmental Ministry (SC/A-11-13, SE/A-55-17) and approved by the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (100625-1008-15-A4, 2017-0601-2020-2-A1).

General vibration playback methods

Three experiments (Experiments I–III; Figures 4.4, 4.6, 4.7) presenting different sets of playback stimuli to egg clutches were conducted in June and July of 2013, and one additional experiment (Experiment IV; Figure 4.8) presenting another set of playback stimuli to eggs in trays was conducted in June and July of 2017. All stimuli were constructed in Matlab as frequency-filtered random white noise, normalized to the same maximum amplitude. Intervals were created by setting values within specified periods to

zero, leaving roughly rectangular amplitude envelopes of vibrational noise. Such synthetic vibrations enable excellent control and independent manipulation of multiple stimulus properties and have been highly informative in animal behavior research (Cocroft et al., 2014; Hill, 2008). We kept all vibration pulse properties the same, designing them with a frequency spectrum and pulse length that elicit high hatching, and manipulated only periods of silence (including either short intervals, longer gaps, or combinations thereof) as the simplest possible test for multi-level assessment of temporal patterns.

To compensate for nonlinearities in the frequency response of the playback equipment, we adjusted playback stimuli using Matlab, based on frequency analysis of recordings (Warkentin et al., In press). We recorded playback stimuli using an accelerometer (AP19 for Experiments I–III and AP32 for Experiment IV; AP Technology International, Oosterhout, The Netherlands) connected via a signal conditioner (AP Technology APC7) and external sound card (Onkyo MSE-U33HB) to a laptop running Raven Pro (Cornell Laboratory of Ornithology, Ithaca, NY). Recordings were made with clutches that were 3 d old clutches, which are structurally similar to the 5 d old clutches used in experiments but the embryos are not yet capable of perceiving vibrational cues (Jung et al., 2019; Jung et al., 2020). Frequency analysis of recordings confirmed that all stimuli, including those presented through tines to clutches in Experiments I–III and those presented through trays to groups of individual eggs in Experiment IV (Figure 4.3), presented frequency distributions similar to those that occur in snake attacks (Caldwell et al., 2009). Playbacks through tines and trays were similar in key features with their dominant frequency under 75 Hz and intensity dropping off at higher frequencies (Figure

4.3). The distribution of energy across the lowest frequencies (below 20 Hz) was limited by shaker capabilities. We also recorded the vibrations comprising each type of stimulus (to clutches and eggs-in-trays) as continuous vibration, with all gaps and intervals between pulses removed, to measure its RMS amplitude; this was 3.2 m/s^2 in Experiments I–III and 6.3 m/s^2 in Experiment IV. Stimuli were played at a consistent amplitude across all experiments using the same playback system.

Vibrations were generated by an electrodynamic minishaker (Model 4810 in Experiments I–III and LDSV203 in Experiment IV; Bruel and Kjær, Nærum, Denmark). The shaker was connected to a custom-made amplifier (E. Hazen, Boston University Electronic Design Facility), which was connected via an external sound card (MSE-U33HB; Onkyo, Osaka, Japan) to a laptop computer. Vibrational stimuli were played using the open-source program Audacity (www.audacityteam.org).

Vibration playback to clutches through tines (Experiments I–III)

In Experiments I–III, we played vibrations to entire clutches through a set of tines inserted among the eggs (Figure 4.3, black inset). All clutches used fit entirely within the grid of the tines. We only used healthy clutches with 20–70 eggs for playbacks because small clutches limit resolution of variation in responses and very large clutches made it difficult to accurately record hatching times of individual embryos. The shaker was hung upside-down from a wooden stand and attached to a minishaker-clutch interface, comprised of a rigid stinger tipped by a perforated clear plate holding a grid of blunt-ended stainless steel tines (Warkentin et al., 2006) (Figure 4.3, black inset). For each trial we

taped a card holding an egg clutch onto a support stand, with the long axis of the clutch oriented vertically, then carefully slid the stand towards the minishaker-clutch interface to embed the tines among the eggs. After connecting a clutch to the shaker, we noted any hatching induced by the setup procedure and allowed five hatching-free minutes (resetting the timer every time an embryo hatching during acclimation) before starting a playback. If 25% or more of a clutch hatched during set up and acclimation, that clutch was not used for playback and was excluded from the experiment. The shaker moved the tines vertically according to the playback stimulus, thus shaking the clutch. We took care that the ends of the tines did not touch the stand, in order to avoid generating additional high frequency vibrations that can inhibit hatching (Caldwell et al., 2009). A tray of aged tap water below the clutch caught hatched tadpoles. To record the hatching times of each embryo, we used a Matlab stopwatch program written for this purpose and run on a separate laptop. We simultaneously started the vibration playback and the Matlab program to record hatching events, then watched the eggs closely through the playback period. Every time an embryo hatched, we recorded the time with the stopwatch program. After the end of the playback, we watched the clutch for two additional minutes, recording the times of any additional hatching. The only exception was for the shortest stimulus ("prefix only"), consisting of just three pulses, which we observed for five minutes post-playback. Any eggs that fell off the clutch during tine playback, and embryos that failed to hatch in response to egg jiggling, or were dead or visibly underdeveloped, were considered not hatching competent and excluded from analysis.

Vibration playback to individual embryos through trays (Experiment IV)

In Experiment IV, we played vibrations to embryos in custom-made trays that held up to 15 eggs in individual funnel-shaped spaces (Figure 4.3, red inset). The shaker was mounted horizontally on a wooden stand and connected to a minishaker-tray interface, consisting of an aluminum alloy shaft with one end threaded for attachment to the shaker and the other terminating in a cone-mount to interface with horizontally oriented egg trays (Warkentin et al., 2019; Warkentin et al., In press) (Figure 4.3, red inset). We transferred eggs to trays at age 3 d, just before the embryos develop sensitivity to mechanosensory cues (Jung et al., 2019; Jung et al., 2020) and reared them on racks in humidified plastic containers until testing (Warkentin et al., In press). For each trial, we connected a tray of eggs to the minishaker-tray interface, supporting the tray edge and shaft with foam to avoid torque on the shaker, and suspending embryos over a container of aged tap water to catch hatchlings (Figure 4.3, red inset). After connecting an egg tray to the shaker, we allowed at least five total minutes of acclimation; since embryos were separated in trays, if any individuals hatched they did not disturb the others and we did not restart the acclimation period. We noted any hatching induced by the setup procedure and only used trays with at least five eggs remaining after acclimation. We simultaneously started the vibration playback and the timer to record hatching events, then watched the tray closely through the playback period to record the latency until the first embryo in the tray hatched. After the end of the playback, we watched the clutch for three additional minutes, noting any additional hatching, then counted the total number hatched and manually jiggled any remaining eggs with a blunt probe to assess hatching competence (Jung et al., 2019; Jung

et al., 2020; Warkentin et al., 2017).

Stimulus design

We used a series of four playback experiments to test whether and begin to assess how embryos use a second level of temporal pattern information in their hatching decisions. Our base stimulus was a standard, rhythmic pattern — 0.5 s vibration/1.5 s silence — previously shown to elicit high hatching (Warkentin et al., 2006b). This stimulus was included in all four experiments to facilitate comparisons, and each experiment tests the effect of one or more 30 s periods of silence inserted into this rhythmic base pattern (replacing a short interval).

In Experiment I, we tested whether temporal pattern elements that themselves elicit little or no hatching can function, contextually, to increase the hatching response. Specifically, we added a brief vibrational "prefix" — 3 pulses separated by 1.5 s intervals — followed by a 30 s gap (Figure 4.4a, "prefix + gap") before the rhythmic base stimulus. We also included two controls to confirm that neither the 3-pulse prefix alone (Figure 4.4a, "prefix only"), nor a rhythmic pattern of 0.5 s vibration/1.5 s silence elicits substantial hatching (Figure 4.4a, "gaps only"). These controls were included in the stimulus sets during Experiment II as well, to confirm that embryo responses were consistently low across testing periods.

In Experiment II, we asked whether the propensity to hatch accumulates solely with actual vibration pulses, or if it can also increase during intervening periods of silence, in association with certain vibration patterns. Ten pulses are sufficient to elicit hatching in

response to the base stimulus (Figure 4.5, "early hatching (10-pulse groups)"). Thus, by inserting a sequence of 30 s gaps between 10-pulse groups (Figure 4.6a, "10-pulse groups"), we can ask if hatching increases only in response to rhythmic playback or if and to what extent it also increases during gaps. The first 3 pulses elicited little or no hatching in playbacks of the base stimulus (Figure 4.5, "assessment (3-pulse groups)") or when presented alone (Experiment I). By inserting a sequence of 30 s gaps between 3-pulse groups (Figure 4.6a, "3-pulse groups"), we can ask if embryos combine or integrate information from a series of 3-pulse sequences, each of which individually elicits little or no hatching, into a cumulative perception of risk based on a relatively small amount of vibration. The base stimulus contained 150 total pulses, while the "10-pulse groups" stimulus contained 70 total pulses and the "3-pulse groups" stimulus contained 30 total pulses.

In Experiment III, we examined whether the effect of a 30 s period of silence on embryos' hatching response depends on the timing of the silent period in relation to their risk assessment, decision, and hatching process. We tested three vibration stimuli that differed in the timing of a single long gap, based on hatching timing in response to our base pattern (Figure 4.5), as well as our base pattern as a control (Figure 4.7a). In one stimulus we placed the gap after 3 pulses, during risk assessment before embryos begin hatching (Figure 4.5 “assessment (gap at 3 pulses)”). In another, we placed the gap after 17 pulses, at the center of the 10-s period where the most hatching occurred in response to the base stimulus (Figure 4.5 “peak hatching (gap at 17 pulses)”). In the third, we placed the gap after 43 pulses, in the first 10-s period with consistent low hatching (Figure 4.5

"post peak hatching (gap at 43 pulses)"). Some hatching continued after "post peak hatching", but most subsequent 10-s periods contained very low levels of hatching (Figure 4.5). All stimuli in Experiment III contained 150 vibration pulses (Figure 4.7a).

In Experiment IV, to assess how long embryos can retain information that increases hatching after a brief initial stimulus, we tested hatching responses to stimuli featuring various lengths of silence. Our stimuli included our base pattern with no long gap and three stimuli with a single 3-pulse prefix followed by a 30 s gap, 45 s gap, and 60 s gap (Figure 4.8a).

Combining data for stimuli presented across experiments

To assess if hatching response varied across the field season for stimuli that were included in the playback series for multiple experiments, we tested for the effect of "experiment" (testing period) using Kruskal–Wallis tests, following Warkentin et al. (2006b). Since all stimuli that were repeated across multiple experiments with the same (tine-based) playback system yielded consistent hatching responses ($P>0.16$ for all repeated stimuli), hatching data for those repeated stimuli were pooled across Experiments I–III in subsequent analyses. We also pooled hatching response data from both stimuli that included a 30-s gap placed after 3 pulses within a base pattern, since the difference only between the stimuli was 3 pulses at the end of one stimulus (primer + gap = 153 total pulses) but not the other (assessment = 150 pulses), only 2 of the 340 embryos that hatched (0.588%) in response to the 153 pulse stimulus did so during or after the last 3 pulses, and hatching responses between the two stimuli were not statistically different (Wilcoxon rank

sum test, P=0.2543). Hatching timing data are not pooled.

Statistical analysis

To compare the proportion of hatched embryos and hatching timing across stimuli, we used Kruskal–Wallis tests and pairwise Wilcoxon rank sum tests for non-parametric data. To assess hatching timing across stimuli, we compared the midpoint of hatching, defined as the point at which 50% of all eventually-hatched embryos in a clutch had hatched. To assess hatching synchrony, across stimuli, we compared the period encompassing the second and third quartiles of hatching. To compare the accumulation of hatching over time (including long gaps) vs. over the number of vibration pulses, we used Matlab to create copies of the data with the cumulative times of prior long gaps (28.5 extra s per gap) subtracted for each time point. Hatching that occurred during a gap was condensed to the point just before the subsequent vibration. This analysis allowed us to assess whether hatching propensity accrues only with vibration pulses or also during intervening periods of silence. All statistics were carried out in the R statistical environment (version 3.6.2, R Development Core Team 2019, http://www.r-project.org) in RStudio (version 1.4.869, RStudio Team 2020).

RESULTS

Experiment I

Hatching responses varied considerably with stimulus treatment (χ^2= 72.03, df=3, P=1.56e-15). As expected, the rhythmic base pattern stimulus elicited substantial hatching (Figure 4.4b). However, adding a 3-pulse group and 30 s gap before it (prefix + gap) increased the hatching response (P=0.0037, Figure 4.4b). The 3-pulse prefix alone (prefix only) and the stimulus comprised of single pulses separated by 30 s intervals (gaps only) elicited little to no hatching (0.0295 ± 0.005, mean ± SE), well below the hatching response to the base pattern (P<1.6e-09), and the hatching responses to these controls were not significantly different from each other (P=0.6162, Figure 4.4b).

The midpoint of hatching was statistically earlier for our base pattern than for the stimulus with a prefix + gap added (P=5.9e-06, Figure 4.4c). However, measured over accumulated vibration pulses (i.e., removing the period of the long gap), the timing of hatching midpoints no longer differed between these stimuli (P=0.7399, Figure 4.4c). Our measure of hatching synchrony, the period encompassing the middle 50% of hatching, did not differ between the base pattern and the stimulus with a prefix + gap (P=0.38). As embryos did not start hatching until after the gap, considering synchrony over accumulated pulses rather than time did not alter this (P=0.38). Although the prefix + gap stimulus included 3 pulses more than the base pattern (153 vs. 150 pulses), the vast majority of hatching occurred well before the end of the stimulus and the cumulative hatching curves reached a plateau, such that only 2 of the 340 embryos that hatched (0.588%) did so during

or after the last 3 pulses in this 153-pulse stimulus (Figure 4.4c).

Experiment II

Embryos showed equally strong hatching responses to stimuli with 10-pulse groups or 3-pulse groups separated by 30 s gaps as they did to a continuous rhythmic pattern (70, 30, and 150 total pulses, respectively; χ^2= 0.75, df=2, P=0.6859). The 10-pulse groups and 3-pulse groups stimuli elicited similar hatching responses to each other (P=0.46), and to the base pattern (3-pulse groups: P=0.48, 10-pulse groups: P=0.74; Figure 4.6b). However, if considered on a per-pulse basis (normalizing by the number of pulses in each stimulus) hatching responses increased with the amount of silence relative to pulses (Figure 4.6b; χ^2= 33.789, df=2, P=4.601e-08).

The midpoint of hatching was later in time in response to the 3-pulse groups stimulus, compared to the base pattern (P=5.7e-06) and the 10-pulse groups stimulus (P=0.00014), but did not differ between base and 10-pulse groups stimuli (P=0.0595, Figure 4.6c). However, considering hatching over accumulated vibration pulses rather than time, the midpoint of hatching was significantly earlier for both the 10-pulse groups and 3-pulse groups stimuli compared to the base pattern (P=0.03364 and 0.00016, respectively), and similar for two stimuli with gaps (P=0.12094, Figure 4.6c). The middle 50% of hatching was slightly faster (more synchronous) for the base pattern than for our 10-pulse groups and 3-pulse groups stimuli (P=0.04388 and 0.01587, respectively), but hatching synchrony did not differ between the two stimuli with gaps (P=0.3897). Considering hatching over vibration pulses, rather than time, hatching synchrony differed for all three

stimuli. The middle 50% of hatching occurred over the fewest pulses in the 3-pulse groups stimulus (vs. 10-pulse groups, P=0.00152; vs. base P=5.7e-06) and was also more synchronous for the 10-pulse groups stimulus compared to the base pattern (P=0.03174).

Experiment III

Placing a 30-s gap during embryo risk assessment elicited more hatching than the base pattern (see Experiment I results). However, the hatching responses to stimuli with a gap located later, either at peak hatching or post peak hatching, were intermediate (Figure 4.7b). They did not differ from either the response to the base stimulus (P=0.4052 and P=0.6427, respectively) or the response to stimuli with a gap before hatching began (P=0.131 and P=0.076, respectively), nor from each other (P=0.664).

As in Experiment I, the midpoint of hatching was slightly earlier for our base stimulus than for the stimulus with a gap during assessment (P=0.0169), but there was no difference when the gap time was subtracted to compare across accumulated pulses (Figure 4.7c, P=0.2543). Placing a gap at peak hatching did not alter the midpoint of hatching from the base pattern in time (P=0.97593), but it did occur after fewer accumulated pulses (Figure 4.7c, P=0.01045). Unsurprisingly, placing a gap after peak hatching did not alter the midpoint of hatching from the response to the base pattern either in time (P=0.88011) or number of pulses accumulated (Figure 4.5, P=0.84284). Hatching synchrony over time or pulses was not altered from the response to the base stimulus by including a gap during embryo assessment (both P=0.72234). Including a gap at peak hatching did not alter hatching synchrony in time (P=0.60752) but did increase synchrony over pulses, compared

with the base pattern (P=0.00129). Including a gap after peak hatching did not alter hatching synchrony in time (P=0.67066), or over accumulated pulses (P=0.88739).

Experiment IV

In motion-only playbacks to individual eggs in trays hatching responses are typically lower than in playbacks presenting the same vibration pattern as a combination of motion and tactile cues (Figures 4.4, 4.6, and 4.7 vs. 4.8) (Fouilloux et al., 2019; Jung et al., 2019; Jung et al., 2020; Warkentin et al., In press); thus we do not directly compare data across tray and tine playbacks. Hatching responses varied across stimuli within the tray-playback experiment (χ^2= 15.02, df=3, P=0.001798). Inserting 30- or 45-s silent gaps after the first 3 pulses of a rhythmic stimulus increased hatching, compared to the base stimulus (P=0.00616 and 0.00046, respectively), and the hatching response to both of these stimuli with gaps was similar (Figure 4.8b, P=0.17084). The hatching responses to the stimulus with a 60-s gap and the base pattern were similar (P=0.20139) and significantly lower than the response to the stimulus with a 45-s gap (Figure 4.8b, P=0.02450).

The latency for the first individual in each tray to hatch, measured in seconds, increased with gap length (Figure 4.8c). Measured in vibration pulses, latency to hatch was similar across stimuli with different gap lengths and with no gap in the rhythmic vibration pattern (Figure 4.8d, P>0.13).

DISCUSSION

This study substantially extends our understanding of how *A. callidryas* embryos process temporal pattern information for risk assessment in escape-hatching decisions. An earlier analysis showing that embryos use temporal pattern cues in risk assessment addressed only the most basic level of temporal pattern, simplifying synthetic stimuli to a consistent rhythm of intervals and durations (Warkentin et al., 2006b). However, comparing this simple analysis with measured mean properties of common natural vibrations suggested that embryos would not distinguish between stimuli that, in fact, they routinely distinguish (snakes vs. rain, Figure 4.2). Motivated by this mismatch, here we test whether embryos parse temporal pattern information on more than just one level, in a way that is more complex and context-dependent than previously considered. In a series of vibration playback experiments with synthetic stimuli featuring three core elements – short pulses, short intervals, and long gaps – we determined that embryos faced with the challenge of deciding to hatch, or not to hatch, in response to incidental egg vibrations appear to assess the sensation of intermittent stimulation on multiple levels.

Experiment I – The effect of temporal pattern elements on hatching is contextual

We began by asking if a long silent interval, added near the beginning of a rhythmic vibration pattern to separate a short prefix from the subsequent vibration series, might increase hatching in response to the subsequent vibrations. Essentially, this is a question about contextual interpretation, assessing if a pattern element that decreases or fails to elicit hatching in one context can increase it in another. Results from these playbacks suggest

that identical periods of silence can act either to dampen responses to associated vibrations or to potentiate or increase them, depending on context.

Prior rhythmic playbacks found that the pulse duration that elicited the most hatching was 0.5 s, and the inter-pulse interval that elicited the most hatching was 1.5 s; however, combining either of these cue properties with longer or shorter values of the other property elicited less hatching (Figure 4.2)(Warkentin et al., 2006b). Based on that work, we expected that a rhythm with long (30 s) intervals would result in very little hatching (Figure 4.2). Our playbacks confirmed that a stimulus with a long and consistent inter-pulse interval elicits little hatching, even using the pulse duration that elicits the most hatching (Figure 4.4a-b, gaps only). Thus, the perception of risk that can be generated by properties of these vibration pulses appears to be substantially reduced by the long intervals. Nonetheless, the same long interval inserted early in a rhythmic 0.5 s vibration/1.5 s silence temporal pattern actually increased the already strong hatching response (Figure 4.4a-b, prefix + gap). Another way to consider this pattern manipulation is that the addition of the long gap to the base rhythm creates a separated group of three pulses, or prefix. By itself, this pulse group elicited very little hatching (Figure 4.4a-b, prefix only), yet its inclusion before the base stimulus had a larger impact in augmenting hatching.

The start of a stimulus may provide particularly salient information for animals faced with a behavioral decision. In *A. callidryas*, periods of vibration with gradually increasing intensity at the start of storms are known to reduce embryos' hatching response to subsequent more intense rain vibrations (Caldwell et al., 2010). The response to our

prefix + gap stimulus suggests that isolated vibrations preceding a larger disturbance may similarly modulate the response to subsequent vibrations, but in a positive or sensitizing manner. While the response to edited recordings may reflect multiple properties of rain vibrations that vary over the course of storms (Caldwell, McDaniel & Warkentin, 2010), the current results are based on very simple synthetic stimuli, comprised of standardized pulses combined with intervals of two lengths. Results from these simple playbacks support that *A. callidryas* embryos incorporate multiple levels or scales of temporal pattern analysis in their risk assessment process, and that temporal patterns influence their hatching decisions in more complex and nuanced ways than can be captured by a bivariate analysis of vibration durations and intervals (Figure 4.2).

Experiment II – Risk perception can accumulate during long periods of silence

Considering that the effect of long periods of silence is to increase hatching in some contexts yet decrease it in others, depending on the associated vibrations, we investigated the extent to which long periods of silence may contribute to risk perception. We found that stimuli with long gaps interspersed with groups of 3 or 10 pulses elicited the same high hatching rates as the rhythmic base pattern (Figure 4.6b), despite containing much less vibration (Figure 4.6a). Thus, the majority of a hatching-inducing vibrational pattern can be replaced by silence without reducing hatching, as long as some minimal amount of the pattern remains. When data are normalized to consider hatching relative to the number of vibrational pulses in each stimulus and to the hatching responses of compared stimuli, we see that the proportion of embryos hatched per pulse is much higher in response to stimuli

with more silence (Figure 4.6b). The inclusion of multiple long gaps in the playback stimuli also changed the timing of hatching. We plotted cumulative hatching curves with the long gaps removed (i.e., over pulses) to visualize proportion hatched as vibrational stimulation accumulated. In real time, hatching occurs more slowly in response to stimuli with shorter pulse groups; however, embryos exposed to shorter pulse groups hatch much more rapidly relative to their experience of vibration pulses (Figure 4.6c). This suggests that embryos' perception of risk continue to increase through these long silent intervals, or that information pertinent to hatching decisions is at least retained and perhaps updated for relatively long periods without vibrational stimulation.

These results strengthen and extend the interpretation, from prior work with *A. callidryas* embryos, that more vibrational stimulation does not necessarily elicit more hatching. One might expect that more vibration, accumulated over time or relative to silence, would elicit more vibration-cued hatching. However, in playbacks of synthetic stimuli with regular rhythms, combinations of long vibrations and short intervals elicit relatively low hatching rates; peak hatching occurs in response to stimuli with relatively short vibration durations and longer intervals (Warkentin et al., 2006). Also, when presented with stimuli varying in cycle length, so slower or faster temporal patterns, embryos sampled the fewest cycles of the slowest pattern, using less information to make their hatching decision (Warkentin et al., 2007; Warkentin et al., 2019), and in general duty cycle is not a good predictor of hatching (Warkentin, 2005; Warkentin et al., 2006b). Here, using the same 0.5 s vibration pulses across stimuli, we found that removing 120 (80%) of the pulses did not reduce hatching. Moreover, a much smaller change, adding 20 total

pulses to a 1-pulse to create a 3-pulse pattern, both comprised largely of long gaps, elicited dramatically different hatching responses (Figure 4.4b, Figure 4.6b).

A key role for periods of silence among vibrations, for risk assessment by *A. callidryas* embryos, is consistent with the fact that snake attacks are intermittent, and their recorded vibrations contain substantially more silence than do comparable periods of hard rain (Warkentin 2005). Snake attacks on egg clutches always include periods where the snake is biting and pulling at eggs, generating intermittent vibrations with relatively short intervals between movements that, in the time domain, may be challenging to distinguish from hard rain. However, these attacks often also include periods when the snake is chewing and swallowing eggs without touching the clutch, generating long gaps between series of closely spaced vibrations. Such gaps are unlikely to occur during hard rain, thus the presence or absence of a long gap, or gaps, might help embryos to distinguish the vibrations caused by heavy rain vs. snake attacks.

The fact that *A. callidryas* embryos interpret a long silence following or interspersed among vibrations as a risk cue is not unique. In other anurans as well, the "sound of silence" or lack of acoustic stimulus can indicate risk and serve as a cue to cease chorusing, or vocally advertising for mates (Dapper et al., 2011). The adult frogs in these choruses assess predation risk based on a rapid change in the behavior of other frogs, whereas frog embryos assess predation risk based on a sudden cessation of egg clutch vibrations. In both cases, specific patterns of amplitude modulation may serve to distinguish a behaviorally mediated change in stimulation from abiotic background noise. Gaps in a signal or cue can make communication either more or less difficult depending

on the overall temporal structure of the acoustic environment (Gomes et al., 2021). In many species, from fish to humans, an intermittent stimulus that is less predictable or regular is generally more distracting, stressful, and difficult to habituate to than if it were continuous or regular (Debusschere et al., 2016; Glass and Singer, 1972; Kjellberg et al., 1996; Matthews et al., 1980; Neo et al., 2014). Thus, in many contexts, silence can provide useful information for receivers faced with a behavioral decision.

Experiment III – Does gap timing matter?

Since periods of silence spaced throughout a sequence of intermittent vibration appear to contribute substantially to the overall hatching response, we examined how the timing of a single long silent gap affects hatching responses. Clearly a gap near the onset of vibrations, during the risk-assessment period for most embryos, increases the hatching response to subsequent vibrations (Figures 4.4b, 4.7b). We asked if gaps at the peak of hatching or later — after most embryos had either hatched or, presumably, decided not to in response to the base pattern — might also increase hatching responses. A late gap might, for instance, function to dishabituate the remaining embryos, increasing later hatching. Our results, however, were inconclusive; responses to the stimuli with a gap during or after peak hatching were intermediate between responses to stimuli with an early gap and no gap (Figure 4.7b), and different from neither. While our data do not entirely rule out a role for late gaps in increasing hatching, they suggest that gaps during the initial assessment period may be more influential. Some evidence from other anurans suggests that, in the context of mating, females assess differences in temporal patterns by comparing the first

few pulses of each call rather than by averaging over the entire call (Gerhardt and Schul, 1999). Other species exhibit an iterative and open-ended decision-making process, as seen in mate choice behaviors in túngara frogs (Baugh and Ryan, 2010). In the context of egg-predator attacks, embryos that more rapidly reach a decision to hatch would be less likely to be eaten. This suggests that initial vibration patterns may be heavily weighted in hatching decisions. However, snakes may take many minutes to consume an egg clutch (Warkentin, 2005); thus, embryos that initially decide not to hatch could also benefit from continuing to gather information and using it to update their risk assessment. Determining if they do will require further investigation.

Experiment IV – Embryos retain information through 45 s of silence

Our final experiment examined how the priming, or hatching-increasing, effect of a silent period after a few initial vibrations might vary with gap length. Playing motion-only vibrational cues to individual embryos in trays, we corroborated the initial result from our tine playbacks that presented motion and tactile cues. Inserting a single 30-s long gap into our rhythmic base stimulus significantly increased hatching rates but had no impact on the timing of hatching relative to pulse accumulation. This hatching-increasing effect of the gap was similar or even stronger when it was extended to 45 s, but waned when the gap was extended to 60 s. Thus, embryos clearly have a mechanism to retain information over at least 45 s without vibrational stimulation. Adult female túngara frogs also retain acoustic information from male advertisement calls through a 45 s interval of silence, as evidenced by their subsequent behavioral choices, but with longer intervals this effect

wanes (Akre and Ryan, 2010). In the context of naturally intermittent chorusing (mean silent gap 25 s), this period of working memory would allow females to make choices informed by male behavior in their last calling bout (Akre and Ryan, 2010). Our results suggest that the working memory of *A. callidryas* embryos, at least for certain critical types of information, might be comparable to that of adult frogs.

Over half of the inter-vibration intervals in cat-eyed snake attacks and almost 30% of those in parrot snake attacks on egg clutches are over 10 s long (Warkentin, 2005). While embryo responses to simple rhythmic playbacks (Figure 4.2; Warkentin et al. 2006) suggest that these long intervals contribute little or nothing to inform embryos of their impending risk, the absence of intervals over 5 s and extreme rarity of those over 2 s in recordings of rain suggests that long intervals could serve to indicate that associated strong vibrations are not benign. The present study shows that intervals as long as 45 s can function to increase perceived risk, and hatching likelihood, but that this effect is only evident when the long gaps are combined with periods of vibration that are intermittent or amplitude-modulated on shorter time-scales.

Our improved understanding of the temporal complexity incorporated into embryos' risk-assessment strategies opens new avenues for investigation. For instance, in the case of túngara frog females, it appears that not all types of calls have an active time that extends to influence females' choices after a period of silence; this extended influence depends on call complexity (Akre and Ryan, 2010). Similarly, embryos might retain information through a silent period for only a subset of possible vibrational patterns. The fact that they do retain such information at all, showing an effect of earlier vibrations on

their response after a period of silence, makes further exploration of such questions worthwhile. Moreover, the present data motivate a re-analysis of temporal patterns in vibration recordings from snake attacks and rainstorms on clutches, separating finer-scale patterns of amplitude-modulation from longer gaps. Such analysis might resolve the mismatch evident between responses to synthetic stimuli and oversimplified natural disturbance patterns (Fig. 2; Warkentin, 2005 vs. Warkentin et al. 2006).

Insights from animal communication, applications to use of incidental cues

Our prior and current playback experiments, collectively, establish that *A. callidryas* embryos exhibit a complex, multi-faceted risk assessment process based on incidental egg-clutch vibrations, contextually incorporating at least two levels of temporal pattern. The unitary and irrevocable nature of the hatching decision, combined with strong risk trade-offs between egg and larval environments (Gomez-Mestre and Warkentin, 2007; Warkentin, 1995; Warkentin, 2011a; Warkentin et al., 2019), has likely refined their risk-assessment mechanisms. Undoubtedly, many other animals also practice a range of more or less sophisticated assessment processes using incidental cues to inform their behavioral decisions – as we know they do for decisions based on communication signals.

In communication contexts, when assessing signals such as advertisement or aggressive calling, many animals attend to multiple levels of temporal pattern information, across different time scales. These include properties of individual pulses such as rise-time and duration, the spacing or repetition rate of pulses, the number of pulses per call, the positions of different pulse types or other elements within calls, as well as larger-scale

patterns such as call repetition rate. Female gray treefrogs prefer male mating calls with pulses that have longer, more gradual rise-times (Gerhardt and Schul, 1999). In addition, these treefrogs attend to larger-scale temporal properties, showing a preference for mating calls with four attractive pulses placed at the beginning of the call rather than at the end (Gerhardt and Schul, 1999). Female grey treefrogs attend to even larger scale temporal properties, since different combinations of a conspecific advertisement call and acoustic appendages led to higher and lower mating preferences (Gerhardt et al., 2007). For another example in anurans, the acoustic signals of male cricket frogs have a complex temporal structure, with varying numbers of pulses grouped within calls and produced at varying rates, and varying numbers of calls grouped within calling bouts. These frogs adjust multiple temporal properties of these signals in response to their social environment and the properties of the calls they hear (Burmeister et al., 1999). In primates, a large body of research has revealed that temporal features are critical for encoding the call identity (Ghazanfar and Hauser, 2001; Ghazanfar et al., 2001a; Ghazanfar et al., 2001b; Hauser, 1998; Hauser et al., 1998; Le Prell and Moody, 1997; May et al., 1988; May et al., 1989). Macaques in particular can use multiple temporal cues on multiple levels – including harmonic temporal features, durations of interpulse interval, or temporal positions of the peak fundamental frequency inflection – to categorize different calls of their species repertoire (Ghazanfar et al., 2001a; Hauser et al., 1998; May et al., 1988).

The highly stereotyped structure of animal signals, evolved under selection for efficacy in communication, has enabled researchers to identify and characterize distinct signal elements and how they are combined into larger patterns, then test the effect of

variation in specific properties and pattern elements on receivers. This has generated a large and productive area of animal behavior research, based on recordings and playbacks of animal signals (Bradbury and Vehrencamp, 2011; Gerhardt and Huber, 2002; Hill et al., 2019; Hill, 2008; Maynard-Smith and Harper, 2003).

In principle, we should be able to apply the same kinds of playback-based approaches to learn how animals extract information from the temporal patterns of non-stereotyped, incidental cues. In practice, and by comparison, such studies of incidental cues are still in their infancy. One challenge is that incidental cues, such as egg-clutch vibrations caused by snake attacks or rainstorms, can present relatively broad and continuous distributions of basic temporal properties, such as vibration durations and interval lengths (Warkentin 2005). Thus, it may not be clear if or how such cues might be parsed into multi-level temporal patterns. Our initial analysis of how *A. callidryas* embryos use temporal pattern cues in risk assessment (Warkentin et al., 2006b) addressed only the most basic level of variation. Nonetheless, it revealed that both vibration durations and inter-vibration intervals affect hatching, functioning as non-redundant elements of a composite cue (Warkentin et al., 2006b). Our present results reveal that this basic level of pattern analysis, simplified to a consistent rhythm, is insufficient to understand how temporal patterns inform *A. callidryas*' hatching decision. They indicate that embryos parse vibrations using at least two temporal levels and suggest new approaches to analyses of temporal patterns in recorded egg-clutch vibrations of natural stimuli. This reanalysis of recordings could address the current conceptual mismatch we see in embryos responses to synthetic and natural stimuli (Fig. 2) and update our understanding of temporal pattern processing in

embryos. Applying the power of playback experiments more broadly to study the specific ways in which animals extract behaviorally relevant information from non-stereotyped incidental cues has the potential to expand our understanding of both animal behavior and information processing.

FIGURE LEGEND

Figure 4.1 – Sample recording of vibrations from a clutch of 5 day old *Agalychnis callidryas* eggs being attacked by a snake *Imantodes inornatus*. This predation event presents an amplitude-modulated incidental vibrational cue to embryos. Embryos, assessing risk, and researchers, recording the cue, may perceive different temporal patterns of "vibration" (supra-threshold amplitude) and "silence" (sub-threshold amplitude), depending on the sensitivity of their mechanosensory system. For illustration, two arbitrarily different thresholds are shown as shades of gray. Green line highlights a single 1.5 s interval in stimulus.

Figure 4.2 – Contour plot of *Agalychnis callidryas* hatching response to vibration playback stimuli varying in disturbance duration and interval. Blue to yellow shading indicates mean proportion hatched in response to test stimuli of various durations and intervals, shown in black points. Figure is adapted with permission from Warkentin et al. (2006). Average durations and intervals measured by Warkentin (2005) for vibrations recorded from egg clutches in three natural disturbances are plotted (labelled enlarged dots). Measures for a common benign natural disturbance (rainstorms) and common threats (parrot snake, *Leptophis ahaetulla*, and cat-eyed snake, *Leptodeira annulata*, attacks) all fall outside the area of high hatching (Warkentin, 2005). The temporal pattern that elicits peak hatching (0.5 s duration, 1.5 s interval) is marked with a yellow star, and a green line highlights the 1.5 s and 30 s intervals.

Figure 4.3 – Playback systems used to test *Agalychnis callidryas* embryo responses to vibrational cues, showing the relative frequency spectra of vibrations presented through each system. The tine-based playback system (black inset) used in Experiments I–III featured an array of blunt metal tines that were inserted among eggs, presenting a combination of motion and tactile cues to embryos. The tray-based playback system (red inset) used in Experiment IV presented motion cues only, without concurrent tactile cues, to trays of up to 15 eggs in individual funnel-shaped spaces. The lines represent the distribution of energy across frequencies in vibration playback stimuli presented through tines (black) and trays (red). Lines are fast Fourier transforms of a period of vibration recorded from a playback stimulus via an accelerometer attached to the back of the tines inserted in an egg clutch or to the top of a tray. Vibrations used in all stimuli were constructed to have high energy below 75 Hz, congruent with snake attack vibrations, although equipment limitations reduced the energy below 20 Hz

Figure 4.4 – Experiment I – Vibration playbacks to *Agalychnis callidryas* embryos testing if temporal pattern elements that themselves elicit little hatching can increase the response to a subsequent hatching-inducing pattern. (a) Stimuli included a simple rhythmic pattern of 0.5 s vibration / 1.5 s silence, presented in all four experiments (base pattern), the base pattern preceded by a 3-pulse prefix and 30 s gap (prefix + gap), a 3-pulse (prefix only) stimulus, and a rhythmic pattern of single pulses separated by 30 s intervals (gaps only). (b) Proportion of embryos hatched in response to each stimulus. Data points represent values per clutch, with sample sizes indicated. Boxplots show quartiles and 1.5x the

interquartile range (IQR) across clutches with medians per stimulus in gold. Different letters indicate significant differences in response among stimuli. (c) Cumulative hatching curves showing proportion of clutch hatched on the y-axis over time or accumulated number of vibration pulses (scale is equivalent to time with gap replaced by a standard 1.5 s interval). The blue curve shows the mean proportion hatched at each time point; red curves show the standard error across all trials of the stimulus. The vertical lines indicate the mean time across trials at which 25% (gray), 50% (gold), and 75% (gray) of all eventually-hatched embryos in a clutch hatched. Significant differences in midpoints of hatching between the prefix + gap stimulus and the base pattern are indicated, measured in time or pulses (ns = $P > 0.05$, *** = $P < 0.001$). Green vertical lines show the end of the playback period

Figure 4.5 – Timing of *Agalychnis callidryas'* embryo hatching in response to playbacks of a regular series of 0.5-s pulses of vibrational noise, separated by 1.5-s silent intervals (base pattern). Bars show the mean proportion, out of the total embryos hatched, that hatched in each 10-s period from the start of the playback. Error bars are SE across trials. Colored are the 10-s periods when the start of long gaps were placed in the stimuli for Experiments II and III

Figure 4.6 – Experiment II – Vibration playbacks to *Agalychnis callidryas* embryos comparing hatching responses to a simple rhythm vs. short sections of it separated by periods of silence. (a) Stimuli include a rhythmic base pattern used as a control across

experiments (150 total pulses), 10-pulse groups separated by 30 s gaps (70 total pulses), and 3-pulse groups separated by 30 s gaps (30 total pulses). (b) Embryo hatching responses to each stimulus in proportion of clutch hatched and normalized by number of pulses in each stimulus and to the hatching responses of compared stimuli (in pink). Data points represent values per clutch, with sample sizes indicated. Boxplots show quartiles and 1.5x the interquartile range (IQR) across clutches with medians per stimulus marked. Different letters indicate significant differences in response among stimuli. (c) Cumulative hatching curves showing proportion of clutch hatched on the y-axis over time or accumulated number of vibration pulses (scale is equivalent to time with gaps replaced by standard 1.5 s intervals). The blue curve shows the mean proportion hatched at each time point, and the red curves show the standard error across all trials of the stimulus. The vertical lines indicate the mean time across trials at which 25% (gray), 50% (gold), and 75% (gray) of all eventually-hatched embryos in a clutch have hatched. Significant differences in midpoints of hatching between each stimulus and the base pattern are indicated (ns = $P > 0.05$, ** = $P<0.01$, *** = $P < 0.001$). Green vertical lines show the end of the playback period

Figure 4.7 – Experiment III – Vibration playbacks to *Agalychnis callidryas* embryos testing the effect of the timing of a long gap on hatching responses. (a) Stimuli include a base rhythmic pattern with no gap, used as a control across experiments, and with a single 30 s gap after 3 pulses (assessment), after 17 pulses (peak hatching), and after 34 pulses (post peak hatching). (b) Proportion of embryos hatched in response to each stimulus. Data

points represent values per clutch, with sample size indicated. Boxplots show quartiles and 1.5x the interquartile range (IQR) across clutches with medians per stimulus in gold. Different letters indicate significant differences among stimuli. (c) Cumulative hatching curves showing proportion of clutch hatched on the y-axis over time or accumulated vibration pulses (scale is equivalent to time with gaps replaced by standard 1.5 s intervals). The blue curve shows the average proportion hatched at each time point, and the red curves show the standard error across all trials of the stimulus. The vertical lines indicate the mean time across trials at which 25% (gray), 50% (gold), and 75% (gray) of all eventually-hatched embryos in a clutch have hatched, and the end of the playback period (color). There was no significant difference in midpoints of hatching between each stimulus and the base stimulus, measured over accumulated pulses (ns = $P > 0.05$). Green vertical lines show the end of the playback period

Figure 4.8 – Experiment IV – Vibration playbacks to *Agalychnis callidryas* embryos testing the effect of the length of a long gap on hatching responses. (a) Stimuli include a base rhythm without a long gap (used as a control across experiments, base pattern), and the same pattern with a silent gap of 30, 45, or 60-s inserted after the first 3 pulses. Green vertical lines show the end of the playback period. Data points represent (b) proportions of embryos hatched from trays and (c) latency in time (s) and (d) number of vibration pulses for the first individual to hatch per tray. N of trays (and of sibships) exposed to each stimulus are shown above the plot. Boxplots show quartiles and 1.5x the interquartile range (IQR) across trays with medians per stimulus in gold. Different letters indicate significant

differences in responses among stimuli

Figure 4.1

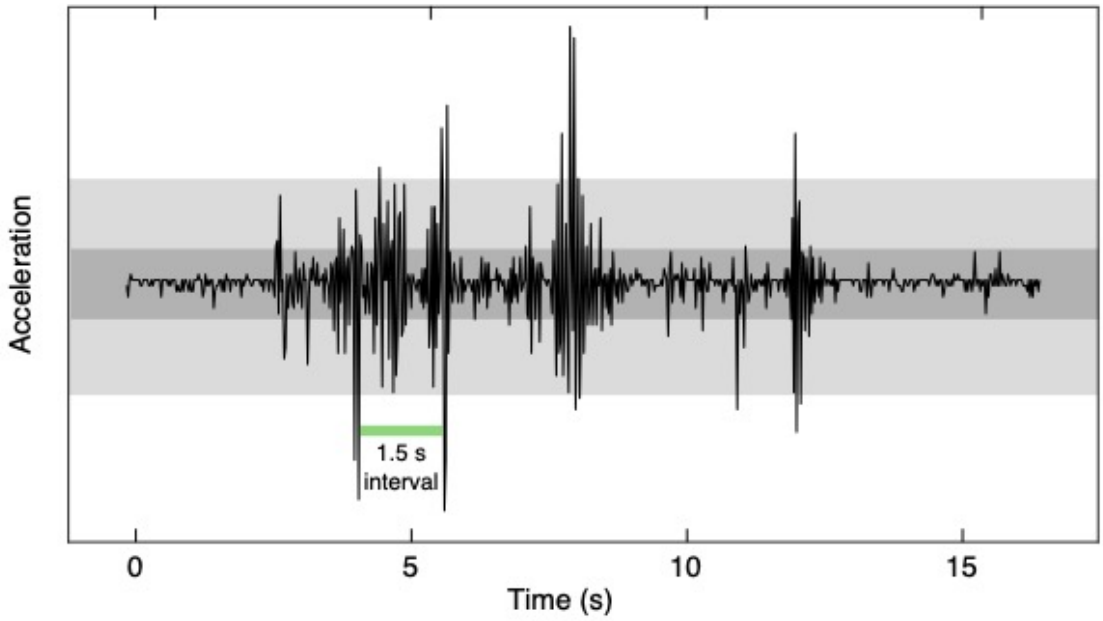


Figure 4.2

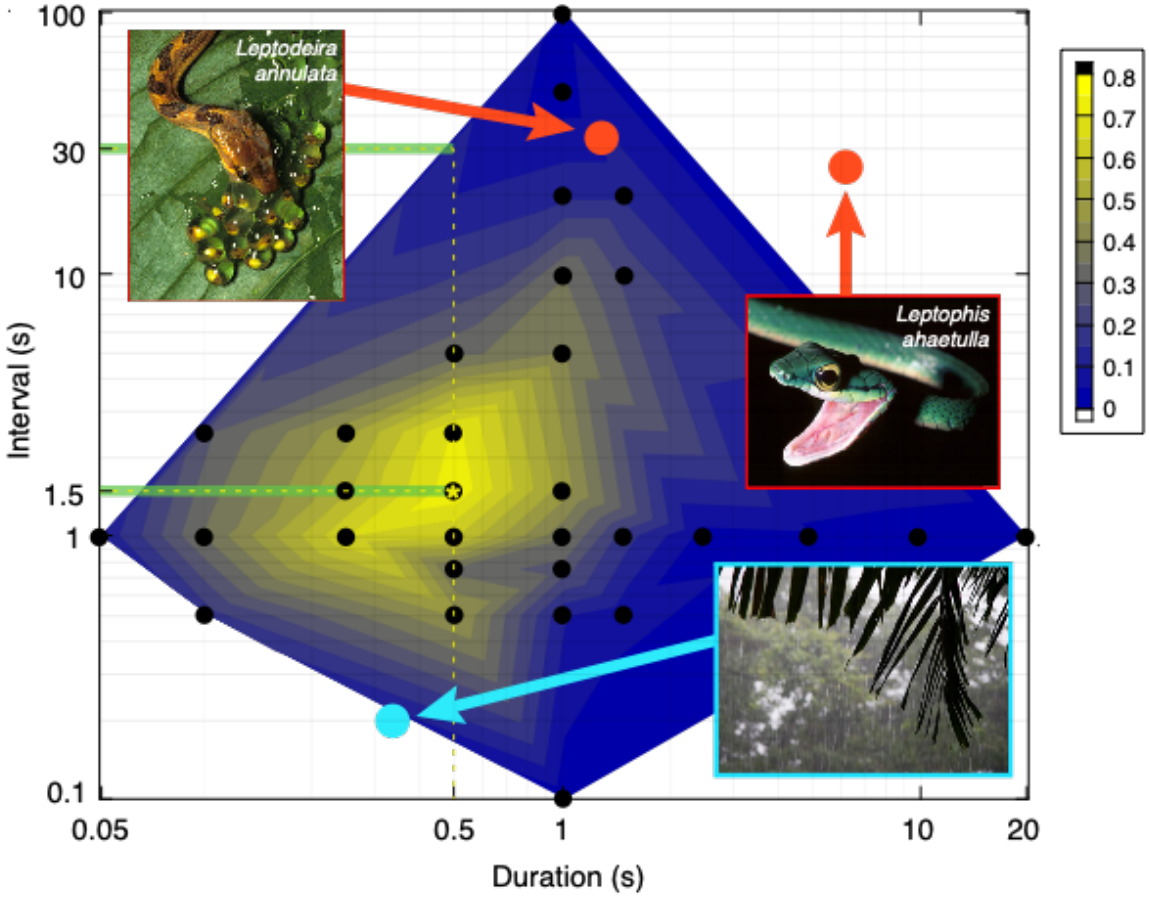

Leptodeira annulata
Leptophis ahaetulla
Interval (s)
100
30
10
1.5
1
0.1
0.05
0.5
1
10
20
Duration (s)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0

Figure 4.3

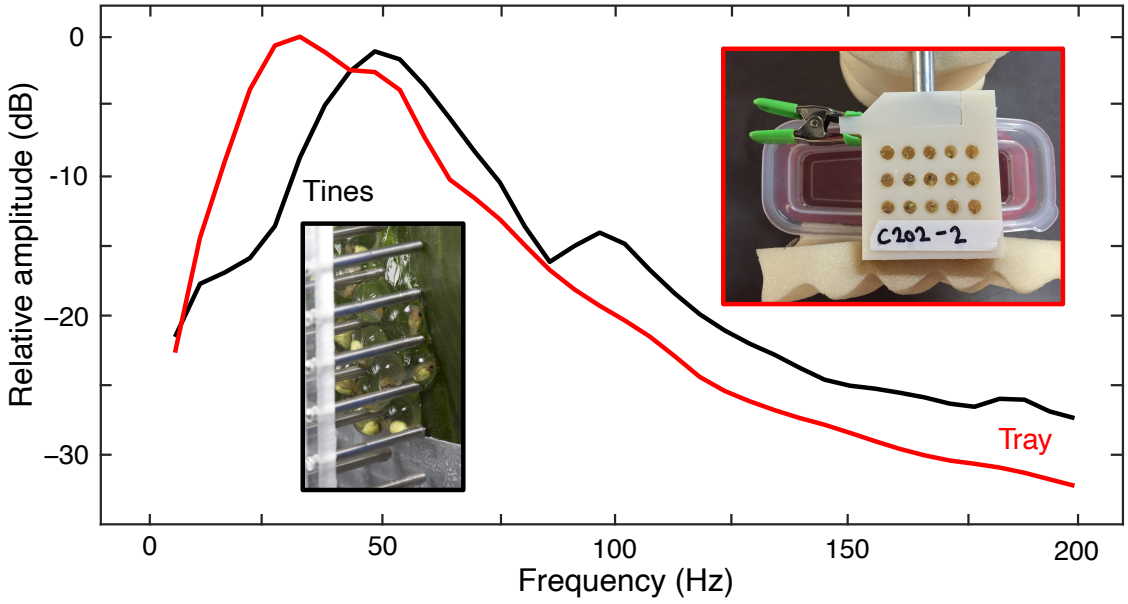

0
-10
−20
−30
Relative amplitude (dB)
0
50
100
150
200
Frequency (Hz)
Tines
Tray
C202-2

Figure 4.4

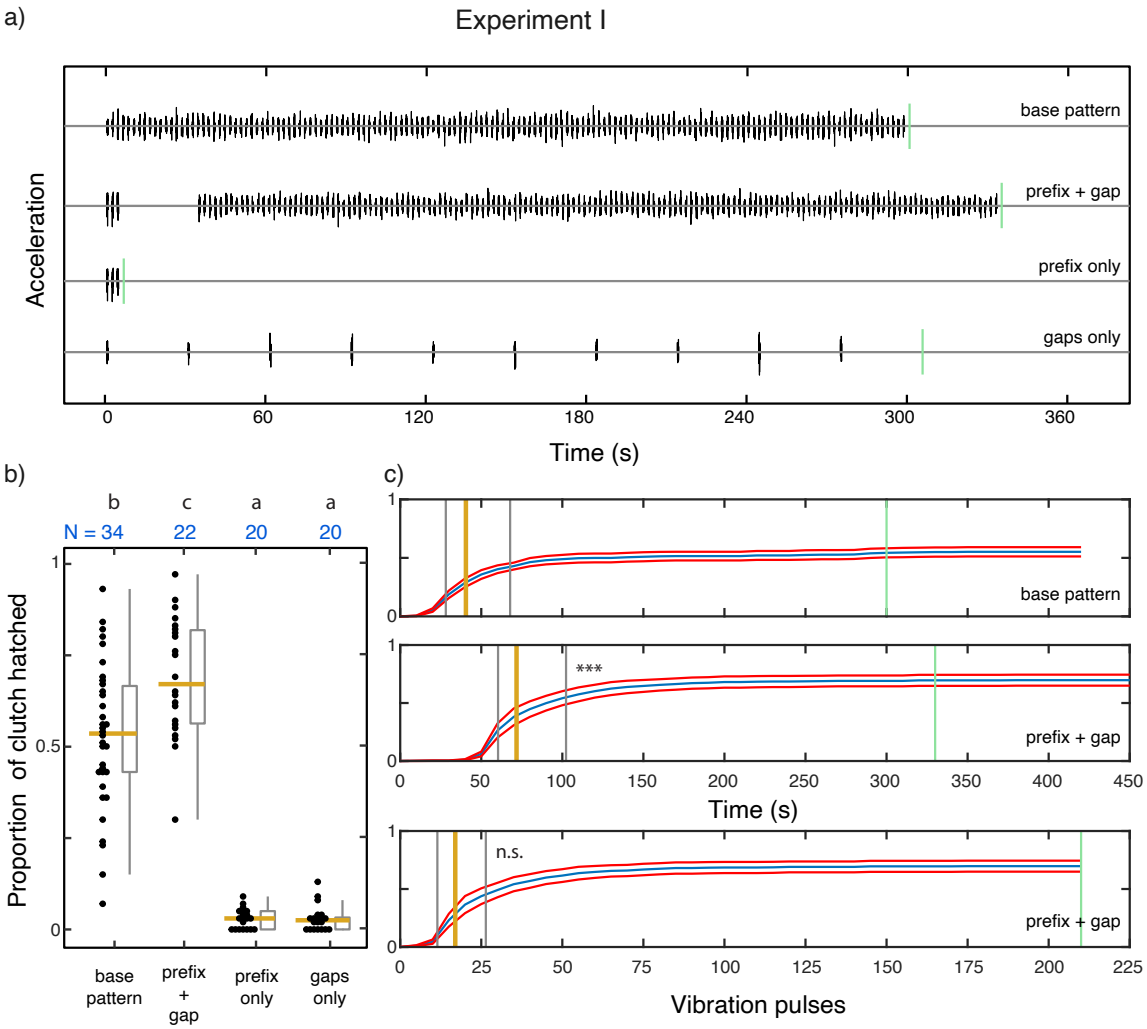

a)
Experiment I
Acceleration
base pattern
prefix + gap
prefix only
gaps only
0
60
120
180
240
300
360
Time (s)
b)
b
c
a
a
N = 34
22
20
20
Proportion of clutch hatched
1
0.5
0
base pattern
prefix + gap
prefix only
gaps only
c)
base pattern
***
prefix + gap
0
50
100
150
200
250
300
350
400
450
Time (s)
n.s.
prefix + gap
0
25
50
75
100
125
150
175
200
225
Vibration pulses

Figure 4.5

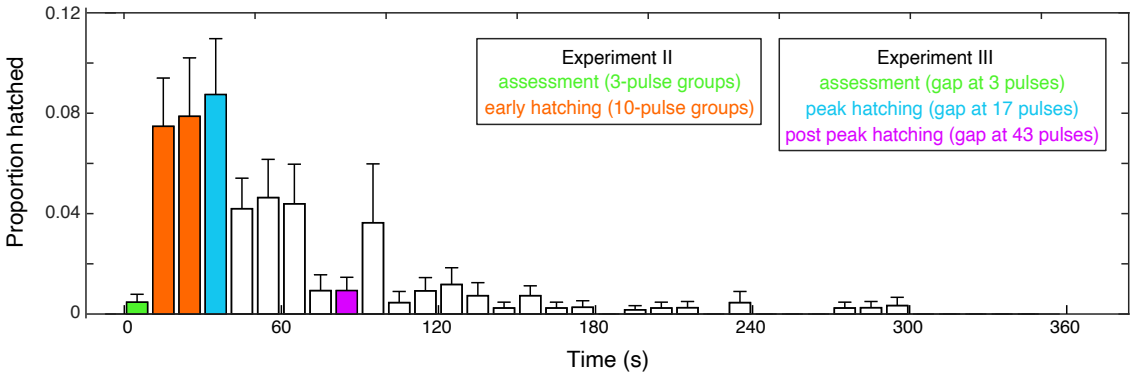

0.12
0.08
0.04
0
Proportion hatched
0
60
120
180
240
300
360
Time (s)
Experiment II
assessment (3-pulse groups)
early hatching (10-pulse groups)
Experiment III
assessment (gap at 3 pulses)
peak hatching (gap at 17 pulses)
post peak hatching (gap at 43 pulses)

Figure 4.6

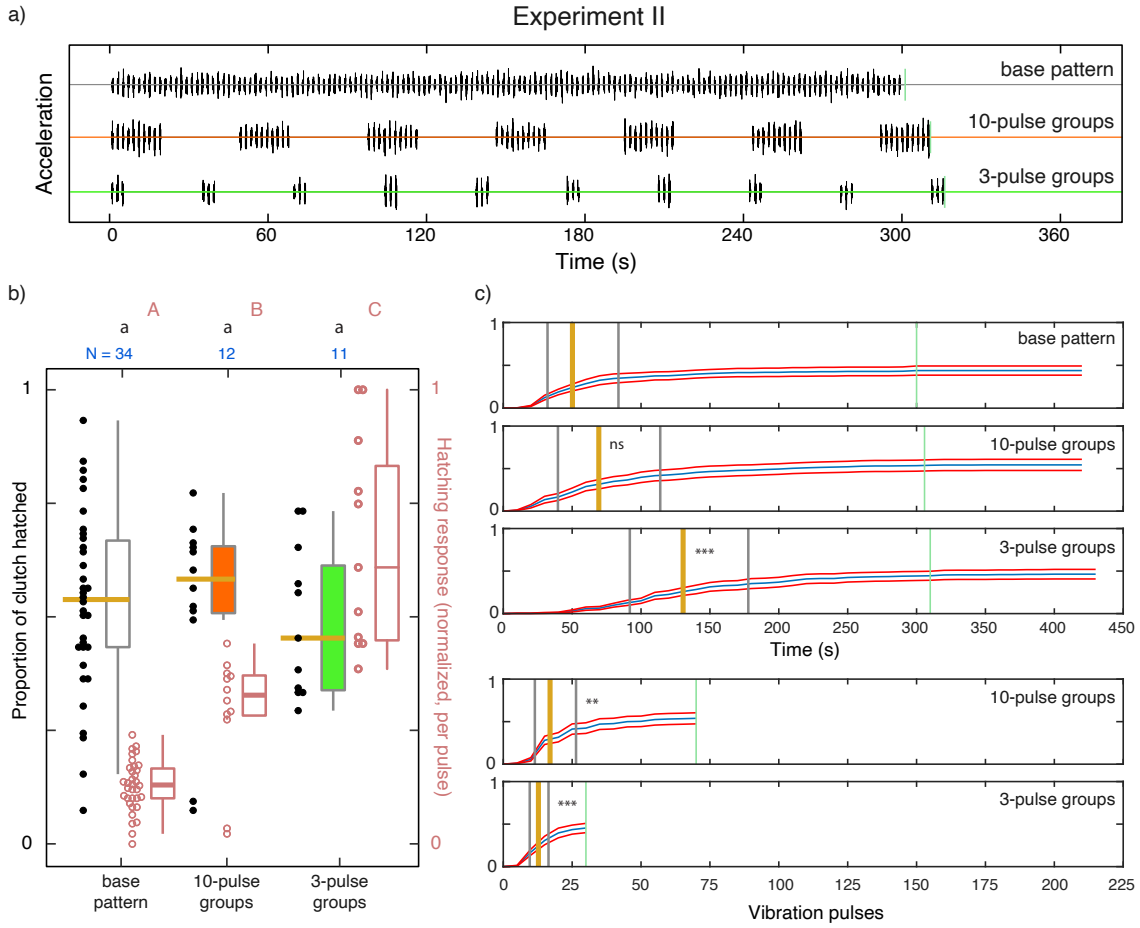

a)
Experiment II
Acceleration
base pattern
10-pulse groups
3-pulse groups
0
60
120
180
240
300
360
Time (s)
b)
A
B
C
a
a
a
N = 34
12
11
1
0
Proportion of clutch hatched
Hatching response (normalized, per pulse)
base pattern
10-pulse groups
3-pulse groups
c)
base pattern
ns
10-pulse groups
***
3-pulse groups
0
50
100
150
200
250
300
350
400
450
Time (s)
**
10-pulse groups
***
3-pulse groups
0
25
50
75
100
125
150
175
200
225
Vibration pulses

Figure 4.7

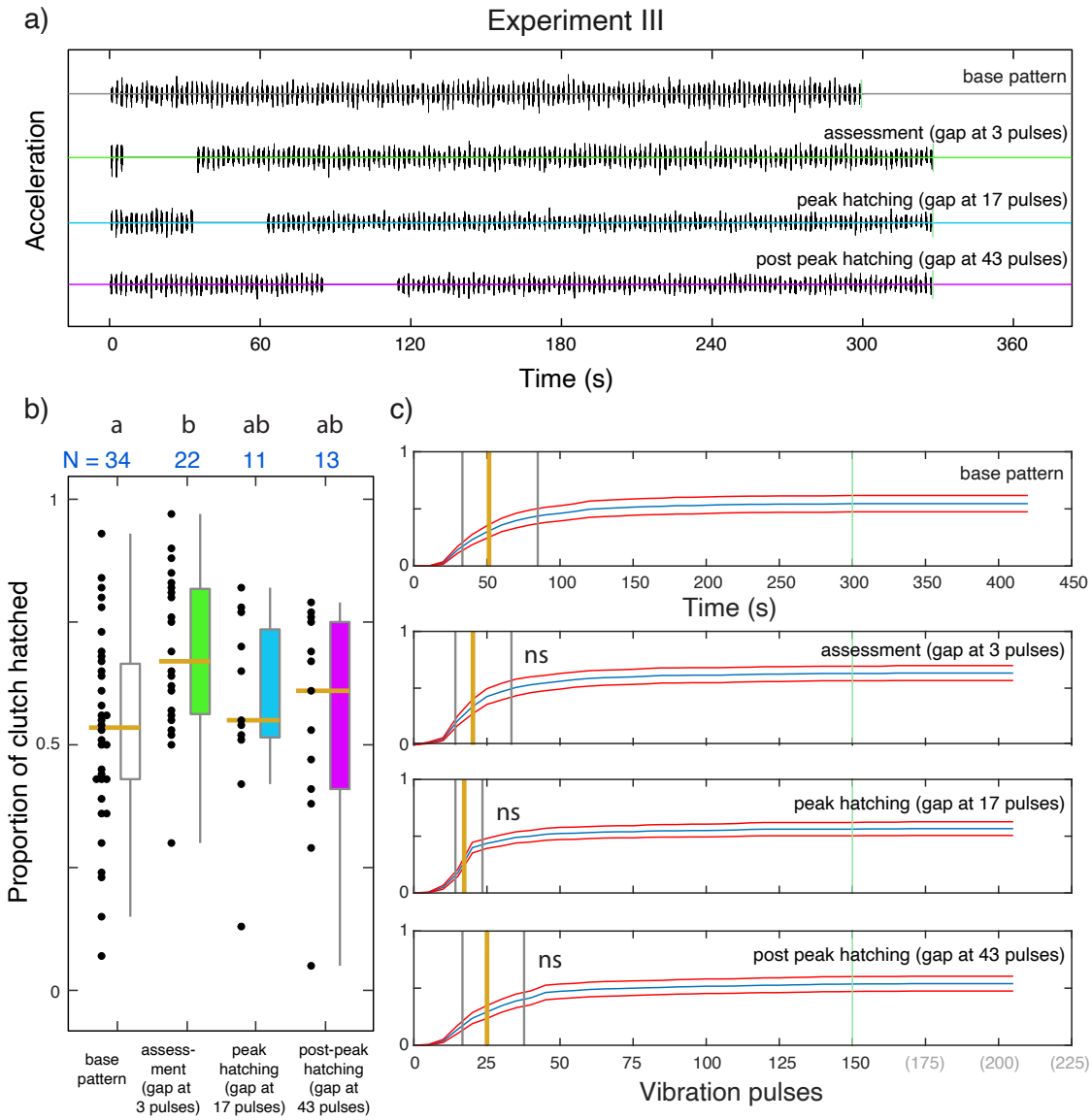
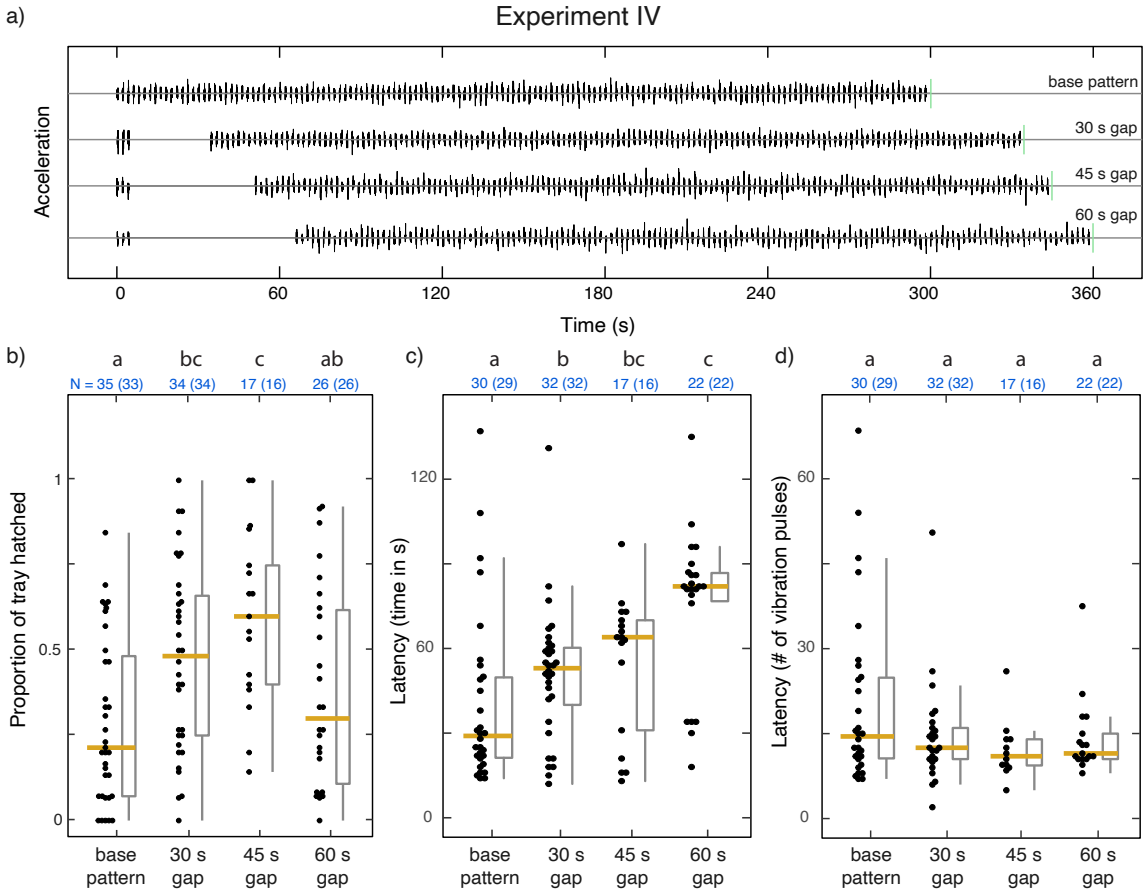

a)
Experiment III
Acceleration
base pattern
assessment (gap at 3 pulses)
peak hatching (gap at 17 pulses)
post peak hatching (gap at 43 pulses)
0
60
120
180
240
300
360
Time (s)
b)
a
b
ab
ab
N = 34
22
11
13
Proportion of clutch hatched
1
0.5
0
base pattern
assess-ment (gap at 3 pulses)
peak hatching (gap at 17 pulses)
post-peak hatching (gap at 43 pulses)
c)
base pattern
0
50
100
150
200
250
300
350
400
450
Time (s)
ns
assessment (gap at 3 pulses)
peak hatching (gap at 17 pulses)
post peak hatching (gap at 43 pulses)
0
25
50
75
100
125
150
(175)
(200)
(225)
Vibration pulses

Figure 4.8


a)
Experiment IV
Acceleration
base pattern
30 s gap
45 s gap
60 s gap
0
60
120
180
240
300
360
Time (s)
b)
a
bc
c
ab
N = 35 (33)
34 (34)
17 (16)
26 (26)
Proportion of tray hatched
1
0.5
0
base pattern
30 s gap
45 s gap
60 s gap
c)
a
b
bc
c
30 (29)
32 (32)
17 (16)
22 (22)
Latency (time in s)
120
60
0
base pattern
30 s gap
45 s gap
60 s gap
d)
a
a
a
a
30 (29)
32 (32)
17 (16)
22 (22)
Latency (# of vibration pulses)
60
30
0
base pattern
30 s gap
45 s gap
60 s gap

CHAPTER 5. ESCAPE-HATCHING DECISIONS SHOW ADAPTIVE ONTOGENETIC CHANGES IN HOW EMBRYOS MANAGE AMBIGUITY IN PREDATION RISK CUES

ABSTRACT

Behavior is perhaps the most flexible part of an animal's phenotype and depends critically on development. As animals develop, so do their capacities to sense cues, assess threats, and perform actions. Since development may also affect the relative costs and benefits that underlie behavioral decisions, we tested if the hatching decisions of red-eyed treefrogs, *Agalychnis callidryas*, in response to ambiguous cues follow adaptive predictions based on the changing costs of decision errors across development. These arboreal embryos hatch prematurely to escape from egg predators, cued by vibrations in attacks. Young embryos modulate hatching based on multiple frequency and temporal properties of cues, reducing false alarms that unnecessarily expose them to risk in the water. The cost of false alarms decreases developmentally. Therefore, we hypothesized that hatching responses to ambiguous cues would increase, accepting more false alarms. We tested this using vibration playbacks at two ages. We designed two sets of 3 stimuli, with matched sampling costs, so one elicited high hatching (positive control) and two elicited similarly low hatching but differed in ambiguity, based on prior results with younger embryos. Separately, we varied ambiguity in temporal pattern and in frequency spectrum. Older embryos hatched more and showed lower latency to hatch, indicating reduced cue sampling. They had similarly high hatching responses to both clear threat cues

and ambiguous stimuli but little response when either frequency spectrum or temporal pattern provided a clear indication of low risk. In both experiments, we saw the greatest ontogenetic change in response to the more ambiguous stimulus. Vibration-cued hatching allows us to use the power of playback experiments to improve our understanding of adaptive embryo behavior in response to ambiguity. Ambiguity in incidental cues is ubiquitous and developmental changes in behavior due to ontogenetic adaptation of decision processes are likely to be widespread.

INTRODUCTION

Behavioral decisions are pervasive and critical for survival in many contexts. However, responses to a given context often change as animals develop (Wiedenmayer, 2009). This occurs for at least four reasons. First, sensory development enables an animal to perceive, and thus respond to, a wider range of environmental stimuli (Romagny et al., 2012). Second, the development of motor capabilities first enables and subsequently improves the performance of many actions, from respiration and locomotion to communication (Bate, 1999). Both fundamentally change how animals can interact with their environments, gather information, and effect behavioral decisions. Third, experience enables learning to alter subsequent behavioral responses (Guibé et al., 2012; Harshaw and Lickliter, 2011; Wiedenmayer, 2009). Finally, the costs and benefits of behavioral decisions change developmentally; thus, ontogenetic adaptation can lead to stage-specific behavioral responses (Hall and Oppenheim, 1987). In this study, we test for adaptive ontogenetic change in behavioral decisions in the context of information ambiguity.

Information ambiguity in an uncertain world

The information from biotic and abiotic environments that animals use to inform their behavior is almost never perfect (Dall et al., 2005; Schmidt et al., 2010; Trimmer et al., 2011). Even stereotyped signals that are initially clear and honest because they have evolved to influence the behavior of others (Krebs and Dawkins, 1984; Tinbergen, 1952) can change and degrade during transmission due to the physical structure of the environment, and signals adapted for transmission in ancestral contexts may suffer greater degradation in altered environments (Boncoraglio and Saino, 2007; Elias and Mason, 2014; Mortimer, 2017; Morton, 1977; Richards and Wiley, 1980; Tobias et al., 2010). A receiver perceives this attenuated and degraded signal against a background of irrelevant energy that shares some features with the signal, such as frequencies, intervals, or other patterns. Moreover, not all signals are honest, since animals can evolve to deceive each other (Mokkonen and Lindstedt, 2016).

A large body of work addresses ambiguity in contexts such as dishonest signals or effects of environmental noise on communication (Maynard-Smith and Harper, 2003; Mokkonen and Lindstedt, 2016). However, we understand less about how animals cope with ambiguity in incidental cues generated by non-communicative activity. Such cues are critical for foraging and defense, although selection on the cue-producer may act to reduce their clarity or perceptibility (Velilla et al., 2020). Thus, compared to stereotyped signals, incidental cues may be fundamentally more variable, ambiguous, or cryptic (Getty and Krebs, 1985; Oberst et al., 2017; Wilcox et al., 1996). The perception of these non-stereotyped, incidental cues is also subject to and hampered by the same gamut of natural

and anthropogenic noises as stereotyped signals (Brumm and Slabbekoorn, 2005; Geipel et al., 2019; Ord et al., 2007; Schaub et al., 2008; Wu and Elias, 2014). Thus, animals face substantial variation in both signal and cue ambiguity when making decisions.

Signals and cues can be characterized by multiple parameters and properties, which may serve different purposes or differentially influence receiver responses (Hebets and Papaj, 2005; Ryan and Rand, 2001). For instance, in insects and anurans certain properties of acoustic signals function in species identification while others provide information about individual quality (Bailey, 1991; Ewing, 1989; Gerhardt, 1991; Gerhardt and Huber, 2002). Moreover, signal components can interact to affect receiver responses (Narins and Capranica, 1976; Taylor et al., 2011). Ambiguity may occur in multiple ways (Leavell and Bernal, 2019), including the state of individual cue properties or incongruence between different properties. Ambiguity affects the value of environmental information (Dall et al., 2005; Stevens, 2013) and imperfect information leads to increased uncertainty regarding local conditions among information receivers (Dall et al., 2005; Munoz and Blumstein, 2012). As a result, animals make imperfect estimates (Bouskila and Blumstein, 1992; Roitberg, 1990) and are prone to costly decision errors. We use a defensive behavior deployed based on incidental vibrational cues to test if responses to ambiguous cues depend more on the costs of defense than do responses to stimuli that more clearly indicate high or low risk level.

Environmentally cued hatching as a plastic behavior

When developing animals exit their egg capsule and enter the outside world, they exchange one set of risks and opportunities for another. Thus, hatching can serve as a defense against threats to eggs. The ability to alter hatching timing in response to environmental cues is taxonomically widespread (Warkentin, 2011b; Warkentin, 2011a), and physical disturbance is a particularly common cue for hatching. Vibrations from biotic sources such as siblings (Doody et al., 2012; Endo et al., 2019), parents (Mukai et al., 2012; Mukai et al., 2014), and hosts (Whittington and Kearn, 1988; Whittington and Kearn, 2011) or abiotic sources such as waves or weather (Griem and Martin, 2000; Martin et al., 2011; Roberts, 2001) can cue hatching. Physical disturbance might be a particularly salient cue in the context of egg predation, since predators cannot eat eggs without touching or moving them (Doody and Paull, 2013; Gomez-Mestre et al., 2008; Oyarzun and Strathmann, 2011; Warkentin, 2005). In some species, cued hatching occurs as a rapid response to a transient stimulus, enabling embryos to escape egg predators (Cohen et al., 2016; Warkentin, 1995) and exploit brief environmental opportunities (Martin et al., 2011; Whittington and Kearn, 1988). However, hatching at the wrong time can be costly, even lethal (Chivers et al., 2001; Li, 2002; Vonesh, 2000), and encapsulated embryos experience stimuli from many sources, not all associated with risk or opportunity. Thus, embryos deciding whether or not to hatch in response to a stimulus must assess information and balance fitness trade-offs across life stages.

Development alters the costs and benefits of hatching in several ways. As animals develop, their ability to cope with challenges outside the egg improves. For instance, more

developed hatchlings often have better locomotor performance (Buckley et al., 2005; Delia et al., 2019; Sih and Moore, 1993; Touchon et al., 2006) and better sensory abilities (Ball et al., 2016; Hempleman and Pilarski, 2011; Jung et al., 2019; Jung et al., 2020; Kawamura and Ishida, 1985; Romagny et al., 2012; Sandeman and Sandeman, 2003; Sisneros et al., 1998). In addition, the potential egg-stage benefits lost by hatching decrease developmentally; for instance, the remaining yolk reserves that may be lost by hatching decrease (Doody and Paull, 2013; Pezaro et al., 2013). Moreover, the egg environment imposes constraints on gas exchange, energy reserves, waste disposal, and space. Embryos that remain unhatched must, at some point, slow or cease development and eventually they will die upon exhaustion of energy reserves (Andrewartha, 1952; Gyllström and Hansson, 2004; Wourms, 1972). As the costs and benefits of hatching change across development, embryos should tailor their hatching responses to stimuli accordingly.

Red-eyed treefrogs as a study system to assess ontogenetic adaptation

The arboreal embryos of red-eyed treefrogs, *Agalychnis callidryas*, provide an excellent system for research on developmental changes in behavioral decisions. Undisturbed embryos typically hatch at age 6 or 7 d (Gomez-Mestre et al., 2013), but they can hatch in seconds to escape from egg-eating snakes, cued by vibrations in attacks (Cohen et al., 2016; Warkentin, 1995). They develop the ability to hatch at age 3 d (Warkentin et al., 2017) and the ability to sense egg motion at 4 d (Jung et al., 2019; Jung et al., 2020). This enables them to flee from egg predators and creates a discrimination challenge, because snakes are not the only force that shakes gelatinous egg clutches on

rainforest plants. In particular, rainstorms generate intense vibrations in egg clutches but pose no threat to eggs (Warkentin, 2005). Since vibrations are strongly shaped by their substrates (Michelsen et al., 1982), there is substantial overlap between the properties of egg clutch vibrations caused by predators and those caused by benign disturbances (Caldwell et al., 2009; Caldwell et al., 2010). Embryos must navigate this environmental ambiguity in order to make an informed decision to hatch.

To assess risk and distinguish among types of physical disturbance, embryos utilize both the temporal pattern (Warkentin et al., 2006b) and frequency spectrum of vibrations (Caldwell et al., 2009). We can create synthetic stimuli from pulses of noise that vary in temporal pattern and frequency spectrum, present them to embryos, and observe hatching responses (Warkentin et al., In press). Embryos hatch in response to low frequencies, excited in all egg-clutch disturbances, and not to high frequencies, which are prevalent in rain, absent in attacks, and inhibit hatching responses (Caldwell et al., 2009). Embryos also attend to temporal pattern elements, including the duration of and interval between vibration pulses (Warkentin et al., 2006b). Each of these vibration properties adds information, reducing the risk of decision errors (Warkentin and Caldwell, 2009). Moreover, embryos are sensitive to the cost of information sampling, basing decisions on more information when it is less costly (Warkentin et al., 2007).

What causes ontogenetic change in hatching responses?

Warkentin et al. (2019) proposed a framework associating patterns of ontogenetic change in environmentally cued hatching with different potential causes (Figure 5.1). Six

scenarios for how embryos could respond to different stimuli encompass a developmental increase in hatching ability or tendency (age effect, Figure 5.1a), effects of cues on hatching (stimulus effect, Figure 5.1b), their combination (age and stimulus effects, Figure 5.1c), and three kinds of non-independence of age and stimulus effects (age and stimulus interaction effects) due to increased discrimination (Figure 5.1d) and/or sensory ability (Figure 5.1e), as well as ontogenetic adaptation (Figure 5.1f). The critical prediction of the ontogenetic adaptation hypothesis is non-independence of stage and stimulus effects on hatching, with greater developmental change in response to more costly-to-assess and to more ambiguous stimuli.

Prior work using stimuli that varied in sampling cost found evidence of ontogenetic adaptation (Warkentin et al., 2019). For embryos passively gathering information *in ovo*, sampling costs vary when information accrues over time at variable rates and predation might occur at any moment during sampling (Warkentin et al., 2007; Warkentin et al., 2019). More generally, sampling costs may vary when animals gather information from the temporal patterns in stimuli, or when animals must perform certain actions, which take time and effort or entail risk, to gather information (Schoener, 1971; Warkentin and Caldwell, 2009; Warkentin et al., 2007). Ambiguity, however, may vary for a broader range of cue types and contexts; indeed, most information that animals receive can be unclear or ambiguous to some degree — even if sampling is cheap or if sampling costs are invariant — and greater sampling does not always lead to greater clarity (Cole, 1993; Getty and Krebs, 1985). If the cause of egg vibration is unclear, optimal embryo behavior depends on the relative mortality risks of missed cues and false alarms. Younger embryos, facing

higher false alarm costs, should rarely hatch in response to an ambiguous stimulus, while embryos near spontaneous hatching, facing low false alarm costs, should often hatch in response to the same stimulus (Warkentin et al., 2019). Thus, we expect younger embryos to treat ambiguous cues more like a clearly benign disturbance, and older ones to treat it more like a clear threat cue.

Here we test for ontogenetic adaptation in embryo hatching responses to vibrational cues that vary in ambiguity, controlling for sampling costs. Distinguishing adaptive ontogeny from simple increases in sensory, motor, or cognitive abilities requires the measurement of developmental changes in response to at least three stimuli (Warkentin et al., 2019). Thus, we used vibration playback experiments presenting sets of three stimuli, matched for sampling costs, to embryos at two ages. Our two experiments both included the same positive control, a clear threat cue that elicits high hatching of younger embryos. Each included a different negative control, which differed from the clear threat cue in either temporal pattern or frequency in ways that substantially reduce the hatching response of younger embryos. Each also included an ambiguous stimulus, with properties intermediate between the positive and negative controls. Some investigators have generated ambiguity by adding noise or mixing conflicting cues in the same stimulus (Brumm and Slabbekoorn, 2005; Geipel et al., 2019; Ord et al., 2007; Schaub et al., 2008; Wu and Elias, 2014). Others have used stimuli synthesized as multi-property intermediates between two signals known to elicit different responses or generated such signals to be intermediate in one property while keeping other properties constant (Bonachea and Ryan, 2011; Ryan et al., 2003; Winters et al., 2020). We followed the latter strategy to create ambiguity in two distinct

cue property domains: temporal pattern (E1) and frequency spectrum (E2), while controlling for sampling costs. Finding a greater developmental increase in hatching in response to an ambiguous stimulus, compared to positive and negative controls, would reveal adaptive ontogenetic change in embryos' use of information, and finding similar effects of ambiguity across two cue property domains would strengthen this case for ontogenetic adaptation.

METHODS

Egg collection and care

We collected leaves with newly laid (0–1 d) *A. callidryas* egg clutches from the Experimental Pond in Gamboa, Panama (9°7′15″N, 79°42′14″W) and transported them to a nearby ambient temperature and humidity laboratory at the Smithsonian Tropical Research Institute. There, clutches were mounted on plastic cards for support, positioned over aged tap water, and misted with rainwater at regular intervals to maintain hydration. All tested individuals were confirmed to be morphologically normal and staged based on external morphology (Warkentin et al., 2017). Since most clutches are laid between 10 pm and 2 am, we report developmental timing starting from midnight of their oviposition night (Warkentin, 2002; Warkentin, 2005). The first experiment varying ambiguity in temporal pattern (E1) was conducted from July 24 to August 4, 2017, and the second experiment varying ambiguity in frequency (E2) was from June 7 to June 22, 2018.

We tested embryos at two developmental periods, in the middle of their plastic

hatching period and close to spontaneous hatching, differing by about 20% in age since oviposition. Mechanosensory-cued hatching of *A. callidryas* begins almost a day before our "younger" test period (Jung et al., 2019; Jung et al., 2020; Warkentin et al., 2017), thus all tested embryos were clearly hatching-competent and highly vibration-sensitive. After running playback experiments (details below), we confirmed hatching ability in all tested individuals and returned them to the pond from which they were collected. This research was conducted under permits from the Panamanian Environmental Ministry (SE/A-55-17 and SC/A-10-18) and approved by the Institutional Animal Care and Use Committee of the Smithsonian Tropical Research Institute (2017-0601-2020-2).

Playback system

To determine if embryos exhibit adaptive ontogeny in their escape-hatching responses, we performed vibration playbacks to groups of embryos held in custom-made egg-trays (Warkentin et al., 2019; Warkentin et al., In press). This vibration playback system allows us to see and track individual embryo behavior, and work with eggs across a broad range of development. Trays held up to 15 eggs (minimum 6, mean ± SE, 11.58 ± 0.18 eggs per tray) in individual funnel-shaped spaces, allowing hatched tadpoles to slide through the tray to water below. We tested 1519 individuals from 128 trays and 64 clutches for E1 and 1422 individuals from 126 trays and 69 clutches for E2. For both experiments we tested at 2 points in development: midway through their plastic hatching period (E1 age: 5.16 ± 0.01 d, range 5.01–5.33 d; E1 stage: 30.32 ± 0.06, 30.0–32.0; E2 age: 5.34 ± 0.01 d, range 5.17–5.58 d; E2 stage: 30.90 ± 0.09, 30.0–32.0) and more developed

embryos, closer to spontaneous hatching, when false alarm costs are lower (E1 age: 6.01 ± 0.01 d, range 5.83–6.12 d; E1 stage: 33.63 ± 0.15, 31.33–35.67; E2 age: 6.28 ± 0.01 d, 6.18–6.39 d, E2 stage: 34.39 ± 0.14, 30.67–37.0). We moved eggs from clutches to individual spaces in egg-trays at embryonic age 3 d, while eggs could be easily handled without inducing hatching. Full trays were maintained on racks over aged tap water until testing, with regular misting until age 5 d (Warkentin et al., In press).

Playback methods followed published work detailing vibration presentation to embryos in egg-trays (Warkentin et al., 2019; Warkentin et al., In press). For testing, we clamped egg-trays holding embryos to a custom-made interface on a rigid post attached to an electrodynamic minishaker (E1: Model 4810, E2: LDS V203, Brüel & Kjær, Nærum, Denmark). The shaker, post, and tray were horizontally leveled, with foam supports under the post and tray edge and water under the embryos. Thus, embryos were moved horizontally and hatchlings fell into the water below. Shaker output was controlled by Audacity 2.1.0 on a 2014 Macbook Air, via an external sound card (MSE-U33HB, Onkyo, Japan) and a custom-made amplifier (E. Hazen, Boston University Electronic Design Facility). We constructed a transfer function for the playback system by recording playbacks using an AP32 accelerometer powered by an APC7 signal conditioner (AP Technology International, Oosterhout, The Netherlands), Onkyo MSE-U33HB external sound card (Onkyo Corporation, Osaka, Japan), and RavenPro 1.3 (Cornell Lab of Ornithology, Ithaca, NY) on a MacBook Pro. We recorded any hatching induced by the set-up procedure during a five-minute acclimation period before starting the playback (N=272 individuals). Individuals that hatched before the stimulus started were not

considered part of the test. We noted if and when (to the nearest second) each embryo hatched during the playback period and a 3-min post-playback observation period (N=39 individuals hatched post-playback). We confirmed hatching competence of embryos remaining unhatched by manually stimulating them with a blunt metal probe (N=1702 individuals). We staged 3 haphazardly selected hatchlings from each tray (Warkentin, 2017).

Experimental design and stimulus creation

For each experiment, we designed a set of three synthetic vibration stimuli based on prior playback results with 5 d old embryos, which revealed that low frequencies stimulate while high frequencies inhibit hatching (Caldwell et al., 2009; Caldwell et al., 2010) and that both vibration pulse duration and interval (but not cycle length or duty cycle) strongly and non-redundantly affect hatching (Warkentin et al., 2006b; Warkentin et al., 2007). All stimuli consisted of vibration pulses with roughly rectangular amplitude envelopes, separated by intervals of silence. To equalize sampling costs, all stimuli across both experiments had a cycle length of 2 s (Warkentin et al., 2019).

Stimuli for the first experiment (E1), varying ambiguity in temporal pattern (Figure 5.2a-b), all used a relatively narrow spectrum of low frequencies, characteristic of predator attacks (Figure 5.2c), but varied vibration pulse duration and interval length. A pattern of 0.5 s vibration: 1.5 s interval, which represents the peak of the hatching response surface based on prior playbacks to 5-day-old embryos (Jung et al., 2019; Warkentin et al., 2006b; Warkentin et al., 2017; Warkentin et al., 2019), served as our clear cue for high risk (Figure

5.2a-b). The other two stimuli included relatively longer vibrations, each designed to yield less hatching at the earlier test age (Figure 5.2a-b). A 1.5 s: 0.5 s pattern was chosen to elicit low proportions of embryos hatching, based on a systematic study of hatching responses to various temporal patterns (Warkentin et al., 2006b), and served as our clear cue for low risk, while the 1 s: 1 s pattern acted as our ambiguous cue, as in both hatching response and temporal pattern space it is intermediate between the two clear cues (Figure 5.2a-b). We specifically chose stimuli for which temporal patterns with longer periods of vibration (i.e., longer duty cycles) elicited less hatching to ensure that any increases in hatching response could be attributed to the specific pattern rather than cumulative stimulation.

Stimuli for the second experiment (E2), varying ambiguity in frequency, all used a 0.5 s vibration: 1.5 s interval temporal pattern but varied in frequency spectrum (Figure 5.2d). As the clear cue for high risk, we used a relatively narrow band of low frequencies, characteristic of predator attacks and chosen to elicit high proportions of embryos hatching based on prior playbacks to 5-day-old embryos (Figure 5.2c) (Caldwell et al., 2009). As the clear cue for low risk, we used a broader distribution of frequencies resembling rain but with the lowest frequencies attenuated, designed to yield low hatching at the earlier test age (Figure 5.2c). As our ambiguous cue, we designed an intermediate stimulus with frequencies intermediate between the two clear cues, similar to the broadband stimulus, but with some attenuation of high frequencies (Figure 5.2d).

Both sets of stimuli were generated as bandpass-filtered white noise with the desired duration and interval structure in Matlab. We then adjusted frequency ranges as

needed to achieve desired spectra (Caldwell et al., 2009) and set playback amplitude in Audacity to equalize the RMS acceleration of the noise portions of the stimuli, based on analysis of recordings in RavenPro 1.3 (Warkentin et al., 2019; Warkentin et al., In press). From test playbacks of continuous noise, RMS acceleration of the stimuli in both experiments was 14 ms^{-2}. For comparison, the amplitude threshold for vibration-cued hatching is less than 0.1 ms^{-2} at 5 d and even lower at 6 d (Jung et al. in prep).

Statistics

We used generalized linear mixed models within the 'lme4' package (Bates et al., 2015) with clutch as a random effect and likelihood ratio tests to compare nested models for fixed (age, stimulus) and interaction effects (age x stimulus) on hatching responses (binomial) and latency to first hatching in each tray (gamma). We followed this with multiple comparisons of planned contrasts to test hypothesized patterns using simultaneous tests for general linear hypotheses. All statistical tests were carried out in the R statistical environment (version 3.6.2, R Development Core Team 2019, http://www.r-project.org) in RStudio (version 1.2.5033, RStudio Team 2019).

RESULTS

In trays where embryos hatched during playback, latency until first hatching ranged from 7 to 252 s in E1 (Figure 5.3a) and 5 to 240 s in E2 (Figure 5.3b), and no embryos attempted to hatch but failed. In both experiments, latency to hatch decreased with age (E1

χ^2=72.89, df=1, P<0.0001; E2 χ^2=44.13, df=1, P<0.0001), such that older embryos hatched sooner (Table 5.1, Figure 5.3a-b). In E1, there was no stimulus effect (χ^2=1.15, df=2, P=0.56) or interaction effect (χ^2=0.15, df=2, P=0.93) on latency to hatch (Table 5.1, Figure 5.3a). In E2, there was a stimulus effect (χ^2=28.08, df=2, P<0.0001) but no interaction effect (χ^2=0.90, df=2, P=0.64) on latency to hatch (Table 5.1, Figure 5.3b). Younger embryos hatched sooner in response to very low frequencies as compared to intermediate and broad frequencies (Figure 5.3b, Z=4.27, P<0.001), which were themselves different from each other (Figure 5.3b, Z=3.60, P=0.0013); however, this distinction changed with development such that older embryos hatched sooner in response to both low and intermediate frequencies (Figure 5.3b, Z=5.17, P<0.001), which had similarly low latency (Figure 5.3b, Z=1.95, P=0.13).

Hatching responses of younger embryos were detectable for all stimuli in both experiments (95% CI of proportion hatched did not include 0) and no stimulus in either experiment elicited complete hatching of older embryos (95% CI did not include 1). Stimulus, age, and their interaction affected the hatching response of embryos to playbacks in both experiments (Table 5.1, Figure 5.3c-d). Within each set of stimuli, the differences in temporal pattern (Figure 5.3c, E1 χ^2= 16.21, df=2, P=0.0003) and frequency spectrum (Figure 5.3d, E2 χ^2=64.17, df=2, P<0.0001) elicited variation in hatching responses, as planned (Table 5.1). In both experiments, as expected, older embryos hatched more than did younger ones (Table 5.1, Figure 5.3c, E1 χ^2=99.67, df=1, P<0.0001; Figure 5.3d, E2 χ^2=35.92, df=1, P<0.0001). However, the developmental increase in hatching was not

uniform across stimuli (Table 5.1, interaction: E1 χ^2=12.80, df=2, P=0.0017; E2 χ^2=7.89, df=2, P=0.0193). In E1, younger embryos hatched more in response to the clear cue for high risk (0.5:1.5) as compared to the clear cue for low risk (1.5:0.5) and the ambiguous (1:1) cue (Figure 5.3c, Z=3.05, P=0.0072), which were themselves different from each other (Figure 5.3c, Z=2.49, P=0.0364). Older embryos hatched less in response to the clear cue for low risk than both the clear cue for high risk and the ambiguous cue (Figure 5.3c, Z=3.89, P<0.001), which had similarly high hatching rates (Figure 5.3c, Z=1.47, P=0.3163). In E2, younger embryos hatched more in response to the low-frequency (clear cue for high risk) than to the intermediate and broad frequencies (clear cue for low risk) (Figure 5.3d, Z=7.28, P<0.001), which were themselves different from each other (Figure 5.3d, Z=2.92, P=0.0107); while older embryos hatched less in response to the broad frequencies than to both the low frequencies and the ambiguous intermediate frequencies (Figure 5.3d, Z=6.75, P<0.001), which were themselves different from each other (Figure 5.3d, Z=5.23, P<0.001).

DISCUSSION

Embryos adjust hatching decisions to ambiguous cues in predictably adaptive ways

Our results reveal adaptive ontogenetic change in the hatching response of *A. callidryas* embryos to ambiguous vibrational cues. We report a developmental increase in overall hatching response, decrease in cue sampling, and specific patterns of discrimination among stimuli that change as predicted based on levels of ambiguity in cues. When faced

with an ambiguous cue, younger embryos hatch relatively little, as predicted from the high cost of false alarms early in development. Because false alarms become less costly with development (Touchon et al., 2013; Warkentin, 1995; Warkentin, 1999; Willink et al., 2014) older embryos should, and did, hatch more readily in response to ambiguous cues. The interaction effect revealing greater developmental change in response to stimuli with greater ambiguity, in both temporal and frequency domains, provides strong evidence for ontogenetic adaptation in behavioral decision rules.

To assess effects of cue ambiguity on hatching, we designed our experiment to minimize the possibility that older embryos would hatch more and faster simply due to better hatching ability or sensory ability (Figure 5.1a, e). First, developmental differences in hatching response and latency between our younger and older embryos clearly do not reflect differences in hatching ability. Our younger embryos were tested at age 5 d, two days after the onset of hatching competence (Warkentin et al., 2017) and 1 d after the transition between the early and late-appearing hatching gland cell types (Cohen et al., 2019). Thus, both younger and older embryos were using the same type of well-developed hatching effectors. Moreover, all tested individuals were confirmed to be fully hatching-competent. No embryo that attempted to hatch during experiments failed to do so, and we manually induced hatching of all remaining embryos at the end of each experiment. Although hatching performance and speed do increase over the plastic hatching period, and the duration of cue sampling in physical disturbance and the fraction of hatching latency that this sampling represents both decrease from age 5–6 d, no element of hatching performance improves during this timeframe (Güell, B.A., J. Jung, A. Almanzar, J.

Cuccaro-Díaz, and K.M. Warkentin; in prep). Additionally, if there were developmental effects on hatching ability or performance they would manifest as a difference between ages; differences in hatching response and latency at the same age clearly reflect some other cause.

Second, vestibular and lateral line mechanisms of mechanosensing develop a day or more before our younger test age (Jung et al., 2019; Jung et al., 2020). Although sensitivity to lower amplitude stimuli increases developmentally, we set playback amplitudes over two orders of magnitude higher than the response threshold for 5-d embryos (Jung et al., 2017) to avoid any sensory limitation. For our temporal pattern experiment, we designed a stimulus set in which patterns with higher duty cycle and longer periods of vibration elicited less hatching of 5-d embryos (Figure 5.2a-b) to ensure that lower hatching responses (Figure 5.3c) reflected embryo decisions and could not simply be attributed to a lower amount of stimulation. We also designed our frequency experiment based on prior playbacks, using ecologically relevant frequencies for which behavioral responses of 5-d embryos have been demonstrated (Caldwell et al., 2009; Caldwell et al., 2010; Warkentin et al., 2006b). For these reasons, we consider it extremely unlikely that lower hatching response rates reflect limitations of sensory ability in either age group.

If sensory or cognitive development had improved embryos' abilities to perceive differences in some cue properties, more developed embryos would show increasingly distinct responses to different cues (Figure 5.1d). However, discrimination among stimuli did not, overall, increase with development in either experiment. Both younger and older embryos discriminate among cues, but they do so in different ways (Figure 5.3). Younger

hatchlings face a high risk of predation in ponds. Nonetheless, as embryos, they showed a substantial hatching response to the stimulus we designed to indicate high risk to eggs. Older embryos, close to spontaneous hatching, face a low cost of false alarms. However, in both experiments, they hatched much less in response to the stimulus we designed as a clear indicator of low risk, providing evidence that their hatching response still involves risk-assessment. While, based on a hypothesis of cognitive development, it might be plausible to argue that younger embryos were unable to discriminate between two cues in each experiment, and later gained that ability, this cannot explain reduced discrimination between some cues as embryos developed.

Across both experiments, embryos' hatching responses to clearer and more ambiguous cues changed developmentally in alignment with *a priori* predictions from the hypothesis of adaptive ontogeny, showing greater developmental change in response to the more ambiguous stimuli. We varied two very different vibration properties — frequency spectrum and temporal pattern — to design stimuli that differed in ambiguity while controlling for sampling costs. These two experiments each independently demonstrated increased stage-dependence of responses to ambiguous stimuli, compared to clearer cues of high or low risk. Prior work had indicated adaptive ontogenetic change in hatching decisions in response to varying sampling costs (Warkentin et al., 2019). This study expands our understanding of adaptive ontogeny to a novel type of variation, addressing the quality of information as distinct from its cost. The consistency of results across these studies, and within the present study, provides strong support for the hypothesis of adaptive ontogenetic change in risk assessment and hatching decision rules.

Different ways to present ambiguity yield different patterns of response latency

The latency from stimulus onset to hatching is a useful indicator of cue sampling. In both experiments, younger embryos delayed hatching for longer than older embryos (Figure 5.3a-b), indicating that older embryos take less time to assess cues and base their decision to hatch on less information. In general, we might expect inverse patterns of behavioral responses and latencies to respond, such that stimuli that elicit higher response rates also elicit faster responses (Charlton et al., 2008; Warkentin et al., 2019). However, latency patterns differed between experiments that varied ambiguity in temporal pattern vs. in frequency spectrum. Information from these vibration properties accrues at different rates; samples of pulse duration and inter-pulse interval accrue event by event at a rate dependent on cycle length (i.e. one sample per cycle), while frequency information is continuously available throughout periods of vibration and absent during intervals. We found an inverse relationship between hatching response and latency when we varied frequency and held temporal pattern constant, such that frequency information accrued at the same rate across stimuli (E2, Figure 5.3b). We found no such relationship when we varied the duty cycle (pulse length) of the temporal pattern, holding cycle length and frequency spectrum constant (E1, Figure 5.3a). In both experiments, the constant property was indicative of risk, and the variable property could indicate high or low risk, or be ambiguous. Thus, in both the clear cue of low risk and (to some extent) the ambiguous cue, frequency and temporal properties provided conflicting information (another form of ambiguity). When faced with conflicting information from two cue properties, animals must choose one to inform their behavioral decision.

The more rapid accumulation of frequency information, compared to temporal pattern information (Warkentin and Caldwell, 2009; Warkentin et al., 2007; Warkentin et al., 2019), may explain two patterns in our data. First, in E1, the cue conflict between frequency and temporal properties seems to decouple the linkage between more and faster hatching (Figure 5.3a, c). In fact, the fastest hatching, for younger embryos, occurred with the pattern that ultimately elicited the least hatching. This pattern had the longest vibration pulses, thus exposed embryos to a rapid accumulation of vibration at frequencies indicating high risk, but a slower accumulation of temporal pattern information indicating low risk. In this situation, assuming some variation in estimation of post-hatching risk, individuals perceiving lower false alarm costs may cross a threshold to hatching based on frequency cues before sufficient temporal pattern information accrues to lower their estimate of egg-stage risk. This would yield low response latencies for the first embryos to hatch, then a decreasing response as conflicting temporal pattern information accumulated to decrease estimated risk. In contrast, hatching would increase over time if accumulating temporal pattern information confirmed initial frequency-based assessment of high risk, pushing more embryos over the decision threshold. Our data are consistent with this scenario; however, it could be tested more directly by comparing latency for all embryos that hatch. Second, older embryos show a difference in latency in response to stimuli varying in frequency (E2) but not for stimuli varying in duty cycle (E1). This may be because older embryos have lower false alarm costs and so choose to hatch quickly in response to most stimuli unless the property indicating low risk is quick to assess, as in frequency or fast temporal patterns with short cycle lengths (Warkentin et al., 2019).

Latency to choose is widely used as an indicator of information processing and sampling in animals (Balci et al., 2009; Judge et al., 2014; Lindström and Lehtonen, 2013; Noorani, 2014; Rhebergen et al., 2015). In earlier work, examining latency showed that embryos make hatching decisions based on less information when that information accrues more slowly (Warkentin et al, 2007). We recently used latency to hatch to detect multimodal vestibular and lateral line contributions to hatching decisions in a context where 100% of tested embryos hatched, and to reject a hypothesized role for lateral line input in another context where most embryos hatched (Jung et al., 2020). Here, latency reveals nuances of how embryos make decisions based on conflicting information from cue properties with different rates of information delivery, as well as changes in how embryos use different vibration properties across development. In other work (Jung, J., M. Guo, M.E. Crovella, J.G. McDaniel, and K.M. Warkentin; in prep.), we used latency to assess how information about risk accrues over silent periods during intermittent vibrational disturbance. While the total proportion of embryos hatched provides a convenient metric and overall indicator of risk perception, the timing of hatching offers additional insight into the decision-making process. This may be generally useful for species with a rapid hatching process, for which cue assessment represents a substantial portion of the period between stimulus onset and response. For such species, latency data can improve our understanding of the risk-assessment process underlying hatching decisions.

Conclusion

Substantial research has shown that *A. callidryas* embryos midway through the plastic hatching period use multiple temporal and frequency properties of vibrational cues to assess risk (Caldwell et al., 2009; Caldwell et al., 2010; Warkentin, 2005; Warkentin et al., 2006b). In addition, these embryos alter their sampling of those cues with the cost of information and the developmentally changing cost of false alarms (Warkentin et al., 2007; Warkentin et al., 2019). Variation in sampling costs is particularly relevant for animals assessing environmental cues that accrue gradually over time at variable rates. However, cue ambiguity is broadly relevant across most information assessment contexts, including communication and use of incidental cues (Wiegmann et al., 2010). Taking advantage of developmentally changing risk trade-offs, and controlling sampling costs, we demonstrate that *A. callidryas* embryos adjust their behavioral decisions in response to cue ambiguity. Thus, embryos modulate their use of information based not just on its cost but also on its quality. This adds a new dimension to the demonstrated adjustments of embryos' hatching decisions in predictably adaptive ways and highlights the value of hatching for research in decision making, information use, and animal behavior more broadly.

This work also motivates further tests of predictions from an ontogenetic adaptation framework. We introduced ambiguity by presenting embryos with synthetic playback stimuli intermediate between indicators of low and high risk, in either temporal or frequency domains. Other forms of ambiguity could be generated by imposing background noise over stimuli (e.g., combining recorded snake and rain vibrations) or by mixing stimulus components and properties that provide conflicting information (Brumm and

Slabbekoorn, 2005; Geipel et al., 2019; Ord et al., 2007; Schaub et al., 2008; Wu and Elias, 2014). Since different cue components likely provide different information in different contexts and potentially interact differently when combined (Hebets and Papaj, 2005; Narins and Capranica, 1976; Taylor et al., 2011), further experimentation exploring different manifestations of ambiguity would likely be informative.

Moreover, across taxa, the development of behavioral capabilities includes changes in multiple underlying mechanisms, affected by multiple selective forces. Here, to assess adaptive change in decision rules, we focused on changes in *A. callidryas* behavior from midway to late in the plastic hatching period. However, other factors may be the dominant causes of changes in behavior across other developmental periods. For instance, the early onset of hypoxia-cued hatching demonstrates that hatching ability develops before the onset of mechanosensory-cued hatching, which is limited by the development of mechanosensory lateral line and vestibular systems (Jung et al., 2019; Jung et al., 2020; Warkentin et al., 2017). Early changes in hatching responses to physical disturbance, therefore, strongly reflect sensory development while later changes seem more likely to reflect ontogenetic adaptation in decision-making processes. Rapid hatching responses to variable environmental conditions or transient stimuli are widespread across taxa (Doody and Paull, 2013; Doody et al., 2012; Endo et al., 2019; Griem and Martin, 2000; Martin et al., 2011; Mukai et al., 2012; Mukai et al., 2014; Warkentin, 2011b; Warkentin, 2011a; Whittington and Kearn, 1988; Whittington and Kearn, 2011). We suggest that researchers examine ontogenetic changes in embryo behavior in other species that exhibit such environmentally cued hatching; such species likely offer other experimentally tractable

opportunities for investigating developmental changes in animal behavior, as well as more general questions about behavioral decisions. Across these diverse taxa, sensory development likely changes how animals perceive and respond to cues over relevant ontogenetic periods (Hamann et al., 2004; Ison et al., 1998; Romagny et al., 2012); maturation of effectors and neural circuitry may improve the performance of critical behaviors (Barsz et al., 2002; Bate, 1999); and/or learning may also affect the likelihood of a response (Guibé et al., 2012; Harshaw and Lickliter, 2011). These diverse factors likely affect behavioral responses across different subsets of ontogeny, and thus could be disentangled using similar approaches as we have employed with *A. callidryas,* collectively improving our understanding of behavioral development.

TABLE AND FIGURE LEGEND

Figure 5.1 – Alternative hypotheses and predicted patterns for how development (age), environmental cues or contexts (stimulus), and/or their interaction may affect hatching responses. Lines within panels represent responses to different stimuli. Embryos might show (a) a developmental increase in hatching, (b) different responses to different stimuli, or (c) a combination of the two. If embryos demonstrate non-independence or interaction of age and stimulus effects, we may see a developmental (d) increase or (e) decrease in discrimination between cues. Testing responses to three different stimuli at two developmental stages allows us to distinguish our (f) hypothesis of adaptive ontogeny from non-adaptive causes of developmental changes in hatching response. Significant sources ($P < 0.05$) are highlighted in bold italics, while non-significant sources ($P > 0.05$) are stricken. Predictions figure is adapted with permission from Warkentin et al. (2019).

Figure 5.2 – Sets of vibration playback stimuli varying ambiguity in either temporal pattern (Experiment 1) or frequency (Experiment 2). (a) Contour plot of *A. callidryas* hatching response to vibration playback stimuli varying in disturbance duration and interval. Blue to yellow shading indicates mean proportion hatched in response to test stimuli of various durations and intervals, shown in black or color-filled (stimuli for E1) points. Figure is adapted with permission from Warkentin et al. (2006). (b) Waveforms of portions of the stimuli for E1, showing shared cycle length of 2-s and varied temporal patterns (vibration durations and intervals). All E2 stimuli had the 0.5: 1.5 s pattern. (c)

Average relative distribution of energy across frequencies for vibrations in *A. callidryas* egg clutches during predator attacks (snakes: *Leptodeira annulata*, N=17; *Leptodeira septentrionalis*, N=11; *Leptophis ahetulla*, N=13; wasp: *Polybia rejecta*, N=18) and rainstorms (N=19). Data are means across recorded clutch disturbances, standardized to peak power, and 95% confidence intervals. Figure is adapted with permission from Caldwell et al. (2010). (d) Frequency spectra for E2 stimuli, showing peaks at very low (25 Hz) vs. low (90 Hz) frequencies and different amounts of energy at higher frequencies.

Figure 5.3 – Hatching responses of *Agalychnis callidryas* embryos to vibration playbacks at two ages (mean ages E1 5.2 and 6.0 days; E2 5.3 and 6.3 days). Experiments were conducted in different years with different stimulus sets, varying in temporal patterns (E1) and frequency spectra (E2). Stimuli were designed based on documented responses of younger embryos with properties to elicit high hatching (red triangles) or lower hatching (orange squares, green circles), and to be either clearer (red triangles, green circles) or more ambiguous (orange squares). Data are mean latency for the first embryo to hatch in a tray (a, b), for trays from which at least one embryo hatched during playback (± SE across egg trays) and mean proportions of eggs hatched per tray (c, d). Results of planned contrasts after binomial GLM are indicated in gray; ns = $P > 0.05$, * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$. Significant sources ($P < 0.05$) are highlighted in bold italics, while non-significant sources ($P > 0.05$) are stricken.

Table 5.1 – Summary of statistical analyses for sources of variation in latency to first hatch and proportion hatched in experiments varying ambiguity in either temporal pattern (E1) or frequency (E2).

Figure 5.1

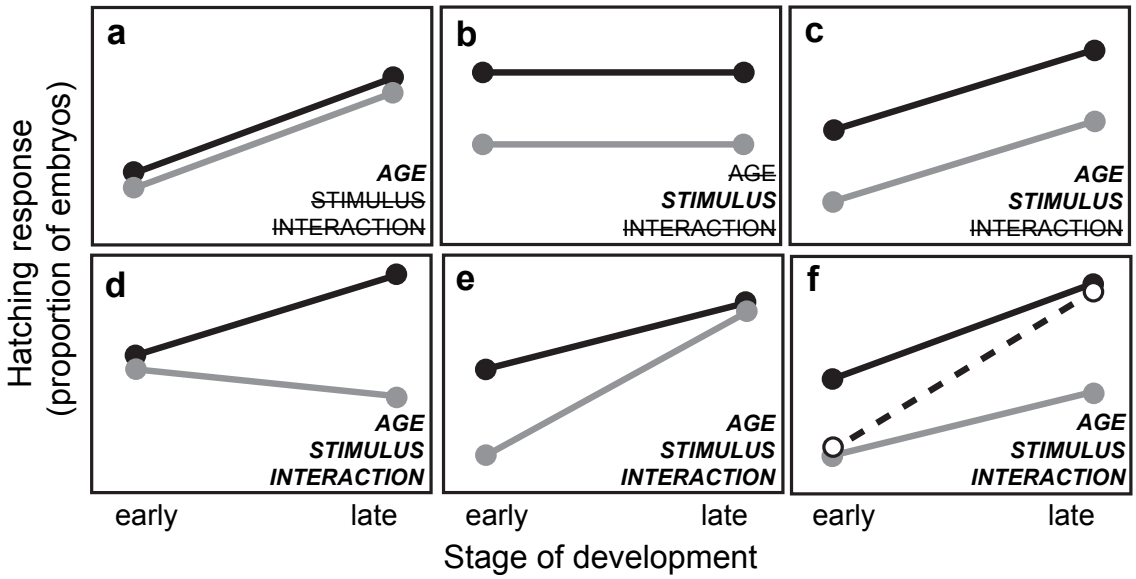

a
AGE
~~STIMULUS~~
~~INTERACTION~~
b
~~AGE~~
STIMULUS
~~INTERACTION~~
c
AGE
STIMULUS
~~INTERACTION~~
d
AGE
STIMULUS
INTERACTION
e
AGE
STIMULUS
INTERACTION
f
AGE
STIMULUS
INTERACTION
Hatching response
(proportion of embryos)
early
late
early
late
early
late
Stage of development

Figure 5.2

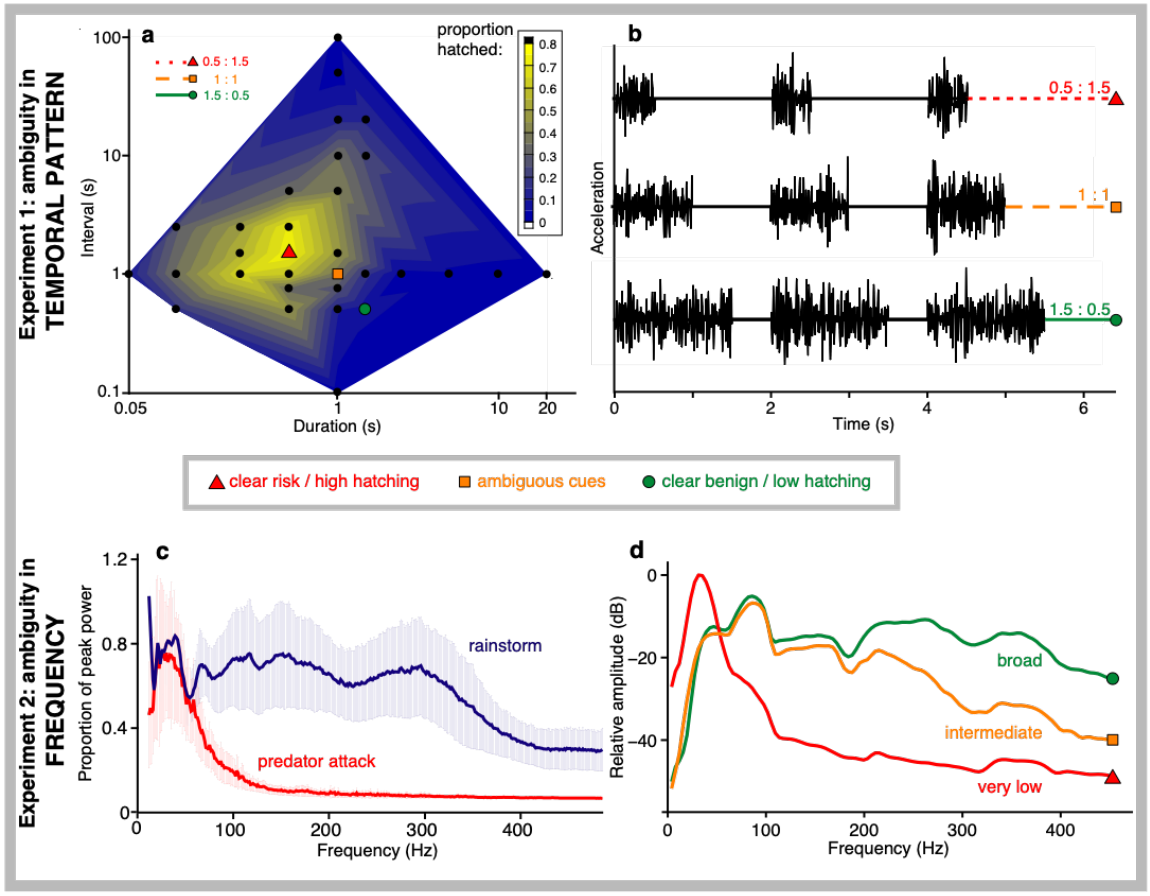

Experiment 1: ambiguity in TEMPORAL PATTERN
a
proportion hatched:
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0.5 : 1.5
1 : 1
1.5 : 0.5
Interval (s)
100
10
1
0.1
0.05
1
10
20
Duration (s)
b
Acceleration
0.5 : 1.5
1 : 1
1.5 : 0.5
0
2
4
6
Time (s)
clear risk / high hatching
ambiguous cues
clear benign / low hatching
Experiment 2: ambiguity in FREQUENCY
c
Proportion of peak power
1.2
0.8
0.4
0
rainstorm
predator attack
0
100
200
300
400
Frequency (Hz)
d
Relative amplitude (dB)
0
−20
−40
broad
intermediate
very low
0
100
200
300
400
Frequency (Hz)

Figure 5.3

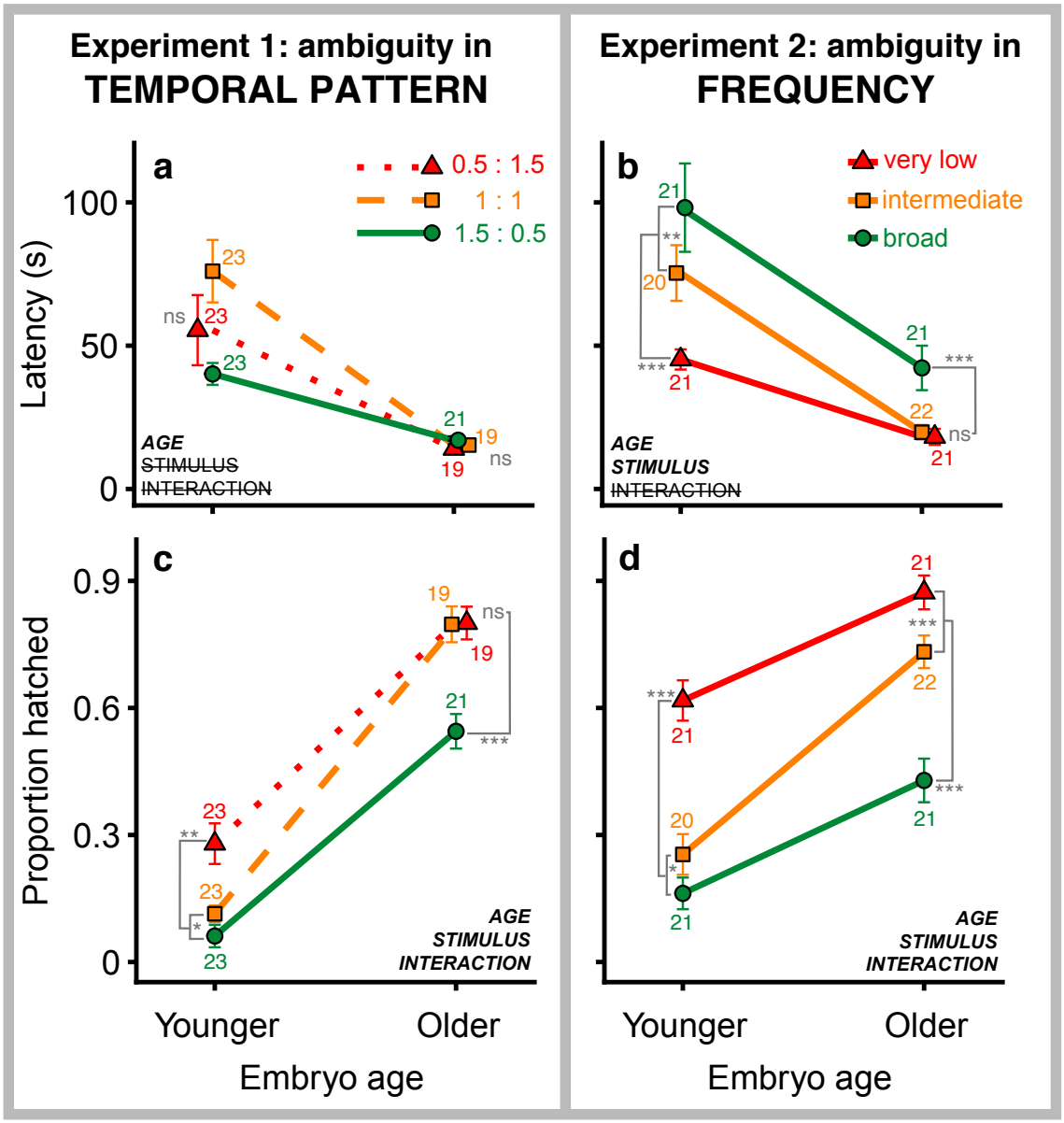

Experiment 1: ambiguity in
TEMPORAL PATTERN
Experiment 2: ambiguity in
FREQUENCY
a
b
c
d
0.5 : 1.5
1 : 1
1.5 : 0.5
very low
intermediate
broad
Latency (s)
Proportion hatched
AGE
STIMULUS
INTERACTION
ns
Younger
Older
Embryo age

Table 5.1

Analysis	Source	df	Chisq	P	Significance Codes
E1 – latency to first hatch	***AGE***	1	72.89	< 2.2e-16	***
	~~STIMULUS~~	2	1.15	0.5614	ns
	~~INTERACTION~~	2	0.15	0.927	ns
E2 – latency to first hatch	***AGE***	1	44.13	3.07e-11	***
	STIMULUS	2	28.08	8.00e-07	***
	~~INTERACTION~~	2	0.90	0.6373	ns
E1 – proportion hatched	***AGE***	1	99.67	< 2.2e-16	***
	STIMULUS	2	16.21	0.0003025	***
	INTERACTION	2	12.80	0.001661	**
E2 – proportion hatched	***AGE***	1	35.92	2.06e-09	***
	STIMULUS	2	64.17	1.16e-14	***
	INTERACTION	2	7.89	0.01933	*

ns = P > 0.05, * = P < 0.05, ** = P < 0.01, *** = P < 0.001. Significant sources (P < 0.05) are highlighted in bold italics, while non-significant sources (P > 0.05) are stricken.

CHAPTER 6. CONCLUSIONS AND SYNTHESIS

This dissertation reveals how sensory development can first constrain, then enable, life-saving responses of developing animals to environmental cues. It identifies key roles for two different mechanosensory systems in the antipredator responses of embryos and describes strikingly precocious development in one of those systems. It demonstrates that, even at early developmental stages, animals can apply nuanced contextual assessment strategies to incidental environmental cues to make important behavioral decisions. Finally, it documents adaptive ontogenetic change in the assessment strategies and decision rules that embryos apply to ambiguous cues in the context of changing cost-benefit trade-offs.

Compared to communication signals, we know much less about how animals use incidental environmental cues to make behavioral decisions. The appreciation that many animals use substrate-borne vibrations as sources of information (Cocroft et al., 2014; Hill et al., 2019; Hill, 2009) created new opportunities to apply methods from communication research to studies of incidental cues (Warkentin et al., In press). Embryos across diverse taxonomic groups use physical disturbance cues from both abiotic sources, indicating habitat conditions, and biotic sources, including egg-predators and larval hosts, to inform their hatching timing (Buckley et al., 2005; Doody and Paull, 2013; Doody et al., 2012; Endo et al., 2019; Gomez-Mestre et al., 2008; Martin et al., 2011; Mukai et al., 2014; Oyarzun and Strathmann, 2011; Touchon et al., 2011; Warkentin, 1995; Warkentin, 2000; Warkentin, 2011a; Whittington and Kearn, 1988). However, the specific assessment and decision rules these embryos apply to make decisions based on vibration properties are

largely unknown. Moreover, the mechanosensory systems mediating their hatching responses had not been assessed for embryos of any species and, across taxa, relatively little was known about the developmental onset of mechanoreception, compared with its mature function. This dissertation advances our understanding of the sensory mechanisms and cue-assessment strategies underlying the vibration-cued hatching of red-eyed treefrogs, *Agalychnis callidryas*, with broader implications for understanding the development of behavior and how animals use incidental cues.

Chapters 2 and 3 demonstrate that both the vestibular system of the inner ear and the lateral line system play important roles in mediating the anti-predator escape-hatching response of *A. callidryas* embryos. The vestibular system is the predominant motion-detection system in vertebrates, thus it might mediate vibration-cued hatching responses for many other vertebrates, from fishes to reptiles and birds. The lateral line system is found in all fishes and aquatic life stages of amphibians, thus it could play a role in hatching responses to physical disturbance cues across anamniote vertebrates. Invertebrates utilize a variety of other mechanosensory mechanisms (Keil, 1997; Keil and Steinbrecht, 1984; Lakes-Harlan and Strauß, 2014; McIver, 1975; Romagny et al., 2012; Urbanek and Kapusta, 2016), and we hope that our work will inspire others to explore the embryonic development of these sensory systems as potential mechanisms for environmentally cued hatching as well. Understanding the sensory mechanism(s) that contribute to behavioral decisions is fundamental for an integrative understanding of behavior and opens possibilities for new avenues of research.

There are several potential avenues for future research stemming from the work

presented here. First, the results specifically motivate continued mechanistic work on *A. callidryas* embryo behavior. For instance, how the vestibular system and lateral line inputs are integrated in the central nervous system to mediate hatching decisions is now an open question. It is also possible that cutaneous mechanoreceptors provide additional information about risk; some results presented here suggest this hypothesis is worth investigating.

Second, these results from *A. callidryas* provide a model for comparative mechanistic work on mechanosensory-cued hatching in embryos of other taxa, particularly for other experimentally tractable vertebrates in which hatching in response to physical disturbance are well documented (Warkentin, 2011b). For instance, in fishes and amphibians where MCH has been demonstrated, such as in California grunion where embryos are cued to hatch by tumbling waves (Griem and Martin, 2000; Martin et al., 2011), it would be worthwhile to investigate whether these embryos use their vestibular systems and/or lateral lines to sense motion cues. The vestibular system could mediate motion-cued hatching responses in embryos of many vertebrate species. Some reptiles, such as pig-nosed turtles (Doody et al., 2012), hatch in response to motion cues. It would be informative to know whether and to what extent their responses are limited by ear development. Finally, for the invertebrate taxa that hatch early in response to environmental cues – such as stink bugs, grasshoppers, mosquitoes, and parasitic flatworms (Warkentin et al., In press) – a fruitful line of inquiry would be to ask what possible sensors are involved in those responses. It is essential to identify these sensors and assess their ontogeny to determine to what extent developmental changes in behavior

reflect the easing of sensory constraints and to understand the information available to embryos at different developmental stages.

A third potential avenue for future research is comparative morphology of embryo sensory systems. For instance, Chapter 3 revealed an extreme disparity between the extent of lateral line development in *A. callidryas* at hatching competence compared to what has been described for other amphibians and fishes. However, lateral line development has been studied in relatively few species, and largely disconnected from research on embryo behavior. Our results could motivate a larger comparative study of lateral line ontogeny. Such a study could assess whether precocious lateral line development or lateral line hypertrophy in embryos is associated with a capacity for MCH across anurans, amphibians, or anamniote vertebrates more broadly. Although ECH appears taxonomically widespread, we know little about the causes of interspecific variation in cued hatching responses (Warkentin, 2011a). For instance, many – perhaps most – phyllomedusid treefrog embryos share *A. callidryas*' escape hatching ability, but the gliding treefrog (*Agalychnis spurrelli*) shows much poorer hatching responses to snake attacks than *A. callidryas* at similar developmental stages (Gomez-Mestre and Warkentin, 2007). Comparative studies may reveal the mechanisms underlying such interspecific variation in behavior.

Behavior changes developmentally due to changes in many underlying traits that alter specific competencies (what animals can do) as well as the effects of those performance traits on natural selection (i.e., changes in what animals should do), which can favor ontogenetic adaptation of decision rules. Understanding how and why behavior changes with development requires determining which of those underlying factors

developmentally matches and functionally contributes to specific changes in behavior. Earlier, we found that the onset of predator-induced mechanosensory-cued escape-hatching lags behind the onset of hypoxia-cued hatching, ruling out hatching ability as the limiting factor on MCH expression (Warkentin et al., 2017). My dissertation research demonstrates that sensory development plays a key role in limiting MCH (Chapters 2 and 3). These results highlight how development can dramatically change what types of environmental information animals have access to – even for embryos that already show substantial behavioral competence and responsiveness to environmental cues. They also highlight how the same behavioral response (escape-hatching) can be subject to very different constraints in different environmental contexts.

The second section of this dissertation addresses how embryos make decisions based on the specific properties of vibrational cues, in the context of risk trade-offs. Chapter 4 addresses the very general, and understudied, issue of how animals parse temporal pattern information from incidental cues. The results suggest that *A. callidryas* embryos perceive temporal patterns of vibrations at more than one scale, such that the same simple pattern elements can function to increase or decrease hatching in different large-scale contexts. Moreover, they strengthen the evidence from prior research, using edited rain vibrations (Caldwell et al., 2010), that initial vibrations can alter the effect or interpretation of subsequent vibrations. This information may enable resolution of the identified mismatch between prior measurements of embryo responses and natural vibration patterns, which were based on simple one-level temporal patterns. Thus, this work motivates re-analysis of temporal patterns in natural benign vibrations and predator

attacks, utilizing more complex decision rules based on these identified embryo capabilities. It may also inform approaches to studying how other animals use incidental vibrational cues to make important behavioral decisions.

Chapter 5 addresses another general issue in information use – how animals make decisions based on ambiguous cues – specifically in the context of developmentally changing costs of false alarms. It documents adaptive ontogenetic changes in decision rules for ambiguous cues. The results are consistent across two different forms of ambiguity; this both extends and generalizes earlier findings of adaptive ontogeny in decision rules based on the cost of information. The extent to which developmental changes in behavior reflect changing constraints or abilities versus adaptive modulation of decisions based on changing fitness consequences is still an open question, especially for early life stages. This work contributes to the body of evidence for adaptive ontogeny, and to our more general understanding of the multiplicity of factors affecting behavioral development.

Collectively, my research elucidates how the development of sensory ability and decision-making processes both affect escape hatching behavior in *A. callidryas* embryos. These embryos combine information from multiple sensory systems to detect and respond to predator cue and deploy impressive cost-benefit risk assessment to determine whether to remain within the confines of their egg or to take their chances with early entry into the outside world. My work on decision rules highlights the potential nuance and complexity of cue assessment and behavioral decisions, even in early life stages. It cautions against oversimplifying assumptions about embryo cognition – at least for species and in contexts where a history of strong selective trade-offs may have honed their capacity to make a key

decision well – and demonstrates the value of embryo hatching behavior for research in animal cognition. On a broader scale, this research models approaches for applying well-established knowledge about and methods for studying animal communication to important but neglected questions in how animals extract information and make decisions based on messy, non-stereotyped incidental cues.

BIBLIOGRAPHY

Akre, K. L. and Ryan, M. J. (2010). Complexity increases working memory for mating signals. *Current Biology* **20**, 502–505.

Almanzar, A. and Warkentin, K. M. (2018). How development changes escape-hatching success in snake attacks: a video analysis of red-eyed treefrog embryo behavior and performance. *Integrative and Comparative Biology* **58**, E267.

Alsina, B. and Whitfield, T. T. (2017). Sculpting the labyrinth: morphogenesis of the developing inner ear. *Seminars in Cell & Developmental Biology* **65**, 47–59.

Andrewartha, H. G. (1952). Diapause in relation to the ecology of insects. *Biological Reviews* **27**, 50–107.

Angelaki, D. E. (2004). Eyes on target: what neurons must do for the vestibuloocular reflex during linear motion. *Journal of Neurophysiology* **92**, 20–35.

Angelaki, D. E. and Cullen, K. E. (2008). Vestibular system: the many facets of a multimodal sense. *Annual Review of Neuroscience* **31**, 125–150.

Appel, H. M. and Cocroft, R. B. (2014). Plants respond to leaf vibrations caused by insect herbivore chewing. *Oecologia* **175**, 1257–1266.

Bacher, S., Casas, J., Wäckers, F. and Dorn, S. (1997). Substrate vibrations elicit defensive behaviour in leafminer pupae. *Journal of Insect Physiology* **43**, 945–952.

Bagger-Sjoback, D. (1997). Effect of streptomycin and gentamicin on the inner ear. *Annals of the New York Academy of Sciences* **830**, 120–129.

Bailey, W. J. (1991). *Acoustic behavior of insects: an evolutionary perspective*. London, UK: Chapman and Hall.

Balci, F., Freestone, D. and Gallistel, C. R. (2009). Risk assessment in man and mouse. *Proceedings of the National Academy of Sciences of the United States of America* **106**, 2459–2463.

Ball, R. E., Oliver, M. K. and Gill, A. B. (2016). Early life sensory ability—ventilatory responses of thornback ray embryos (*Raja clavata*) to predator-type electric fields. *Developmental Neurobiology* **76**, 721–729.

Barsz, K., Ison, J. R., Snell, K. B. and Walton, J. P. (2002). Behavioral and neural measures of auditory temporal acuity in aging humans and mice. *Neurobiology of Aging* **23**, 565–578.

Bate, M. (1999). Development of motor behaviour. *Current Opinion in Neurobiology* **9**, 670–675.

Bateman, P. W. and Fleming, P. A. (2014). Living on the edge: effects of body size, group density and microhabitat selection on escape behaviour of southern leopard frogs *Lithobates sphenocephalus*. *Current Zoology* **60**, 712–718.

Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software* **67**, 1–48.

Baugh, A. T. and Ryan, M. J. (2010). Mate choice in response to dynamic presentation of male advertisement signals in túngara frogs. *Animal Behaviour* **79**, 145–152.

Becerra, M. and Anadon, R. (1993). Development of the inner-ear of the brown trout (Salmo trutta fario). 2. Cytodifferentiation and innervation of sensory cells. *Journal of Morphology* **216**, 241–257.

Becker, E. A., Bird, N. C. and Webb, J. F. (2016). Post-embryonic development of canal and superficial neuromasts and the generation of two cranial lateral line phenotypes. *Journal of Morphology* **277**, 1273–1291.

Bever, M. M. and Fekete, D. M. (2002). Atlas of the developing inner ear in zebrafish. *Developmental Dynamics* **223**, 536–43.

Bever, M. M., Jean, Y. Y. and Fekete, D. M. (2003). Three-dimensional morphology of inner ear development in *Xenopus laevis*. *Developmental Dynamics* **227**, 422–430.

Blaxter, J. H. S. (1984). Neuromasts and cupular growth of cod larvae. *Flodevigen Rapportserie* **1**, 183–188.

Blaxter, J. H. S. (1987). Structure and development of the lateral line. *Biological Reviews* **62**, 471–514.

Blaxter, J. H. S. and Fuiman, L. A. (1989). Function of the free neuromasts of marine teleost larvae. In *The Mechanosensory Lateral Line* (ed. Coombs, S., Görner, P., and Münz, H.), pp. 481–499. New York, NY: Springer.

Bleckmann, H. (1980). Reaction time and stimulus frequency in prey localization in the surface-feeding fish *Aplocheilus lineatus*. *Journal of Comparative Physiology* **140**, 163–172.

Bonachea, L. A. and Ryan, M. J. (2011). Predation risk increases permissiveness for heterospecific advertisement calls in túngara frogs, *Physalaemus pustulosus*. *Animal Behaviour* **82**, 347–352.

Boncoraglio, G. and Saino, N. (2007). Habitat structure and the evolution of bird song: a meta-analysis of the evidence for the acoustic adaptation hypothesis. *Functional Ecology* **21**, 134–142.

Bouskila, A. and Blumstein, D. T. (1992). Rules of thumb for predation hazard assessment: predictions from a dynamic model. *The American Naturalist* **139**, 161–176.

Bradbury, J. W. and Vehrencamp, S. L. (2011). *Principles of Animal Communication*. Sinauer.

Braun, C. B. and Coombs, S. (2000). The overlapping roles of the inner ear and lateral line: the active space of dipole source detection. *Philosophical Transactions of the Royal Society B* **355**, 1115–1119.

Brenowitz, E. A. and Rose, G. J. (1994). Behavioural plasticity mediates aggression in choruses of the Pacific treefrog. *Animal Behaviour* **47**, 633–641.

Brown, R. M. and Iskandar, D. T. (2000). Nest site selection, larval hatching, and advertisement calls, of *Rana arathooni* from Southwestern Sulawesi (Celebes) Island, Indonesia. *Journal of Herpetology* **34**, 404–413.

Brown, J. L., Morales, V. and Summers, K. (2010). A key ecological trait drove the evolution of biparental care and monogamy in an amphibian. *The American Naturalist* **175**, 436–446.

Brownell, P. H. and Leo van Hemmen, J. (2001). Vibration sensitivity and a computational theory for prey-localizing behavior in sand scorpions. *Integrative and Comparative Biology* **41**, 1229–1240.

Brumm, H. and Slabbekoorn, H. (2005). Acoustic communication in noise. In *Advances in the Study of Behavior*, pp. 151–209. Academic Press.

Buck, J. and Buck, E. (1968). Mechanism of rhythmic synchronous flashing of fireflies: fireflies of Southeast Asia may use anticipatory time-measuring in synchronizing their flashing. *Science* **159**, 1319–1327.

Buckley, C. R., Michael, S. F. and Irschick, D. J. (2005). Early hatching decreases jumping performance in a direct-developing frog, *Eleutherodactylus coqui*. *Functional Ecology* **19**, 67–72.

Burmeister, S., Wilczynski, W. and Ryan, M. J. (1999). Temporal call changes and prior experience affect graded signalling in the cricket frog. *Animal Behaviour* **57**, 611–618.

Caldwell, M. S., McDaniel, J. G. and Warkentin, K. M. (2009). Frequency information in the vibration-cued escape hatching of red-eyed treefrogs. *Journal of Experimental Biology* **212**, 566–575.

Caldwell, M. S., McDaniel, J. G. and Warkentin, K. M. (2010). Is it safe? Red-eyed treefrog embryos assessing predation risk use two features of rain vibrations to avoid false alarms. *Animal Behaviour* **79**, 255–260.

Carroll, E. J. and Hedrick, J. L. (1974). Hatching in the toad *Xenopus laevis*: morphological events and evidence for a hatching enzyme. *Developmental Biology* **38**, 1–13.

Castellano, S., Cadeddu, G. and Cermelli, P. (2012). Computational mate choice: theory and empirical evidence. *Behavioural Processes* **90**, 261–277.

Castellanos, I. and Barbosa, P. (2006). Evaluation of predation risk by a caterpillar using substrate-borne vibrations. *Animal Behaviour* **72**, 461–469.

Catton, W. T. (1976). Adaptation in mechanoreceptors of amphibian skin. *Progress in Brain Research* **43**, 227–31.

Chaiyasarikul, A. and Warkentin, K. M. (2017). Escape hatching of red-eyed treefrogs in wasp attacks: how development changes survival. *Integrative and Comparative Biology* **57**, e26.

Charlton, B. D., Reby, D. and McComb, K. (2008). Effect of combined source (F0) and filter (formant) variation on red deer hind responses to male roars. *The Journal of the Acoustical Society of America* **123**, 2936–2943.

Chivers, D. P., Kiesecker, J. M., Marco, A., Devito, J., Anderson, M. T. and Blaustein, A. R. (2001). Predator-induced life history changes in amphibians: egg predation induces hatching. *Oikos* **92**, 135–142.

Cocroft, R. B., Gogala, M., Hill, P. S. M. and Wessel, A. eds. (2014). *Studying vibrational communication*. Berlin Heidelberg: Springer-Verlag.

Cohen, B. (1974). The vestibulo-ocular reflex arc. In *Vestibular System Part 1: Basic Mechanisms* (ed. Kornhuber, H. H.), pp. 477–540. Berlin, Heidelberg: Springer Berlin Heidelberg.

Cohen, K. L., Seid, M. A. and Warkentin, K. M. (2016). How embryos escape from danger: the mechanism of rapid, plastic hatching in red-eyed treefrogs. *Journal of Experimental Biology* **219**, 1875–1883.

Cohen, K. L., Piacentino, M. L. and Warkentin, K. M. (2019). Two types of hatching gland cells facilitate escape hatching at different developmental stages in red-eyed treefrogs, *Agalychnis callidryas* (Anura: Phyllomedusidae). *Biological Journal of the Linnean Society* **126**, 751–767.

Cole, C. (1993). Shannon revisited: information in terms of uncertainty. *Journal of the American Society for Information Science* **44**, 204–211.

Corcoran, A. J., Barber, J. R. and Conner, W. E. (2009). Tiger Moth Jams Bat Sonar. *Science* **325**, 325–327.

Covey, E. and Casseday, J. H. (1999). Timing in the auditory system of the bat. *Annual Review of Physiology* **61**, 457–476.

Daghfous, G., Smargiassi, M., Libourel, P.-A., Wattiez, R. and Bels, V. (2012). The function of oscillatory tongue-flicks in snakes: insights from kinematics of tongue-flicking in the banded water snake (*Nerodia fasciata*). *Chemical Senses* **37**, 883–896.

Dai, C. F., Mangiardi, D., Cotanche, D. A. and Steyger, P. S. (2006). Uptake of fluorescent gentamicin by vertebrate sensory cells in vivo. *Hearing Research* **213**, 64–78.

Dall, S. R. X., Giraldeau, L.-A., Olsson, O., McNamara, J. M. and Stephens, D. W. (2005). Information and its use by animals in evolutionary ecology. *Trends in Ecology & Evolution* **20**, 187–193.

Dapper, A. L., Baugh, A. T. and Ryan, M. J. (2011). The sounds of silence as an alarm cue in túngara frogs, *Physalaemus pustulosus*. *Biotropica* **43**, 380–385.

Debusschere, E., Hostens, K., Adriaens, D., Ampe, B., Botteldooren, D., De Boeck, G., De Muynck, A., Sinha, A. K., Vandendriessche, S., Van Hoorebeke, L., et al. (2016). Acoustic stress responses in juvenile sea bass *Dicentrarchus labrax* induced by offshore pile driving. *Environmental Pollution* **208**, 747–757.

Delia, J., Rivera-Ordonez, J. M., Salazar-Nicholls, M. J. and Warkentin, K. M. (2019). Hatching plasticity and the adaptive benefits of extended embryonic development in glassfrogs. *Evolutionary Ecology* **33**, 37–53.

Diekamp, B. and Gerhardt, H. C. (1995). Selective phonotaxis to advertisement calls in the gray treefrog *Hyla versicolor*: behavioral experiments and neurophysiological correlates. *Journal of Comparative Physiology A* **177**, 173–190.

Doody, J. S. (2011). Environmentally cued hatching in reptiles. *Integrative and Comparative Biology* **51**, 49–61.

Doody, J. S. and Paull, P. (2013). Hitting the ground running: environmentally cued hatching in a lizard. *Copeia* **2013**, 160–165.

Doody, J. S., Stewart, B., Camacho, C. and Christian, K. (2012). Good vibrations? Sibling embryos expedite hatching in a turtle. *Animal Behaviour* **83**, 645–651.

Duursma, R. and Choat, B. (2017). fitplc - an R package to fit hydraulic vulnerability curves. *Journal of Plant Hydraulics* **4**, e002–e002.

Eaton, R. C. and Nissanov, J. (1985). A review of Mauthner-initiated escape behavior and its possible role in hatching in the immature zebrafish, *Brachydanio rerio*. *Environmental Biology of Fishes* **12**, 265–279.

Eaton, R. C., Lee, R. K. and Foreman, M. B. (2001). The Mauthner cell and other identified neurons of the brainstem escape network of fish. *Progress in Neurobiology* **63**, 467–485.

Edwards, C. J., Alder, T. B. and Rose, G. J. (2005). Pulse rise time but not duty cycle affects the temporal selectivity of neurons in the anuran midbrain that prefer slow AM rates. *Journal of Neurophysiology* **93**, 1336–1341.

Elepfandt, A. (1982). Accuracy of taxis response to water waves in the clawed toad (*Xenopus laevis* Daudin) with intact or with lesioned lateral line system. *Journal of Comparative Physiology* **148**, 535–545.

Elias, D. O. and Mason, A. C. (2014). The role of wave and substrate heterogeneity in vibratory communication: practical issues in studying the effect of vibratory environments in communication. In *Studying Vibrational Communication* (ed. Cocroft, R. B., Gogala, M., Hill, P. S. M., and Wessel, A.), pp. 215–247. Berlin, Heidelberg: Springer.

Emlen, S. T. (1972). An experimental analysis of the parameters of bird song eliciting species recognition. *Behaviour* **41**, 130–171.

Endo, J., Takanashi, T., Mukai, H. and Numata, H. (2019). Egg-cracking vibration as a cue for stink bug siblings to synchronize hatching. *Current Biology* **29**, 143–148.

Espmark, Y. and Langvatn, R. (1985). Development and habituation of cardiac and behavioral responses in young red deer calves (*Cervus elaphus*) exposed to alarm stimuli. *Journal of Mammalogy* **66**, 702–711.

Ewing, A. (1989). *Arthropod bioacoustics: neurobiology and behavior*. Ithaca, NY: Cornell University.

Fabrezi, M., Quinzio, S., Goldberg, J. and Sá, R. (2012). The development of *Dermatonotus muelleri* (Anura: Microhylidae: Gastrophryninae). *Journal of Herpetology* **46**, 363–380.

Fay, R. R. and Edds-Walton, P. L. (2008). Structures and functions of the auditory nervous system of fishes. In *Fish Bioacoustics: With 81 Illustrations* (ed. Webb, J. F., Fay, R. R., and Popper, A. N.), pp. 49–97. New York, NY: Springer.

Ferrari, M. C. O. and Chivers, D. P. (2009). Sophisticated early life lessons: threat-sensitive generalization of predator recognition by embryonic amphibians. *Behavioral Ecology* **20**, 1295–1298.

Fitzgibbon, C. D. (1990). Anti-predator strategies of immature Thomson's gazelles: hiding and the prone response. *Animal Behaviour* **40**, 846–855.

Fouilloux, C., Jung, J., Ospina-L, A. M., Snyder, R. and Warkentin, K. M. (2019). Do developmental changes in fitness trade-offs predict how embryos use mechanosensory cues for escape-hatching decisions? Figshare. https://doi.org/10.6084/m9.figshare.9758372.v2

Fritzsch, B. (1996). How does the urodele ear develop? *International Journal of Developmental Biology* **40**, 763–771.

Fritzsch, B. (2003). Development of inner ear afferent connections: forming primary neurons and connecting them to the developing sensory epithelia. *Brain Research Bulletin* **60**, 423–33.

Fritzsch, B. and Straka, H. (2014). Evolution of vertebrate mechanosensory hair cells and inner ears: toward identifying stimuli that select mutation driven altered morphologies. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **200**, 5–18.

Fritzsch, B., Barald, K. F. and Lomax, M. I. (1998). Early embryology of the vertebrate ear. In *Development of the Auditory System* (ed. Rubel, E. W.), pp. 80–145. New York: Springer-Verlag New York, Inc.

Fritzsch, B., Beisel, K. W., Jones, K., Farinas, I., Maklad, A., Lee, J. and Reichardt, L. F. (2002). Development and evolution of inner ear sensory epithelia and their innervation. *Journal of Neurobiology* **53**, 143–156.

Fromy, B., Sigaudo-Roussel, D. and L Saumet, J. (2008). Cutaneous neurovascular interaction involved in tactile sensation. *Cardiovascular & Hematological Agents in Medicinal Chemistry* **6**, 337–42.

Gabriel, M., Frippiat, J.-P., Frey, H. and Horn, E. R. (2012). The sensitivity of an immature vestibular system to altered gravity. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology* **317**, 333–346.

Geipel, I., Smeekes, M. J., Halfwerk, W. and Page, R. A. (2019). Noise as an informational cue for decision-making: the sound of rain delays bat emergence. *Journal of Experimental Biology* **222**,.

Gerhardt, H. C. (1982). Sound pattern recognition in some North American treefrogs (Anura: Hylidae): implications for mate choice. *American Zoologist* **22**, 581–595.

Gerhardt, H. C. (1991). Female mate choice in treefrogs: static and dynamic acoustic criteria. *Animal Behaviour* **42**, 615–635.

Gerhardt, H. C. (2001). Acoustic communication in two groups of closely related treefrogs. In *Advances in the Study of Behavior*, pp. 99–167. Academic Press.

Gerhardt, H. C. and Huber, F. (2002). *Acoustic communication in insects and anurans*. Chicago, IL: University of Chicago Press.

Gerhardt, H. C. and Schul, J. (1999). A quantitative analysis of behavioral selectivity for pulse rise-time in the gray treefrog, *Hyla versicolor*. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **185**, 33–40.

Gerhardt, H. C., Humfeld, S. C. and Marshall, V. T. (2007). Temporal order and the evolution of complex acoustic signals. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1789–1794.

Getty, T. and Krebs, J. R. (1985). Lagging partial preferences for cryptic prey: a signal detection analysis of great tit foraging. *The American Naturalist* **125**, 39–60.

Ghazanfar, A. A. and Hauser, M. D. (2001). The auditory behaviour of primates: a neuroethological perspective. *Current Opinion Neurobiology* **11**, 712–720.

Ghazanfar, A. A., Smith-Rohrberg, D. and Hauser, M. D. (2001a). The role of temporal cues in rhesus monkey vocal recognition: orienting asymmetries to reversed calls. *Brain, Behavior and Evolution* **58**, 163–172.

Ghazanfar, A. A., Flombaum, J. I., Miller, C. T. and Hauser, M. D. (2001b). The units of perception in the antiphonal calling behavior of cotton-top tamarins (Saguinus oedipus): playback experiments with long calls. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **187**, 27–35.

Gilland, E. and Baker, R. (2005). Evolutionary patterns of cranial nerve efferent nuclei in vertebrates. *Brain, Behavior and Evolution* **66**, 234–254.

Gish, M., Dafni, A. and Inbar, M. (2012). Young aphids avoid erroneous dropping when evading mammalian herbivores by combining input from two sensory modalities. *PLoS One* **7**, e32706.

Glass, D. C. and Singer, J. E. (1972). Behavioral aftereffects of unpredictable and uncontrollable aversive events: Although subjects were able to adapt to loud noise and other stressors in laboratory experiments, they clearly demonstrated adverse aftereffects. *American Scientist* **60**, 457–465.

Glover, J. C. (2003). The development of vestibulo-ocular circuitry in the chicken embryo. *Journal of Physiology-Paris* **97**, 17–25.

Gomes, D. G. E., Francis, C. D. and Barber, J. R. (2021). Using the past to understand the present: coping with natural and anthropogenic noise. *BioScience*.

Gomez-Mestre, I. and Warkentin, K. M. (2007). To hatch and hatch not: similar selective trade-offs but different responses to egg predators in two closely related, syntopic treefrogs. *Oecologia* **153**, 197–206.

Gomez-Mestre, I., Wiens, J. J. and Warkentin, K. M. (2008). Evolution of adaptive plasticity: risk-sensitive hatching in neotropical leaf-breeding treefrogs. *Ecological Monographs* **78**, 205–224.

Gomez-Mestre, I., Kulkarni, S. and Buchholz, D. R. (2013). Mechanisms and consequences of developmental acceleration in tadpoles responding to pond drying. *PLoS One* **8**.

Goyes Vallejos, J., Grafe, T. U. and Wells, K. D. (2018). Prolonged parental behaviour by males of *Limnonectes palavanensis* (Boulenger 1894), a frog with possible sex-role reversal. *Journal of Natural History* **52**, 2473–2485.

Griem, J. N. and Martin, K. L. M. (2000). Wave action: the environmental trigger for hatching in the California Grunion *Leuresthes tenuis* (Teleostei: Atherinopsidae). *Marine Biology* **137**, 177–181.

Güell, B. A. and Warkentin, K. M. (2018). When and where to hatch? Red-eyed treefrog embryos use light cues in two contexts. *PeerJ* **6**, e6018.

Guibé, M., Poirel, N., Houdé, O. and Dickel, L. (2012). Food imprinting and visual generalization in embryos and newly hatched cuttlefish, *Sepia officinalis*. *Animal Behaviour* **84**, 213–217.

Gyllström, M. and Hansson, L.-A. (2004). Dormancy in freshwater zooplankton: Induction, termination and the importance of benthic-pelagic coupling. *Aquatic Sciences.* **66**, 274–295.

Haddon, C. and Lewis, J. (1996). Early ear development in the embryo of the zebrafish, Danio rerio. *The Journal of Comparative Neurology* **365**, 113–28.

Hall, W. G. and Oppenheim, R. W. (1987). Developmental Psychobiology: Prenatal, Perinatal, and Early Postnatal Aspects of Behavioral Development. *Annual Review of Psychology.* **38**, 91–128.

Hamann, I., Gleich, O., Klump, G. M., Kittel, M. C. and Strutz, J. (2004). Age-dependent changes of gap detection in the Mongolian gerbil (*Meriones unguiculatus*). *Journal of the Association for Research in Otolaryngology* **5**, 49–57.

Harris, G. G., Frishkopf, L. S. and Flock, Å. (1970). Receptor potentials from hair cells of the lateral line. *Science* **167**, 76–79.

Harshaw, C. and Lickliter, R. (2011). Biased embryos: prenatal experience alters the postnatal malleability of auditory preferences in bobwhite quail. *Developmental Psychobiology* **53**, 291–302.

Hartman, C. G. (1950). Playing 'possum. *Scientific American* **182**, 52–55.

Hashino, E. and Salvi, R. J. (1996). Regenerated hair cells exhibit a transient resistance to aminoglycoside toxicity. *Brain Research* **720**, 172–182.

Hasson, O. (1994). Cheating signals. *Journal of Theoretical Biology* **167**, 223–238.

Hasson (1997). Towards a general theory of biological signaling. *Journal of Theoretical Biology*.

Hauser (1998). Functional referents and acoustic similarity: field playback experiments with rhesus monkeys. *Animal Behaviour* **55**, 1647–1658.

Hauser, Agnetta and Perez (1998). Orienting asymmetries in rhesus monkeys: the effect of time-domain changes on acoustic perception. *Animal Behaviour* **56**, 41–47.

Hebets, E. A. and Papaj, D. R. (2005). Complex signal function: developing a framework of testable hypotheses. *Behavioral Ecology and Sociobiology* **57**, 197–214.

Hempleman, S. C. and Pilarski, J. Q. (2011). Prenatal development of respiratory chemoreceptors in endothermic vertebrates. *Respiratory Physiology & Neurobiology* **178**, 156–162.

Hernández, P. P., Olivari, F. A., Sarrazin, A. F., Sandoval, P. C. and Allende, M. L. (2007). Regeneration in zebrafish lateral line neuromasts: expression of the neural progenitor cell marker sox2 and proliferation-dependent and-independent mechanisms of hair cell renewal. *Developmental Neurobiology* **67**, 637–654.

Higham, J. P. and Hebets, E. A. (2013). An introduction to multimodal communication. *Behavioral Ecology and Sociobiology* **67**, 1381–1388.

Hill, P., Lakes-Harlan, R., Mazzoni, V., Narins, P. M., Virant-Doberlet, M. and Wessel, A. eds. (2019). *Biotremology: studying vibrational behavior*. Springer International Publishing.

Hill, P. S. M. (2008). *Vibrational communication in animals*. Cambridge, MA: Harvard University Press.

Hill, P. S. M. (2009). How do animals use substrate-borne vibrations as an information source? *Naturwissenschaften* **96**, 1355–1371.

Hite, J. L., Hughey, M. C., Warkentin, K. M. and Vonesh, J. R. (2018). Cross-ecosystem effects of terrestrial predators link treefrogs, zooplankton, and aquatic primary production. *Ecosphere* **9**, e02377.

Hoekstra, D. and Janssen, J. (1985). Non-visual feeding behavior of the mottled sculpin, *Cottus bairdi*, in Lake Michigan. *Environmental Biology of Fishes* **12**, 111–117.

Horn, E. R. (2006). Microgravity-induced modifications of the vestibuloocular reflex in *Xenopus laevis* tadpoles are related to development and the occurrence of tail lordosis. *The Journal of Experimental Biology* **209**, 2847–58.

Horn, E. R. and Gabriel, M. (2011). Gravity-related critical periods in vestibular and tail development of *Xenopus laevis*. *Journal of Experimental Zoology. Part A, Ecological genetics and physiology* **315**, 505–11.

Horn, E. and Sebastian, C. (1996). A hypergravity related sensitive period during the development of the roll induced vestibuloocular reflex in an amphibian (*Xenopus laevis*). *Neuroscience Letters* **216**, 25–8.

Horn, E., Mack, R. and Lang, H. G. (1986a). The development of the static vestibulo-ocular reflex in the southern clawed toad, *Xenopus laevis*. II. Animals with acute vestibular lesions. *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology* **159**, 879–85.

Horn, E., Lang, H. G. and Rayer, B. (1986b). The development of the static vestibulo-ocular reflex in the southern clawed toad, *Xenopus laevis*. I. Intact animals. *Journal of Comparative Physiology. A, Sensory, Neural, and Behavioral Physiology* **159**, 869–78.

Hughey, M. C., Rogge, J. R., Thomas, K., McCoy, M. W. and Warkentin, K. M. (2015). Escape-hatching responses of individual treefrog embryos vary with threat level in wasp attacks: a mechanistic analysis. *Behaviour* **152**, 1543–1568.

Humphreys, R. K. and Ruxton, G. D. (2018). A review of thanatosis (death feigning) as an anti-predator behaviour. *Behavioral Ecology and Sociobiology* **72**,.

Hunt, C. C. and McIntyre, A. K. (1960). Properties of cutaneous touch receptors in cat. *Journal of Physiology* **153**, 88–98.

Ison, J. R., Agrawal, P., Pak, J. and Vaughn, W. J. (1998). Changes in temporal acuity with age and with hearing impairment in the mouse: a study of the acoustic startle reflex and its inhibition by brief decrements in noise level. *Journal of the Acoustic Society of America* **104**, 1696–1704.

Jen, J. C. (2009). Bilateral vestibulopathy: clinical, diagnostic, and genetic considerations. *Seminars in Neurology* **29**, 528–33.

Jenkins, B. A. and Lumpkin, E. A. (2017). Developing a sense of touch. *Development (Cambridge, England)* **144**, 4078–4090.

Judge, K. A., Ting, J. J. and Gwynne, D. T. (2014). Condition dependence of female choosiness in a field cricket. *Journal of Evolutionary Biology* **27**, 2529–2540.

Jung, J., McDaniel, J. G. and Warkentin, K. W. (2017). Ontogeny of vibration-cued escape-hatching in red-eyed treefrogs: two reasons older embryos hatch more. *Integrative and Comparative Biology* **57**, e82.

Jung, J., Güell, B. A. and Warkentin, K. M. (2018). Inner ear development across onset and improvement of escape-hatching ability in red-eyed treefrogs: a confocal and μCT analysis. *Integrative and Comparative Biology* **58**, e348.

Jung, J., Kim, S. J., Pérez Arias, S. M., McDaniel, J. G. and Warkentin, K. M. (2019). How do red-eyed treefrog embryos sense motion in predator attacks? Assessing the role of vestibular mechanoreception. *Journal of Experimental Biology* **222**, jeb206052.

Jung, J., Serrano-Rojas, S. J. and Warkentin, K. M. (2020). Multimodal mechanosensing enables treefrog embryos to escape egg-predators. *Journal of Experimental Biology* **223**,.

Karlsen, H. E. and Sand, O. (1987). Selective and reversible blocking of the lateral line in freshwater fish. *Journal of Experimental Biology* **133**, 249–262.

Kawamura, G. and Ishida, K. (1985). Changes in sense organ morphology and behaviour with growth in the flounder *Paralichthys olivaceus*. *Nippon Suisan Gakkaishi* **51**, 155–165.

Kawamura, G., Mukai, Y. and Ohta, H. (1983). Morphology of the eye and free neuromast of larval ayu and pale chub and its relation to behaviour in water flow. *Nippon Suisan Gakkaishi* **49**, 1527–1532.

Kawamura, G., Masuma, S., Tezuka, N., Koiso, M., Jinbo, T. and Namba, K. (2003). Morphogenesis of sense organs in the bluefin tuna *Thunnus orientalis*. *Proceedings of the 26th Annual Larval Fish Conference* 123–135.

Kay, R. H. (1982). Hearing of modulation in sounds. *Physiological Reviews* **62**, 894–975.

Keil, T. A. (1997). Functional morphology of insect mechanoreceptors. *Microscopy Research and Technique* **39**, 506–531.

Keil, T. A. and Steinbrecht, R. A. (1984). Mechanosensitive and Olfactory Sensilla of Insects. In *Insect Ultrastructure: Volume 2* (ed. King, R. C. and Akai, H.), pp. 477–516. Boston, MA: Springer US.

Kjellberg, A., Landström, U., Tesarz, M., Söderberg, L. and Akerlund, E. (1996). The effects of nonphysical noise characteristics, ongoing task and noise sensitivity on annoyance and distraction due to noise at work. *Journal of Environmental Psychology* **16**, 123–136.

Korn, H. and Faber, D. S. (1975). Inputs from the posterior lateral line nerves upon the goldfish Mauthner cell. I. Properties and synaptic localization of the excitatory component. *Brain Research* **96**, 342–348.

Korn, H. and Faber, D. S. (2005). The Mauthner cell half a century later: a neurobiological model for decision-making? *Neuron* **47**, 13–28.

Korn, H., Faber, D. S. and Mariani, J. (1974). Existence of projections of posterior nerves from the lateral line on the Mauthner cell; their antagonistic effect on the activation of this neuron by vestibular afferences. *Comptes Rendus de l'Académie des Sciences* **279**, 413–416.

Korwin-Kossakowski, M. (2012). Fish hatching strategies: a review. *Reviews in Fish Biology and Fisheries* **22**, 225–240.

Krebs, J. R. and Dawkins, R. (1984). Animal signals: mind-reading and manipulation. In *Behavioural ecology: an evolutionary approach* (ed. Krebs, J. R. and Davies, N. B.), p. Oxford: Blackwell Scientific.

Kroese, A. B. A. and van den Bercken, J. (1982). Effects of ototoxic antibiotics on sensory hair cell functioning. *Hearing Research* **6**, 183–197.

Lakes-Harlan, R. and Strauß, J. (2014). Functional Morphology and Evolutionary Diversity of Vibration Receptors in Insects. In *Studying Vibrational Communication* (ed. Cocroft, R. B., Gogala, M., Hill, P. S. M., and Wessel, A.), pp. 277–302. Berlin, Heidelberg: Springer.

Lambert, F. M., Beck, J. C., Baker, R. and Straka, H. (2008). Semicircular canal size determines the developmental onset of angular vestibuloocular reflexes in larval Xenopus. *Journal of Neuroscience* **28**, 8086–8095.

Lang, F., Govind, C. K., Costello, W. J. and Greene, S. I. (1977). Developmental neuroethology: changes in escape and defensive behavior during growth of the lobster. *Science* **197**, 682–685.

Langner, G. (1992). Periodicity coding in the auditory system. *Hearing Research* **60**, 115–142.

Lannoo, M. J. (1987). Neuromast topology in anuran amphibians. *Journal of Morphology* **191**, 115–129.

Lannoo, M. J. (1999). Integration: nervous and sensory systems. In *Tadpoles: The Biology of Anuran Larvae* (ed. McDiarmid, R.) and Altig, R.), pp. 149–169. Chicago, IL: University of Chicago Press.

Le Prell, C. G. and Moody, D. B. (1997). Perceptual salience of acoustic features of Japanese monkey coo calls. *Journal of Comparative Psychology* **111**, 261–274.

Leavell, B. C. and Bernal, X. E. (2019). The cognitive ecology of stimulus ambiguity: a predator–prey perspective. *Trends in Ecology & Evolution* **34**, 1048–1060.

Levri, E. P. (1998). Perceived predation risk, parasitism, and the foraging behavior of a freshwater snail (*Potamopyrgus antipodarum*). *Canadian Journal of Zoology.* **76**, 1878–1884.

Li, D. (2002). Hatching responses of subsocial spitting spiders to predation risk. *Proceedings: Biological Sciences* **269**, 2155–2161.

Liang, J. K., Bok, J. and Wu, D. K. (2010). Distinct contributions from the hindbrain and mesenchyme to inner ear morphogenesis. *Developmental Biology* **337**, 324–34.

Liao, J. C. (2010). Organization and physiology of posterior lateral line afferent neurons in larval zebrafish. *Biology Letters* **6**, 402–405.

Liégeois-Chauvel, C., de Graaf, J. B., Laguitton, V. and Chauvel, P. (1999). Specialization of left auditory cortex for speech perception in man depends on temporal coding. *Cerebral Cortex* **9**, 484–496.

Lima, S. L. and Dill, L. M. (1990). Behavioral decisions made under the risk of predation: a review and prospectus. *Canadian Journal of Zoology* **68**, 619–640.

Lindström, K. and Lehtonen, T. K. (2013). Mate sampling and choosiness in the sand goby. *Proceedings of the Royal Society B: Biological Sciences* **280**, 20130983.

Llanos-Rivera, A., Herrera, G., Tarifeño, E. and Castro, L. R. (2014). Development of free neuromasts in *Engraulis ringens* and *Strangomera bentincki* (Teleostei, Clupeiformes) early larvae. *Latin American Journal of Aquatic Research* **42**, 264–270.

Loftus-Hills, J. J. and Littlejohn, M. J. (1971). Pulse repetition rate as the basis for mating call discrimination by two sympatric species of Hyla. *Copeia* **1971**, 154–156.

Losey, J. E. and Denno, R. F. (1998). The escape response of pea aphids to foliar-foraging predators: factors affecting dropping behaviour. *Ecological Entomology* **23**, 53–61.

Ma, E. Y., Rubel, E. W. and Raible, D. W. (2008). Notch signaling regulates the extent of hair cell regeneration in the zebrafish lateral line. *Journal of Neuroscience* **28**, 2261–2273.

Martin, J., Luque-Larena, J. and Lopez, P. (2005). Factors affecting escape behavior of Iberian green frogs (*Rana perezi*). *Canadian Journal of Zoology* **83**, 1189–1194.

Martin, K., Bailey, K., Moravek, C. and Carlson, K. (2011). Taking the plunge: California grunion embryos emerge rapidly with environmentally cued hatching. *Integrative and Comparative Biology* **51**, 26–37.

Mateo, J. M. (1996). The development of alarm-call response behaviour in free-living juvenile Belding's ground squirrels. *Animal Behaviour* **52**, 489–505.

Mathis, A., Ferrari, M. C. O., Windel, N., Messier, F. and Chivers, D. P. (2008). Learning by embryos and the ghost of predation future. *Proceedings of the Royal Society B: Biological Sciences* **275**, 2603–2607.

Matthews, K. A., Scheier, M. F., Brunson, B. I. and Carducci, B. (1980). Attention, unpredictability, and reports of physical symptoms: eliminating the benefits of predictability. *Journal of Personality and Social Psychology* **38**, 525–537.

May, B., Moody, D. B. and Stebbins, W. C. (1988). The significant features of Japanese macaque coo sounds: a psychophysical study. *Animal Behaviour* **36**, 1432–1444.

May, B., Moody, D. B. and Stebbins, W. C. (1989). Categorical perception of conspecific communication sounds by Japanese macaques, *Macaca fuscata*. *Journal of the Acoustical Society of America* **85**, 837–847.

Maynard-Smith, J. and Harper, D. (2003). *Animal Signals*. Oxford: Oxford University Press.

McCormick, C. A. (1999). Anatomy of the central auditory pathways of fish and amphibians. In *Comparative Hearing: Fish and Amphibians* (ed. Fay, R. R. and Popper, A. N.), pp. 155–217. New York, NY: Springer.

McCormick, C. A., Gallagher, S., Cantu-Hertzler, E. and Woodrick, S. (2016). Mechanosensory lateral line nerve projections to auditory neurons in the dorsal descending octaval nucleus in the goldfish, *Carassius auratus*. *Brain, Behavior and Evolution* **88**, 68–80.

McIver, S. B. (1975). Structure of Cuticular Mechanoreceptors of Arthropods. *Annual Review of Entomology* **20**, 381–397.

Melendez, K. V., Jones, D. L. and Feng, A. S. (2006). Classification of communication signals of the little brown bat. *The Journal of the Acoustical Society of America* **120**, 1095–1102.

Metcalfe, W. K. (1985). Sensory neuron growth cones comigrate with posterior lateral line primordial cells in zebrafish. *Journal of Comparative Neurology* **238**, 218–224.

Meyhöfer, R., Casas, J. and Dorn, S. (1994). Host location by a parasitoid using leafminer vibrations: characterizing the vibrational signals produced by the leafmining host. *Physiological Entomology* **19**, 349–359.

Michelsen, A., Fink, F., Gogala, M. and Traue, D. (1982). Plants as transmission channels for insect vibrational songs. *Behavioral Ecology and Sociobiology* **11**, 269–281.

Mirjany, M., Preuss, T. and Faber, D. S. (2011). Role of the lateral line mechanosensory system in directionality of goldfish auditory evoked escape response. *Journal of Experimental Biology* **214**, 3358–3367.

Mogdans, J. (2019). Sensory ecology of the fish lateral-line system: Morphological and physiological adaptations for the perception of hydrodynamic stimuli. *Journal of Fish Biology* **95**, 53–72.

Mogdans, J. and Bleckmann, H. (2012). Coping with flow: behavior, neurophysiology and modeling of the fish lateral line system. *Biological Cybernetics* **106**, 627–642.

Mokkonen, M. and Lindstedt, C. (2016). The evolutionary ecology of deception. *Biological Reviews* **91**, 1020–1035.

Montgomery, J. C. (1989). Lateral line detection of planktonic prey. In *The Mechanosensory Lateral Line* (ed. Coombs, S., Görner, P., and Münz, H.), pp. 561–574. New York, NY: Springer.

Montgomery, J. C. and Macdonald, J. A. (1987). Sensory tuning of lateral line receptors in Antarctic fish to the movements of planktonic prey. *Science* **235**, 195–196.

Montgomery, M. E. and Nault, L. R. (1978). Effects of age and wing polymorphism on the sensitivity of *Myzus persicae* to alarm pheromone. *Annals of the Entomological Society of America* **71**, 788–790.

Montgomery, J. C., Baker, C. F. and Carton, A. G. (1997). The lateral line can mediate rheotaxis in fish. *Nature* **389**, 960–963.

Moriceau, S., Roth, T. L., Okotoghaide, T. and Sullivan, R. M. (2004). Corticosterone controls the developmental emergence of fear and amygdala function to predator odors in infant rat pups. *International Journal of Developmental Neuroscience* **22**, 415–422.

Mortimer, B. (2017). Biotremology: do physical constraints limit the propagation of vibrational information? *Animal Behaviour* **130**, 165–174.

Morton, E. S. (1977). On the occurrence and significance of motivation-structural rules in some bird and mammal sounds. *The American Naturalist* **111**, 855–869.

Mukai, Y., Tuzan, A. D., Shaleh, S. R. M. and Senoo, S. (2006). Development of sensory organs in larvae of mouse grouper, *Cromileptes altivelis*. *Proceedings of International Conference on Coastal Oceanography and Sustainable Marine Aquaculture.* 169–177.

Mukai, Y., Tuzan, A. D., Shaleh, S. R. M. and Manjaji-Matsumoto, B. M. (2010). Development of sensory organs and changes of behavior in larvae of the sutchi catfish, *Pangasianodon hypophthalmus*. *Fisheries Science* **76**, 921–930.

Mukai, H., Hironaka, M., Tojo, S. and Nomakuchi, S. (2012). Maternal vibration induces synchronous hatching in a subsocial burrower bug. *Animal Behaviour* **84**, 1443–1448.

Mukai, H., Hironaka, M., Tojo, S. and Nomakuchi, S. (2014). Maternal vibration: an important cue for embryo hatching in a subsocial shield bug. *PLoS One* **9**, e87932.

Munoz, N. E. and Blumstein, D. T. (2012). Multisensory perception in uncertain environments. *Behavioral Ecology* **23**, 457–462.

Murakami, S. L., Cunningham, L. L., Werner, L. A., Bauer, E., Pujol, R., Raible, D. W. and Rubel, E. W. (2003). Developmental differences in susceptibility to neomycin-induced hair cell death in the lateral line neuromasts of zebrafish (*Danio rerio*). *Hearing Research* **186**, 47–56.

Narins, P. M. and Capranica, R. R. (1976). Sexual differences in the auditory system of the tree frog *Eleutherodactylus coqui*. *Science* **192**, 378–380.

Neo, Y. Y., Seitz, J., Kastelein, R. A., Winter, H. V., ten Cate, C. and Slabbekoorn, H. (2014). Temporal structure of sound affects behavioural recovery from noise impact in European seabass. *Biological Conservation* **178**, 65–73.

Nieuwkoop, P. D. and Faber, J. (1956). *Normal table of Xenopus laevis (Daudin); a systematical and chronological survey of the development from the fertilized egg till the end of metamorphosis*. North-Holland, Amsterdam: North-Holland Pub. Co.

Nishide, Y. and Tanaka, S. (2016). Desert locust, Schistocerca gregaria, eggs hatch in synchrony in a mass but not when separated. *Behavioral Ecology and Sociobiology* **70**, 1507–1515.

Noguera, J. C. and Velando, A. (2019). Bird embryos perceive vibratory cues of predation risk from clutch mates. *Nature Ecology Evolution* **3**, 1225–1232.

Noorani, I. (2014). LATER models of neural decision behavior in choice tasks. *Frontiers in Integrative Neuroscience.* **8**.

Oberst, S., Bann, G., Lai, J. C. S. and Evans, T. A. (2017). Cryptic termites avoid predatory ants by eavesdropping on vibrational cues from their footsteps. *Ecology Letters* **20**, 212–221.

O'Brien, G. S., Rieger, S., Wang, F., Smolen, G. A., Gonzalez, R. E., Buchanan, J. and Sagasti, A. (2012). Coordinate development of skin cells and cutaneous sensory axons in zebrafish. The *Journal of Comparative Neurology* **520**, 816–831.

Ord, T. J., Peters, R. A., Clucas, B. and Stamps, J. A. (2007). Lizards speed up visual displays in noisy motion habitats. *Proceedings of the Royal Society B: Biological Sciences* **274**, 1057–1062.

Otsuka, M. and Nagai, S. (1997). Neuromast formation in the prehatching embryos of the cod fish, *Gadus macrocephalus Tilesius*. *Zoological Science* **14**, 475–481.

Oyarzun, F. X. and Strathmann, R. R. (2011). Plasticity of hatching and the duration of planktonic development in marine invertebrates. *Integrative and Comparative Biology* **51**, 81–90.

Partridge, B. L. and Pitcher, T. J. (1980). The sensory basis of fish schools: Relative roles of lateral line and vision. *Journal of Comparative Physiology A* **135**, 315–325.

Pezaro, N., Doody, J. S., Green, B. and Thompson, M. B. (2013). Hatching and residual yolk internalization in lizards: evolution, function and fate of the amnion. *Evolution & Development* **15**, 87–95.

Pfannenstiel, R. S., Hunt, R. E. and Yeargan, K. V. (1995). Orientation of a hemipteran predator to vibrations produced by feeding caterpillars. *Journal of Insect Behavior* **8**, 1–9.

Pohlmann, K., Atema, J. and Breithaupt, T. (2004). The importance of the lateral line in nocturnal predation of piscivorous catfish. *Journal of Experimental Biology* **207**, 2971–2978.

Pollack, G. (2000). Who, what, where? Recognition and localization of acoustic signals by insects. *Current Opinion in Neurobiology* **10**, 763–767.

Pollack, G. S. (2001). Analysis of temporal patterns of communication signals. *Current Opinion in Neurobiology* **11**, 734–738.

Poo, S. and Bickford, D. P. (2014). Hatching plasticity in a Southeast Asian tree frog. *Behavioral Ecology and Sociobiology* **68**, 1733–1740.

Precht, W. (1976). Physiology of the peripheral and central vestibular systems. In *Frog Neurobiology: A Handbook*, pp. 481–512. Berlin, Heidelberg: Springer Berlin Heidelberg.

Pyburn, W. F. (1970). Breeding behavior of leaf-frogs *Phyllomedusa callidryas* and *Phyllomedusa dacnicolor* in Mexico. *Copeia* **1970**, 209–218.

Quick, Q. A. and Serrano, E. E. (2005). Inner ear formation during the early larval development of *Xenopus laevis*. *Developmental dynamics: an official publication of the American Association of Anatomists* **234**, 791–801.

Raible, D. W. and Kruse, G. J. (2000). Organization of the lateral line system in embryonic zebrafish. *Journal of Comparative Neurology* **421**, 189–198.

Rayer, B. and Horn, E. (1986). The development of the static vestibulo-ocular reflex in the southern clawed toad, *Xenopus laevis*. III. Chronic hemilabyrinthectomized

tadpoles. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **159**, 887–895.

Remez, R. E., Rubin, P. E., Pisoni, D. B. and Carrell, T. D. (1981). Speech perception without traditional speech cues. *Science* **212**, 947–949.

Rhebergen, F., Taylor, R. C., Ryan, M. J., Page, R. A. and Halfwerk, W. (2015). Multimodal cues improve prey localization under complex environmental conditions. *Proceedings of the Royal Society B: Biological Sciences* **282**, 20151403.

Ribot-Ciscar, E., Vedel, J. P. and Roll, J. P. (1989). Vibration sensitivity of slowly and rapidly adapting cutaneous mechanoreceptors in the human foot and leg. *Neuroscience Letters* **104**, 130–135.

Richards, D. G. and Wiley, R. H. (1980). Reverberations and amplitude fluctuations in the propagation of sound in a forest: implications for animal communication. *The American Naturalist* **115**, 381–399.

Roberts, D. M. (2001). Egg hatching of mosquitoes *Aedes caspius* and *Ae. vittatus* stimulated by water vibrations. *Medical and Veterinary Entomology* **15**, 215–218.

Roberts, D. M. (2018). Predator feeding vibrations encourage mosquito larvae to shorten their development and so become smaller adults. *Ecological Entomology* **43**, 534–537.

Roberts, W. M., Howard, J. and Hudspeth, A. J. (1988). Hair cells: transduction, tuning, and transmission in the inner ear. *Annual Review of Cell and Developmental Biology* **4**, 63–92.

Roberts, A., Feetham, B., Pajak, M. and Teare, T. (2009). Responses of hatchling *Xenopus* tadpoles to water currents: first function of lateral line receptors without cupulae. *Journal of Experimental Biology* **212**, 914–921.

Rogge, J. R. and Warkentin, K. M. (2008). External gills and adaptive embryo behavior facilitate synchronous development and hatching plasticity under respiratory constraint. *Journal of Experimental Biology* **211**, 3627–35.

Roitberg, B. D. (1990). Optimistic and pessimistic fruit flies: Evaluating fitness consequences of estimation errors. *Behaviour* **114**, 65–82.

Roitberg, B. D., Myers, J. H. and Frazer, B. D. (1979). The Influence of Predators on the Movement of Apterous Pea Aphids between Plants. *Journal of Animal Ecology* **48**, 111–122.

Romagny, S., Darmaillacq, A.-S., Guibé, M., Bellanger, C. and Dickel, L. (2012). Feel, smell and see in an egg: emergence of perception and learning in an immature invertebrate, the cuttlefish embryo. *Journal of Experimental Biology* **215**, 4125–4130.

Rose, G. and Brenowitz, E. A. (1997). Plasticity of aggressive thresholds in *Hyla regilla*: discrete accommodation to encounter calls. *Animal Behaviour* **53**(2), 353–361.

Rose, G. J., Zelick, R. and Rand, A. S. (1988). Auditory processing of temporal information in a neotropical frog is independent of signal intensity. *Ethology* **77**, 330–336.

Rosenberg, H. I. and Russell, A. P. (1980). Structural and functional aspects of tail squirting: a unique defense mechanism of Diplodactylus (Reptilia: Gekkonidae). *Canadian Journal of Zoology.* **58**, 865–881.

Ryan, M. J. (1983). Frequency modulated calls and species recognition in a neotropical frog. *Journal of Comparative Physiology.* **150**, 217–221.

Ryan, M. J. and Rand, S. A. (2001). Feature weighting in signal recognition and discrimination by túngara frogs. In *Anuran Communication* (ed. Ryan, M. J.), pp. 86–101. Washington DC: Smithsonian Institution Press.

Ryan, M. J., Rand, W., Hurd, P. L., Phelps, S. M. and Rand, A. S. (2003). Generalization in response to mate recognition signals. *The American Naturalist* **161**, 380–394.

Salazar-Nicholls, M. J., Macias, H. and Warkentin, K. M. (2020). Ontogeny and extent of hatching enzyme accumulation in red-eyed treefrog embryos. *Integrative and Comparative Biology* **60**, E410–E410.

Salica, M. J., Vonesh, J. R. and Warkentin, K. M. (2017). Egg clutch dehydration induces early hatching in red-eyed treefrogs, *Agalychnis callidryas*. *Peerj* **5**.

Sand, O., Ozawa, S. and Hagiwara, S. (1975). Electrical and mechanical stimulation of hair cells in the mudpuppy. *Journal of Comparative Physiology* **102**, 13–26.

Sandeman, R. and Sandeman, D. (2003). Development, growth, and plasticity in the crayfish olfactory system. *Microscopy Research and Technique* **60**, 266–277.

Schaefer, K. P. and Meyer, D. L. (1974). Compensation of vestibular lesions. In *Vestibular System Part 2: Psychophysics, Applied Aspects and General Interpretations* (ed. Kornhuber, H. H.), pp. 463–490. Berlin, Heidelberg: Springer Berlin Heidelberg.

Schaub, A., Ostwald, J. and Siemers, B. M. (2008). Foraging bats avoid noise. *Journal of Experimental Biology* **211**, 3174–3180.

Schmidt, K. A., Dall, S. R. X. and Gils, J. A. V. (2010). The ecology of information: an overview on the ecological significance of making informed decisions. *Oikos* **119**, 304–316.

Schneider, C. A., Rasband, W. S. and Eliceiri, K. W. (2012). NIH Image to ImageJ: 25 years of image analysis. *Nature Methods* **9**, 671–5.

Schoener, T. W. (1971). Theory of feeding strategies. *Annual Review of Ecology and Systematics* **2**, 369–404.

Schulterbrandt, T. G., Kubie, J., von Gizycki, H., Zuri, I. and Halpern, M. (2008). Patterns of tongue-flicking by garter snakes (*Thamnophis sirtalis*) during presentation of chemicals under varying conditions. In *Chemical Signals in Vertebrates 11* (ed. Hurst, J. L., Beynon, R. J., Roberts, S. C., and Wyatt, T. D.), pp. 345–356. New York, NY: Springer.

Schultz, J. C. (1981). Adaptive changes in antipredator behavior of a grasshopper during development. *Evolution* **35**, 175–179.

Schwalbe, M. A. B., Bassett, D. K. and Webb, J. F. (2012). Feeding in the dark: lateral-line-mediated prey detection in the peacock cichlid *Aulonocara stuartgranti*. *Journal of Experimental Biology* **215**, 2060–2071.

Sebastian, C. and Horn, E. (1999). Light-dependent suppression of the vestibulo-ocular reflex during development. *Neuroreport* **10**, 171–6.

Sebastian, C., Esseling, K. and Horn, E. (2001). Altered gravitational forces affect the development of the static vestibuloocular reflex in fish (*Oreochromis mossambicus*). *Journal of Neurobiology* **46**, 59–72.

Shelton, P. M. J. (1970). The lateral line system at metamorphosis in *Xenopus laevis* (Daudin). *Development* **24**, 511–524.

Shine, R., Sun, L.-X., Fitzgerald, M. and Kearney, M. (2002). Antipredator responses of free-ranging pit vipers (*Gloydius shedaoensis*, viperidae). *Copeia* **2002**, 843–850.

Sih, A. and Moore, R. D. (1993). Delayed hatching of salamander eggs in response to enhanced larval predation risk. *American Naturalist* **142**, 947–960.

Simmons, A. M., Costa, L. M. and Gerstein, H. B. (2004). Lateral line-mediated rheotactic behavior in tadpoles of the African clawed frog (*Xenopus laevis*).

Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology **190**, 747–758.

Siniscalchi, M., Lusito, R., Sasso, R. and Quaranta, A. (2012). Are temporal features crucial acoustic cues in dog vocal recognition? *Animal Cognition* **15**, 815–821.

Sisneros, J. A., Tricas, T. C. and Luer, C. A. (1998). Response properties and biological function of the skate electrosensory system during ontogeny. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **183**, 87–99.

Smith, G. R. and Fortune, D. T. (2009). Hatching plasticity of wood frog (*Rana sylvatica*) eggs in response to mosquitofish (*Gambusia affinis*) cues. *Herpetological Conservation and Biology* **4**.

Smith, M. J. and Harper, D. G. C. (1995). Animal signals: models and terminology. *Journal of Theoretical Biology* **177**, 305–311.

Smith, S. C., Lannoo, M. J. and Armstrong, J. B. (1988). Lateral-line neuromast development in *Ambystoma mexicanum* and a comparison with *Rana pipiens*. *Journal of Morphology.* **198**, 367–379.

Song, J., Yan, H. Y. and Popper, A. N. (1995). Damage and recovery of hair cells in fish canal (but not superficial) neuromasts after gentamicin exposure. *Hearing Research* **91**, 63–71.

Speer-Blank, T. M. and Martin, K. L. M. (2004). Hatching events in the California grunion, *Leuresthes tenuis*. *Copeia* **2004**, 21–27.

Spray, D. C. (1976). Pain and temperature receptors of anurans. In *Frog Neurobiology, a Handbook* (ed. Llinás, R.) and Precht, W.), pp. 607–628. New York: Springer-Verlag.

Stern, D. L. (1998). Phylogeny of the tribe Cerataphidini (Homoptera) and the evolution of the horned soldier aphids. *Evolution* **52**, 155–165.

Stevens, M. (2013). *Sensory ecology, behaviour, and evolution*. New York: Oxford Univ Press.

Stone, L. S. (1933). The development of lateral–line sense organs in amphibians observed in living and vital-stained preparations. *The Journal of Comparative Neurology* **57**, 507–540.

Straka, H. (2010). Ontogenetic rules and constraints of vestibulo-ocular reflex development. *Current Opinion in Neurobiology* **20**, 689–695.

Straka, H. and Dieringer, N. (2004). Basic organization principles of the VOR: lessons from frogs. *Progress in Neurobiology* **73**, 259–309.

Straughan, I. R. (1975). An analysis of the mechanisms of mating call discrimination in the frogs *Hyla regilla* and *H. cadaverina*. *Copeia* **1975**, 415–424.

Strelioff, D. and Honrubia, V. (1978). Neural transduction in *Xenopus laevis* lateral line system. *Journal of Neurophysiology* **41**, 432–44.

Szabo, T. M., McCormick, C. A. and Faber, D. S. (2007). Otolith endorgan input to the Mauthner neuron in the goldfish. *The Journal of Comparative Neurology* **505**, 511–525.

Tanaka, S., Harano, K. I., Nishide, Y. and Sugahara, R. (2016). The mechanism controlling phenotypic plasticity of body color in the desert locust: some recent progress. *Current Opinion in Insect Science* **17**, 10–15.

Taylor, R. C., Klein, B. A. and Ryan, M. J. (2011). Inter-signal interaction and uncertain information in anuran multimodal signals. *Current Zoology* **57**, 153–161.

Thomas, E. D., Cruz, I. A., Hailey, D. W. and Raible, D. W. (2015). There and back again: development and regeneration of the zebrafish lateral line system. *Wiley Interdisciplinary Reviews Developmental Biology* **4**, 1–16.

Tinbergen, N. (1952). "Derived" activities; their causation, biological significance, origin, and emancipation during evolution. *The Quarterly Review of Biology* **27**, 1–32.

Tobias, J. A., Aben, J., Brumfield, R. T., Derryberry, E. P., Halfwerk, W., Slabbekoorn, H. and Seddon, N. (2010). Song divergence by sensory drive in Amazonian birds. *Evolution* **64**, 2820–2839.

Tokunaga, E. and Suzuki, N. (2008). Colony growth and dispersal in the ant-tended aphid, *Aphis craccivora Koch*, and the non-ant-tended aphid, *Acyrthosiphon pisum Harris*, under the absence of predators and ants. *Population Ecology* **50**, 45–52.

Touchon, J. C. and Vonesh, J. R. (2016). Variation in abundance and efficacy of tadpole predators in a neotropical pond community. *Journal of Herpetology* **50**, 113–119.

Touchon, J. C. T. C., Gomez-Mestre, I. G.-M. and Warkentin, K. M. W. M. (2006). Hatching plasticity in two temperate anurans: responses to a pathogen and predation cues. *Canadian Journal of Zoology,* **84**(4). https://doi.org/10.1139/z06-058

Touchon, J. C., Urbina, J. and Warkentin, K. M. (2011). Habitat-specific constraints on induced hatching in a treefrog with reproductive mode plasticity. *Behavioral Ecology* **22**, 169–175.

Touchon, J. C., McCoy, M. W., Vonesh, J. R. and Warkentin, K. M. (2013). Effects of plastic hatching timing carry over through metamorphosis in red-eyed treefrogs. *Ecology* **94**, 850–860.

Trimmer, P. C., Houston, A. I., Marshall, J. A. R., Mendl, M. T., Paul, E. S. and McNamara, J. M. (2011). Decision-making under uncertainty: biases and Bayesians. *Animal Cognition* **14**, 465–476.

Tunstall, D. N. and Pollack, G. S. (2005). Temporal and directional processing by an identified interneuron, ON1, compared in cricket species that sing with different tempos. *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **191**, 363–372.

Urbanek, A. and Kapusta, M. (2016). Atypical mechanoreceptors in larvae of biting midges *Forcipomyia nigra* (Diptera: Ceratopogonidae). *Micron* **88**, 68–76.

Valone, T. J., Nordell, S. E., Giraldeau, L.-A. and Templeton, J. J. (1996). The empirical question of thresholds and mechanisms of mate choice. *Evolutionary Ecology* **10**, 447–455.

Van Buskirk, J. (2016). A meta-analysis on facultative responses of embryonic amphibians to predation risk. *Copeia* **104**, 691–696.

Van Netten, S. M. (2006). Hydrodynamic detection by cupulae in a lateral line canal: functional relations between physics and physiology. *Biological Cybernetics; Heidelberg* **94**, 67–85.

Van Trump, W. J., Coombs, S., Duncan, K. and McHenry, M. J. (2010). Gentamicin is ototoxic to all hair cells in the fish lateral line system. *Hearing Research* **261**, 42–50.

Velilla, E., Polajnar, J., Virant-Doberlet, M., Commandeur, D., Simon, R., Cornelissen, J. H. C., Ellers, J. and Halfwerk, W. (2020). Variation in plant leaf traits affects transmission and detectability of herbivore vibrational cues. *Ecology and Evolution* **10**, 12277–12289.

von Helversen, D. (1993). 'Absolute steepness' of ramps as an essential cue for auditory pattern recognition by a grasshopper (Orthoptera; Acrididae; *Chorthippus biguttulus L.*). *Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology* **172**, 633–639.

Vonesh, J. R. (2000). Dipteran predation on the arboreal eggs of four Hyperolius frog species in Western Uganda. *Copeia* **2000**, 560–566.

Wang, Y., Lutfi, Z., Dong, L., Suman, D. S., Sanad, M. and Gaugler, R. (2012). Host cues induce egg hatching and pre-parasitic foraging behaviour in the mosquito parasitic nematode, *Strelkovimermis spiculatus*. *International Journal for Parasitology* **42**, 881–886.

Warkentin, K. M. (1995). Adaptive plasticity in hatching age: a response to predation risk trade-offs. *Proceedings of the National Academy of Sciences of the United States of America* **92**, 3507–3510.

Warkentin, K. M. (1999). Effects of hatching age on development and hatchling morphology in the red-eyed treefrog, *Agalychnis callidryas*. *Biological Journal of the Linnean Society* **68**, 443–470.

Warkentin, K. M. (2000). Wasp predation and wasp-induced hatching of red-eyed treefrog eggs. *Animal Behaviour* **60**, 503–510.

Warkentin, K. M. (2002). Hatching timing, oxygen availability, and external gill regression in the treefrog, *Agalychnis callidryas*. *Physiological and Biochemical Zoology* **75**, 155–164.

Warkentin, K. M. (2005). How do embryos assess risk? Vibrational cues in predator-induced hatching of red-eyed treefrogs. *Animal Behaviour* **70**, 59–71.

Warkentin, K. M. (2011a). Plasticity of hatching in amphibians: evolution, trade-offs, cues and mechanisms. *Integrative and Comparative Biology* **51**, 111–127.

Warkentin, K. M. (2011b). Environmentally cued hatching across taxa: embryos respond to risk and opportunity. *Integrative and Comparative Biology* **51**, 14–25.

Warkentin, K. (2017). Development of red-eyed treefrog embryos: a staging table for integrative research on environmentally cued hatching. *Integrative and Comparative Biology* **57**, E175.

Warkentin, K. M. and Caldwell, M. S. (2009). *Assessing risk: embryos, information, and escape hatching*. (ed. Dukas, R. and Ratcliffe, J. M.) University of Chicago Press.

Warkentin, K. M., Currie, C. R. and Rehner, S. A. (2001). Egg-killing fungus induces early hatching of red-eyed treefrog eggs. *Ecology* **82**, 2860–2869.

Warkentin, K. M., Gomez-Mestre, I. and McDaniel, J. G. (2005). Development, surface exposure, and embryo behavior affect oxygen levels in eggs of the red-eyed treefrog, *Agalychnis callidryas*. *Physiological and Biochemical Zoology* **78**, 956–66.

Warkentin, K. M., Buckley, C. R. and Metcalf, K. A. (2006a). Development of red-eyed treefrog eggs affects efficiency and choices of egg-foraging wasps. *Animal Behaviour* **71**, 417–425.

Warkentin, K. M., Caldwell, M. S. and McDaniel, J. G. (2006b). Temporal pattern cues in vibrational risk assessment by embryos of the red-eyed treefrog, *Agalychnis callidryas*. *Journal of Experimental Biology* **209**, 1376–1384.

Warkentin, K. M., Caldwell, M. S., Siok, T. D., D'Amato, A. T. and McDaniel, J. G. (2007). Flexible information sampling in vibrational assessment of predation risk by red-eyed treefrog embryos. *Journal of Experimental Biology* **210**, 614–619.

Warkentin, K. M., Cuccaro Diaz, J., Güell, B. A., Jung, J., Kim, S. J. and Cohen, K. L. (2017). Developmental onset of escape-hatching responses in red-eyed treefrogs depends on cue type. *Animal Behaviour* **129**, 103–112.

Warkentin, K. M., Jung, J., Rueda Solano, L. A. and McDaniel, J. G. (2019). Ontogeny of escape-hatching decisions: vibrational cue use changes as predicted from costs of sampling and false alarms. *Behavioral Ecology and Sociobiology* **73**, 51.

Warkentin, K. M., Jung, J. and McDaniel, J. G. (In press). Research approaches in mechanosensory-cued hatching and the iterative development of playback methods for red-eyed treefrog embryos. In *Biotremology: Physiology, Ecology, and Evolution* (ed. Hill, P. S. M., Mazzoni, V., Stritih Peljhan, N., Virant-Doberlet, M., and Wessel, A.), p. New York, NY: Springer Nature.

Webb, J. F. and Shirey, J. E. (2003). Postembryonic development of the cranial lateral line canals and neuromasts in zebrafish. *Developmental Dynamics* **228**, 370–385.

Weeg, M. S. and Bass, A. H. (2002). Frequency response properties of lateral line superficial neuromasts in a vocal fish, with evidence for acoustic sensitivity. *Journal of Neurophysiology* **88**, 1252–62.

Weston, J. A. (1970). The migration and differentiation of neural crest cells. In *Advances in Morphogenesis* (ed. Abercrombie, M., Brachet, J., and King, T. J.), pp. 41–114. Elsevier.

Whittington, I. D. and Kearn, G. C. (1988). Rapid hatching of mechanically-disturbed eggs of the monogenean gill parasite *Diclidophora luscae*, with observations on sedimentation of egg bundles. *International Journal for Parasitology* **18**, 847–852.

Whittington, I. D. and Kearn, G. C. (2011). Hatching strategies in monogenean (Platyhelminth) parasites that facilitate host infection. *Integrative and Comparative Biology* **51**, 91–99.

Wiedenmayer, C. P. (2009). Plasticity of defensive behavior and fear in early development. *Neuroscience & Biobehavioral Reviews* **33**, 432–441.

Wiedenmayer, C. P. and Barr, G. A. (1998). Ontogeny of defensive behavior and analgesia in rat pups exposed to an adult male rat. *Physiology & Behavior* **63**, 261–269.

Wiedenmayer, C. P. and Barr, G. A. (2001). Developmental changes in c-fos expression to an age-specific social stressor in infant rats. *Behavioural Brain Research* **126**, 147–157.

Wiegmann, D. D., Weinersmith, K. L. and Seubert, S. M. (2010). Multi-attribute mate choice decisions and uncertainty in the decision process: a generalized sequential search strategy. *Journal of Mathematical Biology* **60**, 543–572.

Wilcox, S. R., Jackson, R. R. and Gentile, K. (1996). Spiderweb smokescreens: spider trickster uses background noise to mask stalking movements. *Animal Behaviour* **51**, 313–326.

Williams, J. A. and Holder, N. (2000). Cell turnover in neuromasts of zebrafish larvae. *Hearing Research* **143**, 171–181.

Willink, B., Palmer, M. S., Landberg, T., Vonesh, J. R. and Warkentin, K. M. (2014). Environmental context shapes immediate and cumulative costs of risk-induced early hatching. *Evolutionary Ecology* **28**, 103–116.

Winklbauer, R. (1989). Development of the lateral line system in *Xenopus*. *Progress in Neurobiology* **32**, 181–206.

Winters, S., Allen, W. L. and Higham, J. P. (2020). The structure of species discrimination signals across a primate radiation. *eLife* **9**, e47428.

Wourms, J. P. (1972). The developmental biology of annual fishes. III. Pre-embryonic and embryonic diapause of variable duration in the eggs of annual fishes. *Journal of Experimental Zoology* **182**, 389–414.

Wu, C.-H. and Elias, D. O. (2014). Vibratory noise in anthropogenic habitats and its effect on prey detection in a web-building spider. *Animal Behaviour* **90**, 47–56.

Yan, H. Y., Saidel, W. M., Chang, J. S., Presson, J. C., Popper, A. N. and Nicol, J. A. C. (1991). Sensory hair cells of a fish ear: evidence of multiple types based on ototoxicity sensitivity. *Proceedings of the Royal Society of London. Series B: Biological Sciences* **245**, 133–138.

[redacted]

CURRICULUM VITAE