The Journal of Experimental Biology 209, 1376-1384
Published by The Company of Biologists 2006
doi:10.1242/jeb.02150

Temporal pattern cues in vibrational risk assessment by embryos of the red-eyed treefrog, *Agalychnis callidryas*

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Accepted 6 February 2006

Summary

The embryos of red-eyed treefrogs, *Agalychnis callidryas*, use vibrations transmitted through their arboreal egg clutch to cue escape hatching behavior when attacked by egg-eating snakes. Hatching early increases the risk of predation in the water, so embryos should avoid it unless they are in danger. We exposed egg clutches to intermittent vibrations with different combinations of vibration duration and spacing to examine the role of simple temporal pattern cues in the escape hatching response. Stimuli were bursts of synthetic white noise from 0 to 100 Hz, including the range of frequencies with substantial energy in snake attacks, and had approximately rectangular amplitude envelopes. Embryos hatched in response to a small range of temporal patterns and not in response to many others, rather than hatching to most vibrations except for certain patterns perceived as safe. Neither cycle length nor duty cycle predicted hatching response, except at extreme values where no hatching occurred; the highest energy stimuli elicited little or no hatching. Both vibration duration and inter-vibration interval strongly affected the hatching response. The highest levels of hatching were to durations of 0.5 s combined with intervals of 1.5–2.5 s, and hatching decreased gradually with increasing difference of either duration or interval from these most effective stimuli. Vibration duration and interval appear to function as two necessary elements of a composite cue, rather than as redundant cues. This increases response specificity and reduces the range of stimuli that elicit hatching, likely reducing the chance of hatching unnecessarily in a benign disturbance. Vibration-cued hatching in *A. callidryas* embryos offers an opportunity to experimentally assess the behavioral decision rules underlying an effective and costly anti-predator defense.

Key words: hatching, predation, predator detection, defense, seismic, playback, duty cycle, duration, spacing.

Introduction

Prey need information about risk in order to balance the trade-offs between anti-predator defense and other activities. They gather this information from cues that their predators inadvertently provide through their presence and foraging activities. In some cases, these cues are stereotyped, such as when a predator releases alarm pheromones from its prey in the course of an attack (Chivers and Smith, 1998). More often, the cues are variable and potentially overlap with sensory stimuli from benign sources – a rustle in the grass may be caused by a carnivore, a herbivore or wind (Narins et al., 1997). This creates an information processing and discrimination problem for prey: they need to correctly identify sources of risk, but must not deploy costly defenses unnecessarily.

Prey commonly use chemical, visual and acoustic cues to assess risk (Lima and Dill, 1989; Kats and Dill, 1998). There is increasing evidence that both prey and their predators can use substrate-borne vibrations in their interactions (Bleckmann, 1985; Pfannenstiel et al., 1995; Bacher et al., 1996; Meyhofer et al., 1997; Randall and Matocq, 1997; Burger, 1998; Brownell and Van Hemmen, 2001; Warkentin, 2005). Indeed, vibrational cues as a means of predator detection offer certain advantages over other sensory modalities. As an inevitable byproduct of movement, vibrations are difficult to conceal. They serve as a direct indicator of current predator activity, their transmission is not obscured by visual barriers and their detection does not require orientation toward the source. However, prey also experience vibrations from many benign sources.

Like airborne sound, substrate vibrations can be distinguished in both time and frequency domains. However, the media through which vibration travels can be highly complex and more variable than air or water. Thus, vibrations may suffer greater or more variable filtering, with consequent degradation of frequency information, as they are transmitted (Michelsen et al., 1982). Characteristics of the temporal pattern

may be more robust to such degradation and often carry the bulk of the information in intraspecific vibrational communication (Randall, 1995; Hill, 2001; Virant-Doberlet and Cokl, 2004).

We examined the use of temporal pattern information by red-eyed treefrog embryos in the context of vibration-cued early hatching induced by egg predators. Red-eyed treefrogs, *Agalychnis callidryas* (Cope 1862), lay gelatinous egg clutches attached to vegetation overhanging ponds and swamps in wet tropical forests from the Yucatan through Panama. Tadpoles fall into the water when they hatch, escaping from egg predators and exposing themselves to a new suite of aquatic predators. Defenses against aquatic predators improve developmentally, so that hatching later and in a more developed stage increases the chance of survival in the water (Warkentin, 1995; Warkentin, 1999a). Undisturbed eggs hatch relatively late, at age 6–7 days in Panama and 7–8 days at our Costa Rican field sites. However, if attacked by egg-eating snakes or wasps or if infected by a fungal pathogen, embryos hatch up to 30% earlier to escape (Warkentin, 1995; Warkentin, 2000; Warkentin et al., 2001). Predator-induced early hatching is an immediate response to direct physical disturbance of an egg clutch – for instance by a foraging snake – but some violent disturbances, such as tropical rainstorms, do not induce hatching. Warkentin used playback experiments to show that vibrations recorded in snake attacks are sufficient to elicit rapid early hatching and that red-eyed treefrog embryos can distinguish between the vibrational patterns of snake attacks and rainstorms (Warkentin, 2005).

The vibrations produced in egg clutches by rainstorms, a common but benign disturbance type, and snake attacks, a common and dangerous disturbance, differ in two simple aspects of their temporal pattern. Rainstorms cause many short disturbance events, generally separated by short intervals, although in hard rain vibrations from individual drops can overlap to create longer continuous disturbances. By contrast, even short snake bites are long in duration compared with raindrop vibrations, and bites in an attack are typically separated by longer intervals than are drops in a storm, since the snake has to swallow a mouthful of eggs between bites. Furthermore, altering the gross temporal pattern of recorded storms and attacks, by moving periods of silence to clump together or divide periods of vibration, alters the hatching response to that stimulus (Warkentin, 2005). These manipulations simultaneously altered three temporal pattern elements: the duration of periods of vibration, the intervals between them and the entire cycle length, which is a function of the first two parameters. Embryos may attend to just one feature or to multiple features of the temporal pattern of vibrations. If embryos do attend to multiple features they may be redundant or non-redundant (Partan and Marler, 1999). Use of redundant cues would reduce the risk of not hatching when in danger. Non-redundant cues could be combined to increase response specificity, decreasing the chance of hatching in response to a benign disturbance.

Here, we ask which temporal pattern elements red-eyed treefrog embryos use to inform their hatching decision in vibrational disturbances and how they combine information from disturbance duration and inter-disturbance interval. We used simple, rhythmic stimuli based on synthetic white noise to isolate effects of temporal pattern elements and to control other vibrational characteristics that vary in natural disturbances.

Materials and methods

Animal collection and care

Young *A. callidryas* egg clutches on leaves were collected from Ocelot Pond, 2 km south of Gamboa, Panama. Clutches were brought to an open-air laboratory in Gamboa, where any dead (possibly unfertilized) or developmentally abnormal eggs were removed. Each clutch was mounted on a 5×10 cm plastic card for support, excess leaf area was trimmed if necessary, and the clutch was set over water in a plastic cup. Eggs were misted with rainwater several times daily to prevent desiccation. All hatchlings were returned to Ocelot Pond after experiments. This research was conducted under permits from the Panamanian Autoridad Nacional del Ambiente and approved by the Institutional Animal Care and Use Committee of Boston University.

Vibration playbacks

To assess embryo responses to vibrational patterns, we experimentally exposed egg clutches to artificial vibrations and monitored their hatching. The vibration playback system consisted of an electrodynamic minishaker (Model 4810; Bruel and Kjær, Nærum, Denmark) controlled by Canary 1.2.4 (Cornell Laboratory of Ornithology, Ithaca, NY, USA) on a Macintosh G3 (2003) or G4 (2005) laptop computer, *via* an external sound card (MSE-U33HB; Onkyo, Osaka, Japan) and a custom-made amplifier designed to have a flat frequency response from DC to 5 kHz (E. Hazen, Boston University Electronic Design Facility).

For most of the data, collected in 2003, the minishaker–clutch interface (MCI) was a stiff wire rod with a set of eight blunt tines at the end. The tines were constructed of 18-gauge galvanized wire in tight loops spaced 8 mm apart vertically, in two columns of four, spaced 10 mm apart. Egg diameters are typically 3–5 mm. The minishaker with attached MCI was hung from a wooden stand above a tray of aged tapwater. Thus, the eggs were moved up and down, and hatchlings fell into the water. Playback clutches on their plastic cards were mounted with the long axis of the clutch oriented vertically on a flat-sided plastic stand (~1.5 kg), then carefully moved forward so that the MCI tines entered the clutch between eggs.

Only healthy clutches that we could set up to contact at least five MCI tines were used for playbacks. After insertion of the MCI, and any hatching induced by that procedure, we allowed five hatching-free minutes for acclimation before the start of a playback. If 25% or more of a clutch hatched during set-up, we did not use that clutch in a playback trial. Stimuli were played to egg clutches for a period of 5 min. Hatched embryos were counted every minute for 10 min from the start of the

playback. Each clutch was only used once, and the MCI was rinsed with rainwater between trials to remove any perivitelline fluid from hatched eggs. To limit variation in the hatching response due to egg development and diel cycle, all playbacks were conducted from 16.30–04.30 h using clutches that were 5 days old at the start of the playback session, i.e. that were laid six nights before the playback night. Development is highly synchronous within clutches and among clutches laid at the same time and developing together at a site (Warkentin, 1995; Warkentin, 1999b).

Sets of stimuli within series were presented in random order within temporal blocks. On each playback day, stimuli were chosen randomly without replacement until each had been used once. The process was then repeated while suitable clutches remained, thus the last set was sometimes incomplete. If we had insufficient clutches to complete a set we did not do playbacks that day. However, our data include a few partial sets that occurred when clutches were excluded from the experiment due to excess hatching during set-up. Most stimuli were played to 10 or more clutches per series. Sample sizes (Table 1) are smaller in a few cases where data were recorded incorrectly or clutches were limited and the first eight replicates showed essentially no hatching.

For an additional subset of data collected in 2005, we used an improved MCI designed to present vibrations more uniformly to embryos throughout the clutch and to allow use of a broader range of clutch sizes. The newer MCI had five columns of blunt-ended stainless steel tines, which were each 1.5 mm in diameter. The columns were centered 6.5 mm apart, and tines were in offset rows of 12, with 6 mm spacing along the row. The tines were mounted in an acrylic plate, which was attached to an acrylic rod. The minimum initial clutch size was 20 eggs, and all clutches fit within the MCI tine field. Otherwise acclimation and testing procedures were as in 2003.

Playback stimuli

All vibration stimuli were constructed from bursts of 0–100 Hz white noise with approximately rectangular amplitude envelopes (i.e. sudden onset and offset) matched for peak acceleration, interspersed with intervals of silence, and were purely rhythmic; i.e. durations and intervals were constant within each stimulus (Fig. 1). We conducted eight series of playback experiments including 32 different stimuli (Table 1). The first six were conducted over a three-month period during 2003 (8 August to 6 November) and the seventh and eighth in 2005 (2 July to 5 August). Series 1 and 2 were transects through duration:interval space. In each series, we kept one parameter constant at 1 s and varied the other between 0.1 and 20 s. The next three series contained a variety of duration:interval patterns selected to delimit the range of temporal patterns that elicit hatching and to locate the pattern that elicited the most hatching; these were informed by the results of prior playback experiments. Each series included a common stimulus (1 s noise:1 s silence) to facilitate comparisons across series.

Series 6 was designed to evaluate potential ‘series effects’ across stimuli, the extent to which response to the 1:1 stimulus captured them, and thus if adjustments based on the 1:1 response would improve estimates of relative hatching response when combining data across series. It included the 1:1 stimulus and five other stimuli selected from series with high (Series 1 and 5) and low (Series 4) hatching responses to the 1:1 stimulus.

In 2005, we conducted a seventh series of playback experiments to better delineate the area of temporal pattern space with highest hatching and the decline in hatching at short vibration durations. This series included 11 stimuli, of which two were shared with the 2003 stimuli (the 1:1 stimulus and the stimulus that elicited the highest hatching in 2003). Because of the large number of stimuli and limited clutch availability on some playback nights, we divided Series 7 into two subsets, each including a group of stimuli across which duration by interval interactions could be tested. When

Table 1. *Vibration playback stimuli, defined by duration of bursts of white noise and the still intervals between them, with the playback series in which each stimulus was included, the total number of clutches to which it was played, and the mean hatching response*

Duration (s)	Interval (s)	Series	*N*	Proportion hatched	s.e.m.
0.0000016	1	3	8	0.02	0.02
0.05	1	7	9	0.11	0.03
0.1	0.5	7	11	0.18	0.05
0.1	1	1, 6, 8	29	0.36	0.04
0.1	2.5	7	11	0.29	0.06
0.25	1	7	10	0.65	0.10
0.25	1.5	7	10	0.55	0.05
0.25	2.5	8	10	0.49	0.07
0.5	0.5	7	10	0.46	0.05
0.5	0.75	4, 6	20	0.34	0.04
0.5	1	1, 6, 7	33	0.60	0.04
0.5	1.5	7	11	0.74	0.08
0.5	2.5	7	12	0.71	0.08
0.5	5	3, 8	22	0.33	0.06
1	0.1	2	12	0.02	0.02
1	0.5	2	10	0.11	0.02
1	0.75	4	10	0.27	0.07
1	1	1–7	79	0.36	0.03
1	1.5	7	11	0.47	0.07
1	5	2	13	0.40	0.05
1	10	2, 8	22	0.35	0.05
1	20	2	12	0.14	0.05
1	50	3	10	0.02	0.01
1	100	3	10	0.03	0.02
1.5	0.5	5	11	0.06	0.01
1.5	1	4	11	0.17	0.06
1.5	10	4, 6	20	0.23	0.04
1.5	20	5, 6	20	0.14	0.04
2.5	1	1	8	0.03	0.01
5	1	1	11	0.04	0.02
10	1	1	10	0.03	0.01
20	1	1	8	0.02	0.01

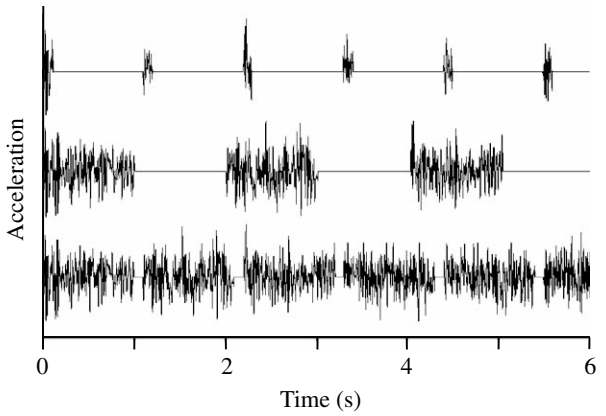

Fig. 1. Examples of stimuli used in vibration playback experiments, constructed from bursts of 0–100 Hz synthetic white noise. Waveforms of 6 s of the pattern of a 0.1 s duration:1 s interval stimulus (top), 1 s duration:1 s interval stimulus (middle), and 1 s duration:0.1 s interval stimulus (bottom).

possible, the entire series was run in randomized order on each playback night, as in 2003. When fewer clutches were available we ran one or the other subseries.

We also include here data from an eighth playback series, conducted in 2005 to address a separate question, which included three stimuli already present in the combined data set plus a fourth unique stimulus. Series 8 did not include the 1:1 stimulus.

Combining data across playback series

Due to the large number of different stimuli involved in this experiment and the iterative process required to identify relevant areas of temporal pattern space, it was not possible to include all stimuli tested in a single, globally randomized playback series. This raises the possibility that variation in hatching response between series, for instance due to seasonal variation in weather or due to the change in MCI between 2003 and 2005, could differentially affect the estimated response to stimuli included in different playback series. We addressed this issue in two ways. First, we tested for series effects on the proportion of eggs hatched in response to each of the stimuli that were represented in more than one series. Significant series effects would preclude simply pooling data across series.

Second, potentially even without statistically significant differences between playback series, the response to a common stimulus might indicate trends in embryo responsiveness at different times that would also alter responses to other stimuli. We used the response to stimuli that were repeated in multiple series to assess this. We calculated an adjusted value for the average proportion hatched (PHadj) in response to each stimulus (i) in each series (s) based on the response to the 1:1 stimuli in the same series with it and the overall response to the 1:1 stimulus (all), averaged across series, as follows:

$$\mathrm{PHadj}_{i,s} = \mathrm{PH}_{i,s}\,(\mathrm{PH}_{1:1,all}/\mathrm{PH}_{1:1,s})\,.$$

For the four stimuli included in two of the series and one stimulus included in three of the series, we then compared the variation in raw average hatching response with the variation in adjusted average hatching response across series, to see which was more consistent.

Statistical analyses

To test for series effects in the hatching response to a common stimulus, and to test for effects of duration and interval separately within series where only one varied, we used Kruskal–Wallis and Mann–Whitney *U* tests in SYSTAT v.5.2 (Systat, Inc., Evanston, IL, USA). We used ANOVA in SAS v.8.00 (SAS Institute, Cary, NC, USA) to test for interaction effects in four subsets of the data with orthogonal combinations of duration and interval. Two were subsets of series 7 (A, durations 0.1 and 0.5 by intervals 0.5 and 2.5; B, durations 0.25, 0.5 and 1 by intervals 1 and 1.5), and two required combining data across series (C, durations 0.1, 0.5 and 1.5 by intervals 0.5 and 1; D, durations 0.5 and 1 by intervals 0.5, 1, 1.5 and 5). Because some stimuli are included in multiple orthogonal combinations tested for interaction effects, we use Bonferroni criteria for the significance of interaction effects. To normalize the proportion-hatched data we used an arcsine square-root transformation. We compensated for heteroscedasticity by specifying the appropriate covariance structure using the REPEATED statement in PROC MIXED.

Results

Combining data across playback series

In 2003, the proportion hatched in response to the 1:1 stimulus was not significantly different across series (Kruskal–Wallis test, H_5=7.41, P=0.19, N=10–15). The within-series averages of proportion hatched ranged from 0.21±0.05 (Series 4, mean ± s.e.m., N=11) to 0.45±0.09 (Series 1, N=11), with an overall average of 0.35±0.03 (N=69). We also compared the hatching response to the same stimulus included in two different series for five other stimuli. In no case were they significantly different (Mann–Whitney *U* tests, duration:interval 0.1:1, $U_{7,12}$=54, P=0.31; 0.5:0.75, $U_{9,11}$=52, P=0.85; 0.5:1, $U_{9,12}$=57, P=0.83; 1.5:10, $U_{9,11}$=63, P=0.30; 1.5:20, $U_{10,10}$=42, P=0.54).

Although we found no significant series effects in 2003, hatching responses in 2005 could have been different due to the improved MCI. Including Series 7 in tests for series effects on the response to the 1:1 stimulus does reduce the P-value (H_6=10.533, N=10–15, P=0.1). However, this marginally significant difference is due entirely to Series 4, the series with the lowest response to the 1:1 stimulus. There is no evidence that Series 7 differs from any other 2003 series (H_5=5.415, N=10–15, P=0.37). As well, for the 0.5:1 stimulus, the proportion hatched in Series 7 is indistinguishable from that in Series 1 and 6 (H_2= 0.07, N=9–12, P=0.96). Series 8 contained three stimuli used in 2003. For two of these, the response was not different across series (0.1:1, H_2=4.038, N=7–12, P=0.13; 1:10, U=47, P=0.39). However, the response to the 0.5:5 stimulus was higher in Series 8 than in Series 3 (U=23,

P=0.015). Overall, this indicates that the new MCI did not fundamentally or consistently change the hatching response to the same stimulus and that hatching responses in different series were usually comparable. However, the hatching response to the same stimulus did sometimes vary among series.

The raw values for the average proportion hatched in response to the same stimulus in different series differed by, on average, 0.03 (±0.03 s.d.), while the adjusted values differed by 0.12±0.08. In six of seven comparisons, the raw values were closer than the adjusted values.

Based on the largely non-significant series effects and the better match of raw than adjusted values, to examine the large-scale pattern of hatching across temporal patterns we pooled the raw data for each stimulus across series and present overall mean values (Table 1; Figs 2, 3, 5). Because of the occasional differences in responses to particular stimuli across series, for statistical tests of interval and duration effects we present comparisons within the same playback series (Fig. 4). For tests of interaction, we address potential series effects in combined data below.

Duty cycle and cycle length

The proportion of *A. callidryas* embryos that hatched in response to vibration playbacks that were matched for frequency, amplitude and duration of the playback period varied from effectively zero (0.02±0.02, mean ± s.e.m.) to 0.74±0.08 (Table 1). These playback stimuli were, however, not matched for total energy because the proportion of the playback period filled with vibration *vs* silence (i.e. duty cycle) varied. To examine if simply introducing more vibration into a clutch over the same playback period induces more hatching, we plotted hatching against duty cycle (Fig. 2). There was no overall trend to higher hatching with a longer duty cycle. Both very short and very long duty cycles elicited little hatching. There was an intermediate range across which embryos showed similar levels of hatching in response to some stimuli with very different duty cycles. Also, some stimuli with similar duty cycles but different temporal patterns elicited different levels of hatching.

As with duty cycle, the hatching response varied substantially across stimuli with similar cycle length (Fig. 3). Very long cycle lengths (>50 s) elicited very little hatching, and moderately long or very short cycle lengths (>20 s or <0.7 s) elicited only moderate hatching. However, cycle lengths from 1 to 11 s elicited a wide range of hatching responses, from essentially none to substantial hatching. For instance, the highest hatching response, 74%, was to a 2 s cycle while another 2 s cycle elicited only 6% hatching (Fig. 3).

Duration and interval cues

Both disturbance duration and the length of intervals between periods of vibration strongly affected the hatching response of *A. callidryas* embryos when the other parameter was held constant at 1 s (Fig. 4; Kruskal–Wallis tests; duration, H_6=51.35, P<0.0001, N=8–12; interval, H_5=37.08, P<0.0001, N=10–15).

Across the combined results of the eight series of temporal pattern playbacks (Fig. 5), we found a single peak of *A. callidryas*' escape hatching response in duration:interval space, surrounded by a range of vibrational stimuli that elicited little or no hatching. The range of intervals that elicited high hatching was wider than the range of durations, and the

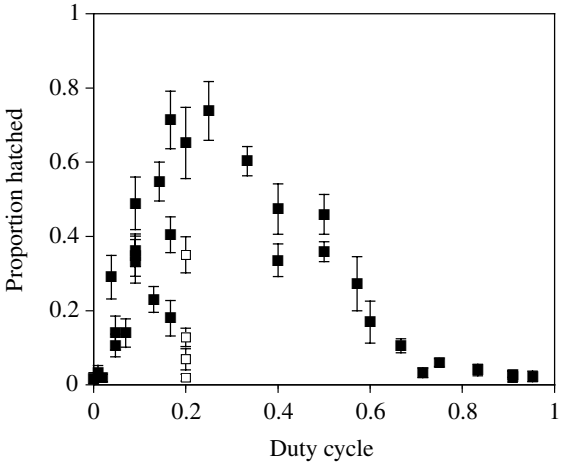

Fig. 2. Hatching response of *Agalychnis callidryas* embryos to 32 different temporal patterns of amplitude-matched 0–100 Hz white noise bursts plotted as a function of duty cycle (the ratio of vibration time to total playback time). Stimuli vary in duration of and intervals between periods of vibration. Data are mean proportion hatched ± s.e.m. for each stimulus. For comparison, we have also included the responses to four patterns of 0–11 kHz noise (open squares) (from Warkentin, 1995).

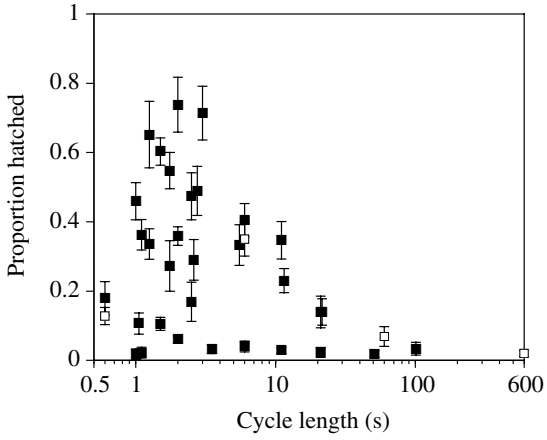

Fig. 3. Hatching response of *Agalychnis callidryas* embryos to 32 different temporal patterns of amplitude-matched 0–100 Hz white noise bursts plotted as a function of cycle length. Stimuli vary in duration of and intervals between periods of vibration. Data are mean proportion hatched ± s.e.m. for each stimulus. For comparison, we have also included the responses to four patterns of 0–11 kHz noise (open squares) (from Warkentin, 1995).

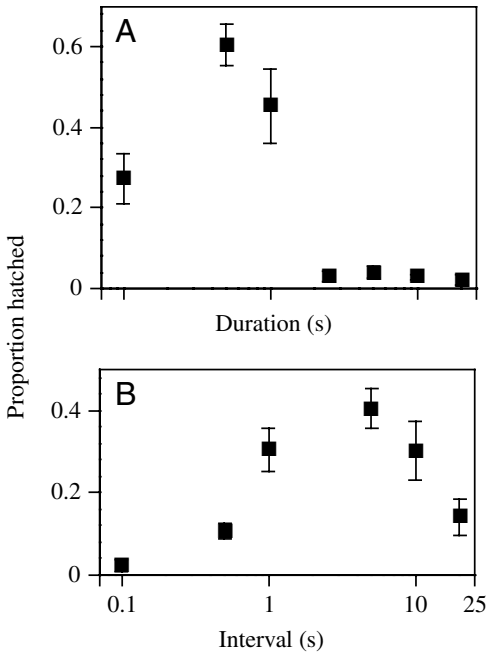

Fig. 4. Hatching response of *Agalychnis callidryas* embryos to vibrational playback stimuli (A) varying in disturbance duration, with a constant interval of 1 s between periods of vibration, and (B) varying in interval, with a constant disturbance duration of 1 s. Data are mean proportion hatched ± s.e.m., from series 1 and 2.

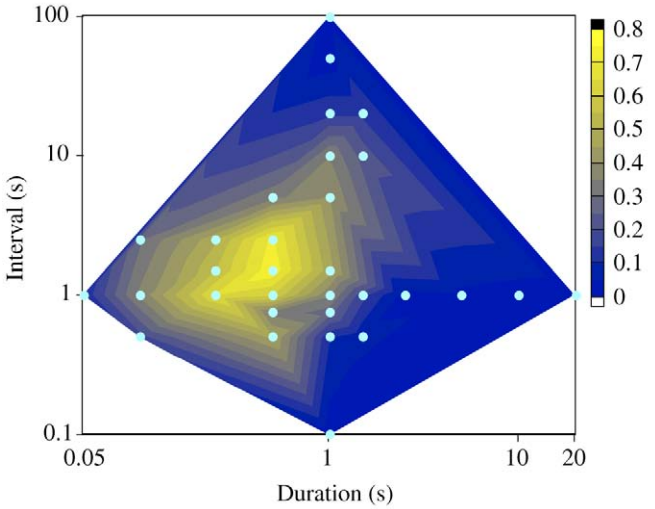

Fig. 5. Contour plot of hatching response of *Agalychnis callidryas* embryos to vibrational playback stimuli varying in disturbance duration and interval. Data are mean proportion hatched for 31 different stimuli, indicated as points. See Table 1 for sample sizes and standard errors. The shortest duration stimulus, clicks, is not included on the graph.

hatching peak included stimuli with shorter vibration durations than intervals (i.e. duty cycles <0.5).

Effects of duration and interval on hatching appear independent across some ranges of the parameter space we examined but show interactions across other ranges. All of the orthogonal subsets of data we tested showed significant main effects of duration (all $P \leqslant 0.001$), and all but B showed significant main effects of interval (A, $P=0.0035$; B, $P=0.22$; C and D, $P<0.0001$). For three of the orthogonal subsets, there was no evidence for a duration by interval interaction effect (A, B and C, $P=0.29$, 0.12, 0.14, respectively). Subsets A and B came from a single series, and there was no evidence for series effects among the data combined in subset C. For one orthogonal subset (D) crossing durations 0.5 and 1 with intervals 0.5, 1, 1.5 and 5, there was a significant interaction ($F_{2,138}=9.22$, $P=0.0002$; Bonferroni corrected $\alpha=0.0125$); the duration that induced the highest hatching was longer at longer intervals. This data set includes the stimulus 0.5:5, to which the hatching response differed in Series 3 and 8. We present results with data from both series included, which increases the variance in response to that stimulus. Excluding Series 8, which contributes no other stimulus to the test, increases the significance of the interaction.

Latency of hatching response

For stimuli that elicited substantial hatching, some embryos hatched in the first or second vibration cycle, but most embryos that hatched did so only after multiple cycles of stimulation, with some waiting for minutes. For the stimulus that caused the most hatching (0.5:1.5 s) 66±5% of the embryos that ultimately hatched did so within the first minute, and 97±1% were hatched within 4 min (Fig. 6). A few hatched in the last minute of playback and immediately after playback ended, and the latest hatching recorded was more than 4 min after playback stopped.

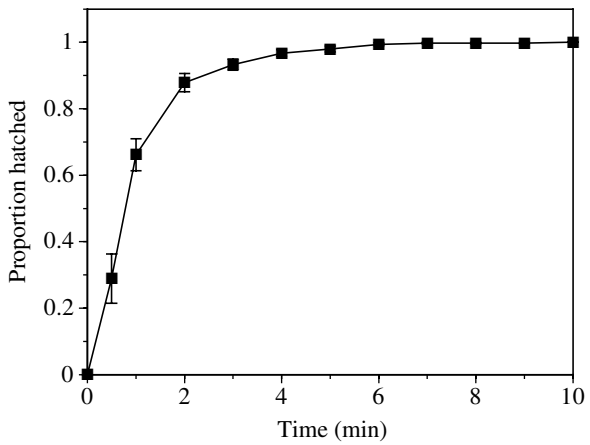

Fig. 6. Hatching response of *Agalychnis callidryas* embryos over time to the vibrational playback stimulus that elicited the strongest hatching response (0.5 s duration:1.5 s interval). A 5 min playback period was followed by 5 min post-playback observation. Data are mean proportion hatched at each time point, out of total hatched, for $N=11$ clutches, except for the 30 s data point where $N=6$ clutches. Error bars are s.e.m.

Discussion

Red-eyed treefrog embryos hatch prematurely to escape from egg predators, but hatching early exposes tadpoles at a less-developed stage to aquatic predators, increasing their risk of mortality in the water (Warkentin, 1995; Warkentin, 1999a; Warkentin, 1999b). Selection by aquatic predators against early hatching should reduce the incidence of unnecessary early hatching, either increasing the specificity of the escape hatching response or perhaps making the embryos generally less responsive to egg disturbance. Warkentin showed that *A. callidryas* embryos can discriminate between different vibrational disturbances and suggested that one or more features of temporal pattern play a role (Warkentin, 2005). Here, we demonstrate that the embryos use a combination of two temporal pattern elements in their hatching response to vibration: the duration of periods of vibration and the spacing or interval length between them.

Specificity of the hatching response

The escape hatching response is not a response to disturbance or vibration in general, nor does more vibration necessarily stimulate more hatching. Indeed, near-continuous vibration elicited almost no hatching. Moreover, neither duration nor interval alone are sufficient to predict the hatching response, except at extreme values that elicit no hatching. The simple composite variables of cycle length and duty cycle are likewise relatively uninformative. In the range of values where hatching may occur, no individual temporal pattern variable predicts the hatching response. Rather, embryos use a specific combination of vibration duration and spacing to inform their escape hatching response.

Duration and interval function as two essential elements of a composite cue, not as two redundant cues that are individually sufficient to induce hatching. A vibration stimulus with a 'scary' duration (i.e. a duration characteristic of stimuli that elicit high hatching) elicits no hatching if the interval is not within an appropriate range. Likewise, a stimulus with a scary interval paired with a duration that is either too short or too long does not elicit hatching (Fig. 5). The requirement that two independently variable temporal pattern elements be within particular ranges substantially reduces the set of clutch disturbance patterns that elicit premature hatching in *A. callidryas*, increasing the specificity of the response. This is consistent with competing selective forces, such as those imposed by aquatic and arboreal predators, having acted to refine the hatching response and reduce the chance of hatching prematurely in the absence of an egg-stage risk. It is inconsistent with vibration-cued early hatching in *A. callidryas* being a general response to '*any movement of, or contact with, the egg mass*' (Savage, 2002).

We found a peak of hatching in temporal pattern space, surrounded by a parameter range across which hatching declined to zero. This indicates that embryos use the temporal patterns of clutch disturbances to recognize danger, and hatch in response to it. They do not use temporal patterns to identify benign disturbances and to refrain from hatching in these while hatching in response to all other patterns. The fact that we found only one hatching peak suggests that embryos do not recognize different species of egg predators by distinct temporal patterns of vibrations, but have a single set of criteria to identify danger. We tested a fairly broad range of duration–interval combinations, informed by prior work on natural disturbance patterns and hatching responses (Warkentin, 2005). Thus, we consider it unlikely, but not impossible, that embryos show strong hatching responses to stimuli outside this range of temporal patterns. If there is indeed just one peak of hatching in temporal pattern space, it suggests that unless the physical properties of the egg clutch impose constraints on predator feeding that affect the temporal pattern of disturbance, a novel predator with a very different feeding pattern could overcome the escape hatching response.

Playback results compared with patterns in natural disturbances

The strongest hatching response is to stimuli with intervals longer than their durations, which is consistent with patterns in snake attacks (Warkentin, 2005). The range of intervals in stimuli that elicit hatching is also larger than the range of durations, which is consistent with the variation of temporal pattern in attacks by egg predators; the spacing between snake bites is more variable than the duration of the bites themselves. Over some ranges of temporal patterns, the effects of vibration duration and interval on the hatching response are statistically independent. Over other ranges, it appears that the interpretation of one parameter is conditioned on the value of the other, with longer durations eliciting more hatching when paired with longer intervals. In rain storms, longer duration vibrations result when multiple drops fall in rapid succession, so that their vibrations overlap in time (Warkentin, 2005). These longer vibrations are associated with shorter, not longer intervals between raindrops. A requirement for longer intervals in association with longer durations would thus reduce the chance of embryos hatching unnecessarily in heavy rain.

The average characteristics of vibrations excited in egg clutches by rain, measured by Warkentin (Warkentin, 2005), fall outside the area of high hatching as expected. However, the peak of hatching is not well matched to the average temporal patterns that Warkentin found in snake attacks. *Leptophis ahaetulla* attacks had an average duration:interval pattern of 1.1:2.7 s, while *Leptodeira annulata* had an average of 0.8:11.3 s. In some ways, this mismatch is not surprising. Our synthetic stimuli were periods of white noise with rectangular amplitude envelopes. For such stimuli, the temporal pattern remains consistent across a wide range of amplitude thresholds for vibration detection. Snake attacks and rain storms, by contrast, cause vibrations with complex and irregular amplitude envelopes. Warkentin's analysis identified periods of vibration using an acceleration amplitude threshold just over the noise threshold of her equipment (Warkentin, 2005). If embryos use a different threshold, they may perceive a very different temporal pattern in these complex stimuli. Moreover, if embryos ignore either intervals or vibration

durations outside a certain range, it may be inappropriate to include these extreme and irrelevant values when calculating average disturbance patterns. For instance, if a snake takes a series of bites, then pauses, then takes another series of bites, embryos may assess the temporal pattern of each bite series but not include the pause between them. A reanalysis of the temporal patterns of natural disturbances using different thresholds or excluding extreme intervals and/or durations may reveal analysis conditions under which there is a better match between hatching responses to natural disturbances and synthetic playback stimuli. If so, synthetic stimuli could be designed to test whether embryos use the same information-processing rules that generated the match.

How do embryos process vibrational information?

We recorded the strongest hatching response to stimuli with a duration of 0.5 s and intervals of 1.5–2.5 s, with embryos hatching from seconds to minutes after the start of stimulation. Hatching itself was very rapid once embryos began hatching movements (usually <1 s; K.M.W. and M.S.C., personal observation), so the bulk of the delay between the stimulus onset and hatching was due to a delay in initiating hatching behavior. Embryos thus appear to integrate information over some period of time or cycles of vibration before initiating hatching.

It is not yet clear how frog embryos sense vibrations. In adult anurans, the saccule of the inner ear and some parts of the amphibian papilla are vibration sensitive, and vibrations are transferred to the inner ear from the pectoral girdle by the operc ularis muscle (Koyama et al., 1982; Lewis et al., 1982; Hetherington, 1985; Narins, 1990; Christensen-Dalsgaard and Narins, 1993). Hatchling tadpoles do not have a pectoral girdle (Shearman, 2005), so the skeletal and muscular coupling that transfers vibrations from the ground to the otic capsule in adults is clearly not present in the embryos. Development of the inner ear has not been examined in *A. callidryas*. However, in the African clawed frog, *Xenopus laevis*, elaboration of the pars inferior, containing the saccule and amphibian papilla, does not occur until a later developmental stage (Bever et al., 2003). The lateral line system is well developed in hatching-competent *A. callidryas* (Warkentin, 1999b) and so is a candidate sensor. Proprioceptive or tactile cues might also be relevant as the entire embryo, floating in perivitelline fluid within the egg capsule, may function as a seismic mass. Regardless of the sensor that embryos use to transduce vibrations, it is likely that central neural processing of temporal pattern information is required for risk assessment. The length of even one cycle of the most effective stimulus (2 s) is long compared with the 150 ms time frame over which neurons in the anuran auditory midbrain are known to integrate temporal patterns in pulsed acoustic stimuli (Adler and Rose, 1998; Adler and Rose, 2000). The processing of patterns of intermittent vibrations in predator attacks may be more akin to assessing call repetition rate in adult anurans than it is to assessing temporal parameters of individual calls.

Mechanosensory cues to risk

Predator detection is crucial for prey, animals produce vibrations as inevitable byproducts of movement, and vibration sensitivity is evolutionarily ancient and phylogenetically widespread (Hill, 2001). Thus, we might expect vibrations to serve as risk cues for many prey. Vibration-cued antipredator defense has, however, received much less research attention than either other modes of predator detection, such as chemoreception (Kats and Dill, 1998), or the role of vibrational signals in intraspecific communication (Hill, 2001; Cocroft and Rodriguez, 2005).

The common observation of singing frogs and insects falling silent as a human observer approaches has been interpreted as a response to vibrations perceived as an indication of risk, although this has rarely been tested (Lewis and Narins, 1985; Narins, 1990). In controlled experiments, hatchling snakes show anti-predator behavior in response to substrate vibrations, without other cues (Burger, 1998). Crickets, cockroaches, caterpillars and spiders respond defensively to nearfield airborne vibrations from predators (Tautz, 1977; Camhi et al., 1978; Tautz and Markl, 1978; Gnatzy and Kämper, 1990; Hieber et al., 2002). Leafmining caterpillars are perhaps the best studied case of antipredator behavior cued by substrate-borne vibrations. Their defensive behavior is elicited by broadband vibrations produced as a parasitoid wasp probes the mine with her ovipositor (Bacher et al., 1996; Bacher et al., 1997; Meyhofer et al., 1997; Djemai et al., 2001).

The escape hatching response of *A. callidryas* differs from the mechanosensory-cued defenses discussed above in that multiple cycles of vibration are usually required to elicit the response. By contrast, adult frogs, hatchling snakes, leafmining caterpillars, and crickets show an immediate defensive response to a single vibration or puff of air (Gnatzy and Kämper, 1990; Narins, 1990; Meyhofer et al., 1997; Burger, 1998). There are, however, two differences between those predator–prey interactions and the red-eyed treefrog case. First, egg-eating snakes take minutes to consume *A. callidryas* egg clutches, allowing embryos more time to escape than in many predator attacks. Second, the fitness cost of hatching prematurely is far higher than that of briefly deploying a defensive posture, pausing in calling or fleeing a short distance. Hatching is an irreversible switch in life stage and ecological niche, and post-hatching performance depends on developmental stage. For instance, the chance of surviving 24 h with a poeciliid fish increases over threefold for tadpoles hatched at the peak of spontaneous hatching, compared with tadpoles hatched 2 days prematurely (Warkentin, 1995). Thus, *A. callidryas* embryos should require a high level of certainty that they are at risk before opting to hatch early. A longer sampling period is likely required for such certainty.

Vibrational risk detection may be important for a wide variety of prey. However, much more biovibrations research is necessary before we will be able to adequately compare the role of vibrations in predator–prey interactions with that of information from other, better-studied sensory modalities across taxa. Vibrations are amenable to detailed signal

manipulation, playback and behavioral or neural assay experiments, like those that have built our knowledge of how animals use acoustic information. Thus, vibration-cued defense offers an excellent opportunity to explore the behavioral decision rules and information processing underlying antipredator behavior. Red-eyed treefrogs are a good study organism for such research since the high selective cost of hatching early is likely to have refined the specificity of vibrational risk assessment. We have begun, in this paper, to address how *A. callidryas* embryos use simple temporal characteristics of vibrations to assess risk. Future papers will address the role of other features of vibrations, individually and in combination. The sensory world and behavioral decisions of embryos may be richer and more sophisticated than we imagined.

This paper is dedicated to the memory of the late A. Stanley Rand, mentor, inspiration and friend, whose practical support and intellectual insight aided this and all our research in Panama. The research and manuscript also benefited from conversations with S. Phelps, G. Rosenthal, I. Schlupp and M. Ryan. We thank the Smithsonian Tropical Research Institute for logistical support. K. Metcalf, A. D'Amato, K. Wright, T. Siok and M. Hughey assisted with playback experiments, and J. Touchon also provided field assistance. We thank I. Gomez-Mestre for assistance with statistics. This work was funded by the National Science Foundation (IBN-0234439), Boston University and the Smithsonian Tropical Research Institute.

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