

Egg vibrations in response to parental calls: scrambled eggs or genuine feedback?

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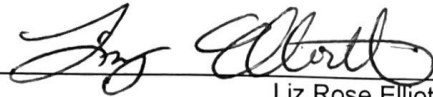
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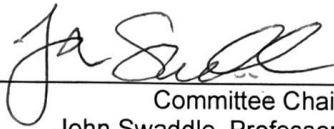
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the requirements for the degree of

Master of Science



Liz Rose Elliott

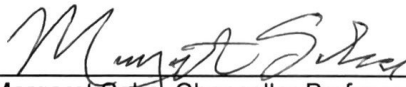
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ABSTRACT

Prenatal acoustic communication affects embryonic development in ways that modify, and potentially optimize, the postnatal phenotype. In zebra finch (*Taeniopygia guttata*), prenatal exposure to a heat-induced parental call affects the behavior, growth, and ultimate fitness of individuals in hotter environments. However, observing the effects of heat calling on the postnatal phenotype does little to inform our understanding of how embryos are immediately perceiving and reacting to this signal. Here, we tested whether prenatal exposure to heat calls induces an immediate response from embryos that can be detected by changes in vibrations at the egg's surface. More specifically, we used laser Doppler vibrometry (LDV) to measure the frequency and amplitude of egg vibrations to assess (i) whether unstimulated eggs exhibit a generalized vibration profile, and (ii) whether this profile changes in response to heat calls played back at four different amplitudes. We found variation in vibration intensity across many frequencies in unstimulated live eggs compared to dead eggs, suggesting randomness or idiosyncrasy in embryonic movements. However, a discernible peak in vibration amplitude between 40 to 45 Hz could be an important identifier for eggs at this stage of development. Importantly, exposure to heat calls affected a two-fold increase in the proportion of eggs exhibiting changes in the intensity of 10 to 15 Hz vibrations. Although the mechanism affecting this response remains unclear, this result provides evidence that developing altricial bird embryos are capable of both perceiving and responding to acoustic stimuli. Moreover, this immediate vibration responses to parental calls supports the potential for communication between parents and their embryos in the nest. Although we acknowledge that anthropogenic sources of noise and other substrate-borne vibrations may affect the transmission and subsequent benefit of this subtle form of communication, egg vibrations may nevertheless be an important tool for measuring embryonic perception.

TABLE OF CONTENTS

Acknowledgements	ii
Dedication	iii
List of Tables	iv
List of Figures	v
Chapter 1. Egg vibrations in response to parental calls: Genuine feedback or scrambled eggs?	1
Bibliography	36

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Finally, I am incredibly thankful for all the emotional support I received from my family, peers, and friends across the globe. Every hug, beer, or jaunt in the woods helped me forward. I know that I could not have done this without you.

This Master's Thesis is dedicated to my cat, Piper.

May we continue to face life's challenges together.

LIST OF TABLES

1. Number of peaks across frequencies for unstimulated eggs	16
2. Proportion of eggs exhibiting changes in vibration amplitude	20
3. P-values for Fisher's exact test pairwise comparisons	21

LIST OF FIGURES

1. Visual representation of heat call playback	7
2. Experimental set-up for playbacks and vibration recording	9
3. Flow chart of data processing steps	12
4. Average FFTs recorded from live and dead ZEFI embryos	18
5. Average change in vibration amplitude before and after playback	19
6. Effect of playback on change in vibration amplitude	22
7. Effect of playback volume on changes in vibration amplitude	23
8. Supplementary Fig. 1: Average number of peaks across frequency bins for unstimulated eggs	35
9. Supplementary Fig. 2: Average change in vibration amplitude across all resolvable frequencies	36

Introduction.

The prenatal environment can influence embryonic development and give rise to lifelong changes in individual behavior, morphology, and fitness (Mariette & Buchanan, 2016; Pessato et al., 2022). In the mammalian womb, the embryo typically experiences variation in the external environment through the mother, with changes in maternal physiology or behavior directly affecting developmental processes (Mousseau & Fox, 1998). For oviparous species, such as birds, the mother's ability to directly influence the prenatal environment is limited after egg laying (Mariette, 2020; Mariette et al., 2021). In such circumstances, selection may favor mechanisms that allow embryos to gain contextual information more directly from their environment.

While there is potential for cues from the external environment to be informative to developing embryos that are inside eggs, these embryos are typically limited in their sensory capacities. In birds, for example, a calcified shell constrains embryonic movement and the perception of visual and chemical cues (Mariette et al., 2021). Sound and vibration, however, may be able to permeate these barriers, and many species appear capable of detecting acoustic signals even without a fully-developed hearing system (Colombelli-Négrel et al., 2014; Höchel et al., 2002; Impekoven, 1976). For example, embryos of yellow-legged gulls (*Larus michahellis*) appear to perceive and appropriately respond to playbacks of parental alarm calls. In this case, the external sound delayed egg hatching and influenced several aspects of post-hatching behavior (reduced vocalization rate, faster antipredatory responses) and physiology (reduced growth rate) (Noguera & Velando, 2019). Such specific, seemingly adaptive responses

to external stimuli would suggest that avian embryos are far less naïve than previously assumed.

As more evidence that acoustic cues from parents can influence embryonic performance, the heat-induced calls (hereafter, “heat” calls) of wild zebra finch (*Taeniopygia guttata*) parents, elicited during incubation, influence the development of specific behaviors and morphologies in embryos that could counter the negative effects of thermal stress. Specifically, nestlings that had been exposed to heat calls showed a decrease in mass in response to higher temperatures and tended to show preference for hotter nesting sites later in life (Mariette & Buchanan, 2016). The relevance of these developmental and behavioral changes to heat tolerance suggests that embryos obtain specific information related to the abiotic environment through reception of the heat calls. This type of prenatal communication may therefore contribute to the species’ ability to survive in the hot, highly-fluctuating temperatures of Australian desert environments.

In general, little is known about how embryos detect and respond to prenatal sounds despite growing evidence that prenatal acoustic communication influences developmental programming in many taxa (Doody et al., 2012; Endo, Takanashi, Mukai, et al., 2019; Mariette, 2016; Noguera & Velando, 2019; Rumpf & Nichelmann, 2007). Indeed, while heat calls affect the post-hatch developmental trajectories and long-term fitness of exposed zebra finch embryos, their immediate perception and response remains assumed rather than documented. Establishing whether and how embryos respond to prenatal sound is important to understanding how embryos function as receivers and, potentially, transmitters of environmental information. In some precocial

birds, for example, embryos are capable of vocalizing or vibrating in the egg to transmit information to their parents or siblings in the nest (Noguera & Velando, 2019; Rumpf et al., 2010); thus, it may be possible that this behavior extends to altricial avian species as well. The detection of tactile stimuli is one of the first senses to develop in altricial ontogeny (Gottlieb, 1968), thus vibrations may provide a good candidate mechanism by which embryos can receive and transmit information.

If *in-ovo* embryos can produce vibrations in response to environmental stimuli, it may be that parent birds and/or adjacent siblings inside other eggs in the clutch are able to detect and respond to these vibrational cues. This potential for bidirectional information flow has yet to be investigated in birds and characterizing the amplitudes and frequencies of vibrations from stimulated eggs is an important first step. Furthermore, demonstrating the importance of prenatal vibrational communication in coordinating postnatal phenotypes could help us understand species' vulnerability to vibro-acoustic interference. Increases in ambient noise and vibrations generated by human structures and activities (e.g., Díaz et al., 2017; Slabbekoorn, 2019) could affect or entirely mask this type of prenatal communication, with repercussions for embryonic development and adaptation. Thus, learning more about whether and how *in-ovo* avian embryos communicate may be relevant for determining conservation and management efforts.

Our research objective was to detect and measure vibrations from *in-ovo* zebra finch embryos that were stimulated by heat calls from conspecific adults. Importantly, our work explores the utility of a non-invasive method (i.e. laser Doppler vibrometry) for assessing behavioral responses whereas many common methods of monitoring

embryonic responsiveness are invasive and terminal (Leandro et al., 2004; Olson et al., 2006; Pitk et al., 2012). Moreover, our methodology allows for real-time assessment of embryonic responses, which are not possible in terminal procedures. While it is possible for a researcher to subjectively observe and categorize egg vibrations in larger, precocial bird species such as gulls (Noguera & Velando, 2019), this is not feasible in smaller, altricial birds such as songbirds where vibrations are too subtle to be observed reliably by eye. Therefore, we used a laser Doppler vibrometer to quantify vibrations of the surface of eggs as their embryos responded to heat calls in real-time.

To address our objective, we investigated the validity of two hypotheses. First, we hypothesized that unstimulated eggs would have a generalized vibration profile, assessed in terms of frequency and amplitude properties, that is similar across all eggs prior to stimulation of the embryo by the presentation of heat calls. We predicted that we would detect multiple peaks in the amplitude of eggs' vibrations at particular frequencies, and that these frequencies would be similar among eggs.

Second, we hypothesized that embryos would show an immediate phenotypic response to the presentation of appropriate heat calls resulting in instantaneous changes in the vibration profile recorded from eggs. Specifically, we investigated whether playbacks of zebra finch heat calls induced an immediate response from embryos that can be detected by vibrations of the outer surface of the egg. We also explored whether the amplitude (i.e., loudness) of the playback influenced the response from the embryo and egg. We predicted that vibrational profiles would change in two ways as the amplitude of playback increased: there would be a change in amplitude of vibrations in the pre-stimulation frequency ranges; and that the range of frequencies

recorded would increase as the embryo is stimulated in novel ways with increasingly loud heat calls.

Methods.

Amplitude calibration of field recordings of heat calls.

Mylene Mariette (Deakin University, Australia) recorded (Wooltana, Australia; March 13, 2022) heat calls from wild (i.e., free-living birds experiencing ambient temperatures above 26 °C) adult zebra finches. The recordings (WAV, at 44.1 kHz 16-bit) were made using a directional microphone (Sennheiser lavalier, MKE 2P-C), located within 5 cm of the focal individual and attached to a solid-state recorder (Tascam DR-40X). The artificially induced heat challenge required powered air flow that created substantial background noise in most recordings, and therefore we decided to use only the calls recorded from the field. Out of all field recordings, however, only one individual produced heat calls. Although heat calls from different individuals would better represent potential variation in call characteristics, using one individual could minimize some of the acoustic variation that might influence variation in embryonic vibrational responses. Moreover, we wanted to isolate the influence of specific acoustic parameters on heat call-induced vibrational responses. Additional variation across playback files would have introduced among-individual call differences that could mask our abilities to detect how embryonic responses might be tuned to specific acoustic parameters.

To calibrate the amplitude of this field recording, we recorded a 7 kHz tone (i.e., within the 6-8 kHz range of heat calls) played at various amplitudes using the same recording equipment and settings as above. We completed all recordings in a sound-proof room, with the sound playing from a speaker located 5 cm away from the recorder

to simulate the distance between the vocalizing bird and the microphone in the recordings. We played the tone from the speaker's lowest to highest volume setting, which ranged from 32 to 80 dBA SPL (saturating above 80) as recorded by handheld sound pressure level (SPL) meter (EXTECH 407730, parameters set to A-weighted and slow response). We then uploaded the recording of the calibration tone to Raven Pro (v 1.6, Cornell Lab of Ornithology) and calibrated the software by relating the recorded amplitudes to the known SPL readings. After calibrating the software, we then measured the sound pressure levels of the recorded heat calls, allowing us to estimate the amplitude at which these calls were experienced by eggs in situ at approximately 5 cm from the vocalizing individual. We used this measured amplitude to inform the design of playbacks used throughout this study.

Generating heat call playbacks.

Using the calibrated recording, described above, we isolated heat calls (i.e., calls ranging between 6 and 8 kHz, with approximately 5 to 8 notes/s) and generated a 2 min playback file to be used in the rest of this study. To reduce background noise and increase the clarity of the playback, we also band-pass filtered the calls between 6 and 8 kHz in Raven Pro.

Using Audacity, we constructed the playback file to comprise four 10 s sequences of heat calls from the same individual, with 20 s of silence at the onset and end of each sequence. Each bout was identical, with 10 s of continuous calling from the focal individual (Figure 1). The file was then exported as a .wav file (1411 kbps bit) and downloaded onto a media player (Samsung Galaxy S22 ®). This playback file was presented to eggs at four amplitudes (42, 45, 54, and 65 dBA SPL, see details below),

with the lowest amplitude representing the lowest possible amplitude attainable in our aviary, 45 dB the average amplitude of heat calls obtained from the field recording, and the highest representing the amplitude of playbacks known to affect embryonic responses (Mariette & Buchanan, 2016).

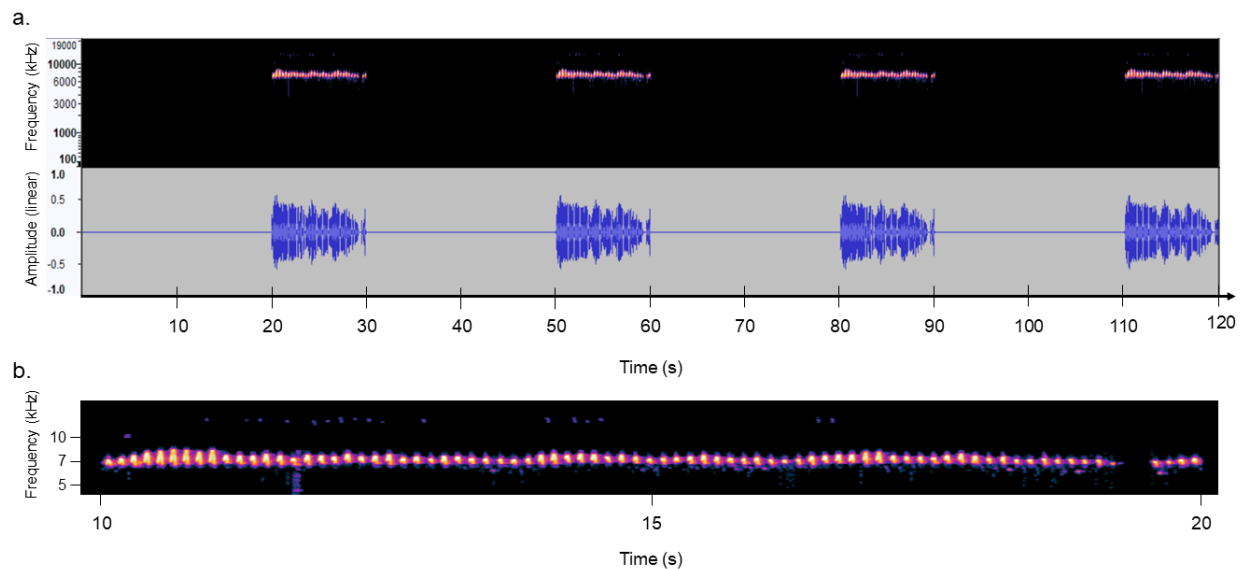


Figure 1. Visual representation of heat call playback. (a) Spectrogram (top) and waveform (bottom) views of the 2 min playback with four 10 s bouts of heat calling. Spectrogram indicates the frequency (kHz) of heat calls falling between 6 to 8 kHz, while the waveform shows the amplitude of each bout using a linear scale, with 1.0 being the maximum value of the positive signals and -1.0 the maximum of negative signals. (b) Close-up view of spectrogram for one 10 s bout, showing modulation and rhythm of individual notes.

Breeding and egg selection.

We established an indoor free-flight breeding room (approximately 4 x 5 x 3 m) of adult domesticated zebra finches (15 males, 15 females, arbitrarily selected from a larger captive colony). All birds received ad libitum seed (Volkman finch super blend), drinking water, and bathing water and had access to numerous perches, 20 nest boxes, and ad libitum Timothy hay for nest construction. These birds experienced a 16:8 light:dark

photoperiod with full spectrum artificial lighting and the room was maintained at a fairly constant 25 °C.

In May 2022, we removed all eggs from the nests so we could determine the lay date of subsequent eggs. Throughout the course of the experiment, each nest box was checked daily (approximately 1200) for eggs. Once an egg was found, it was labeled with a unique three-digit number using a fine-tipped Sharpie and put into an HovaBator (Genesis 1588) incubation chamber, stored in an adjacent room (i.e., the “primary incubator”). After 3 to 5 days in the incubator, eggs were checked for fertilization via the candling method. We continued this process of egg removal and incubation until the end of August 2022. Throughout egg incubation, the incubator was maintained at 37.5 to 37.7°C and 65% humidity to replicate optimal conditions experienced in the nest (Mariette, unpublished data). An automatic rotator was installed inside the incubator to turn the eggs once every 4 h. Although we did not directly play any sounds to the eggs throughout the incubation period, the noise from the colony in the adjacent room ranged between 37 to 42 dBA SPL and may have offered detectable acoustic stimulation. All experimental eggs were kept in this primary incubator when not in use for playback experiments.

Once an egg reached the 10th day of incubation, it was removed and placed in another incubator (i.e., the “trial incubator”; Genesis 1588) in a separate room where we conducted playback trials. The trial incubator was maintained at the same temperature and humidity as the primary incubator, but without an egg rotator to avoid jostling the egg during trials.

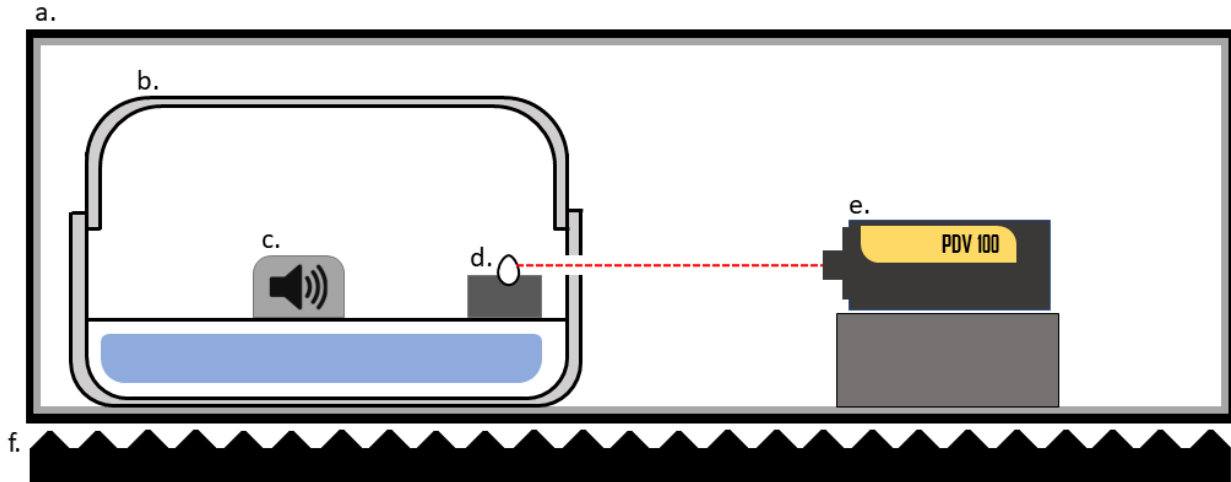


Figure 2. Experimental setup for playbacks and vibration recording. (a) The incubator was stored in a wooden box (121.9 X 30.5 X 30.5 cm) lined with sound-proofing mass-loaded vinyl and sealed with acoustical caulk. (b) The trial incubator was equipped with water troughs (indicated in blue) and an electric thermostat and hygrometer (located at the center of the lid) to maintain optimal humidity and temperature. The incubator housed the speaker for playbacks (c) and a small square of sound-proofing foam (d) on which the egg could be placed for recording. A small hole in the side of the incubator allowed the beam from the laser Doppler vibrometer (e) to reach the egg.

Playback trials and recording of vibrational response.

All playback trials were conducted in the trial incubator (Figure 3). A small hole was cut into the base of the incubator, through which the beam from a laser Doppler vibrometer (LDV, PDV 100, Polytec Inc, Irvine, CA) was pointed directly at the egg for vibration measurements. For each trial, the egg was fit into a small piece of foam for insulation and positioned (pointed end up) in front of the hole. The vibrometer was adjusted so that the egg was 234 mm (i.e., optimal stand-off distance; Polytec, 2017) from the base of the laser's focusing ring. All vibratory signals were recorded using a sampling rate of 0.24 kHz, with a low pass filter of 5 kHz and the high pass filter off (i.e., passing frequencies below 100 Hz). Signals were processed using the Vibsoft data acquisition

software (v5.4, Polytec Inc, Irvine, CA, USA), which measured the velocity ($\mu\text{m/s}$) of the vibrations overtime and stored data in a .txt file.

To broadcast the playbacks, a speaker (© Alpatronix AX400) was situated inside the incubator, 5 cm from the egg, and connected to a Samsung Galaxy S22® phone via Bluetooth. To limit background noise, we placed the set-up in an insulated wooden box (121.9 x 30.5 x 30.5 cm; Figure 3) lined with sound-proofing mass-loaded vinyl (Acoustimac LLC) and acoustical caulk (Green Glue, © Saint-Gobain Group Entities). We placed the entire apparatus on a concrete floor, which was the foundation slab for the aviary building. The aviary was located more than 100 m from the closest road. Hence, we took several steps to minimize background noise and vibrations in this study.

Effect of playback volume on egg vibrations.

Prior to initiating playback trials, each egg was left undisturbed in the incubator for 20 mins, i.e., the minimum amount of time required for embryonic heart rate to return to normal after disturbance (Mariette, unpublished work). This accommodation period preceded all trials. We then presented each egg with the unmanipulated heat call playback file at all four amplitudes (i.e., 42, 45, 54, and 65 dBA SPL playbacks), played in random order with 20 min of silence in between each subsequent playback. The vibrometer began recording at the same time as we initiated each of the four playbacks, allowing us to measure the embryo's immediate vibrational response to the stimulus. Every egg ($n = 37$) received all four playbacks in a randomized order, after which we removed each experimental egg from the trial incubator and placed them in a freezer at $-18\text{ }^{\circ}\text{C}$. The freezing terminated the embryos without altering the surface properties of the eggs. After the completion of all trials with live-embryo eggs, we thawed each egg at

room temperature for approximately 20 mins and repeated each playback and recording trial with dead-embryo eggs (n = 20). Comparison of vibratory recordings from the same egg when the embryo was alive compared to when it was dead allowed us to investigate which vibrations resulted from the playback sound resonating the outer surface of the egg independent of any movements initiated by the live embryo. At the conclusion of the experiments, we converted all .txt files, which contained the velocity ($\mu\text{m/s}$) of the egg's surface vibrations at each second of the 2 min (140 s) playback period, to .csv files for data processing in Microsoft® Excel.

We did not use eggs that were unfertilized, had failed to develop, or were accidentally damaged during handling. Furthermore, the presence of unanticipated background noise during recording (e.g., thunder, rain, or human activity in the building) eliminated some trials from further analysis.

Ethics statement.

All procedures were approved by the William & Mary Institutional Animal Care and Use Committee (2022-0067).

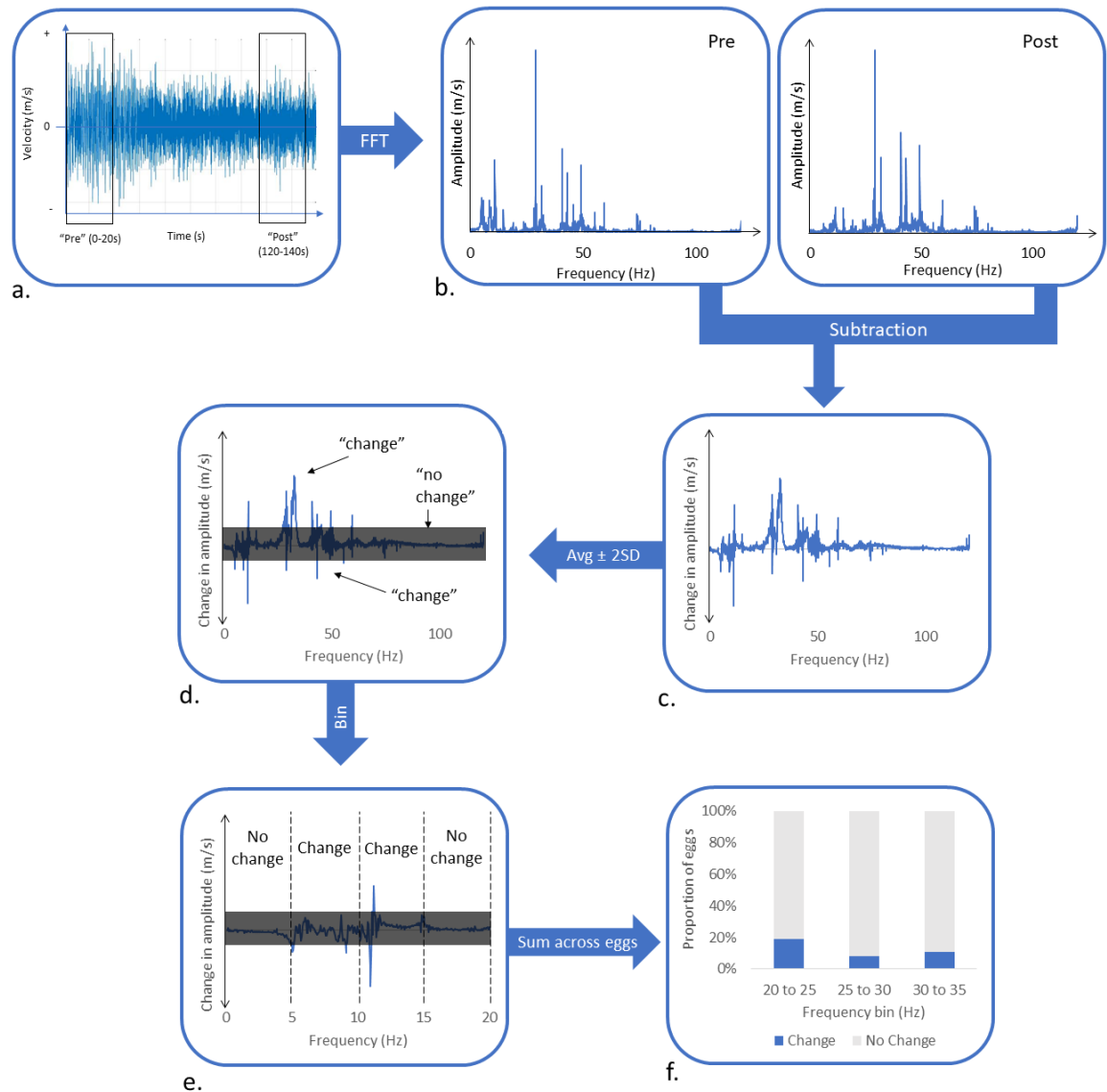


Figure 3. Flow chart illustrating data processing steps (see *Data processing*). (a) Each recording was segmented according to timing of playback and response periods (i.e. pre-playback and post-playback). (b-c) Each recorded segment was fast Fourier transformed and each post-playback (Post 1, etc.) segment was subtracted by the baseline (pre-playback) signal. (d) The average difference in amplitude $\pm 2SD$ for each vibration signal was calculated to generate upper and lower thresholds (gray bar). (e-f) Each signal was divided into discrete frequency bins (in intervals of 5 Hz) so that the number of points above and below the calculated thresholds could be counted to identify larger changes (beyond confidence intervals) in vibration activity within each frequency range. We processed all recordings to estimate the number of eggs that showed “change” or “no change” in vibration intensity within each frequency bin.

Data processing.

Our data processing steps are summarized in Figure 2. First, we split each .csv file into two 20 s segments that represented the following sections of the recording: pre-playback (*pre-playback*) and immediately following the fourth and final 10 s of the heat call playback (*post-playback*; Figure 3a). To analyze the spectral characteristics (e.g., frequency) of the recorded vibrations, we applied a fast Fourier transform (FFT; Hanning window, 120 Hz bandwidth, frequency resolution (dF) = 0.05859) to both segments using enDAQ data acquisition software (enDAQ VibrationData Toolbox v13.3, Mide Technology Corp., Woburn, MA) (Figure 3b). To characterize the baseline vibrations of eggs, we identified “peaks” in the pre-playback FFT as points at which the amplitude was at least $\pm 2SD$ above the average amplitude (i.e., amplitude averaged across all resolvable frequencies; May et al., 2007; Xiao & Braun, 2008). Then, to estimate how the amplitude of the egg’s vibrations changed across all resolvable frequencies (0.1 to 119.9 Hz; Figure 3c), we simply subtracted the pre-playback FFT from the post-playback FFT. Given that we predicted that each egg would have a different baseline vibratory output (or signature), calculating the difference between the baseline and post-playback FFTs would make each egg comparable across time and over treatments. This method also served to reduce background noise present in the recording of both signals and would have removed signals generated by movements of the embryo that were unchanged from pre- to post-playback. To distinguish notable changes in amplitude from minor fluctuations in the egg’s baseline vibration signal, we estimated the average change in amplitude $\pm 2 SD$ as a cut-off point for our confidence

intervals (Figure 3d). In this way, we determined any changes in amplitude within this range to be zero and any changes above (or below) to be of significance.

Without substantial precedent for assessing differences in the frequency of embryonic vibrations, we decreased the resolution of the signal to encompass larger frequency bins (bin width = 5, Figure 3d). This method allowed us to identify broader regions of overlap in each egg's vibrational response to heat calls, since finer resolutions of frequency bins are less likely to be of biological importance (i.e., detectable by parents or siblings in the nest). We then counted the number of eggs that showed a notable change (as explained, above) in amplitude within each 5 Hz frequency bin, with at least one change in amplitude required to classify the vibration signal of an egg to be changing in that bin (i.e., "change"). If there were no notable changes in the amplitude of an egg's vibration within a given bin, we determined there was no reliable change (i.e., "no change") in that frequency range in response to heat calls. We continued this process for all eggs and calculated the proportion of eggs that showed a change or no change in each frequency bin (Figure 3f).

To generate an appropriate comparison for the above analysis, we processed the signals for the thawed-out, dead eggs in the same way. This was done across all treatments to determine whether the calculated differences between the pre- and post-playback signals were, indeed, of biological significance rather than a consequence of any background vibrations or resonance through the egg (i.e., a negative control). Any eggs that broke in the freeze-thaw cycles were not used in the experiments, reducing our effective sample size ($n = 16$). Furthermore, given that the baseline vibrations of an egg are likely to change even without stimulation (i.e., due to random changes in

movement and/or sensitivity to minor changes in temperature, humidity, or background noise), we also generated a positive control for comparison. We did this by comparing the FFTs for the first and last ten seconds of the pre-playback signal, estimating any notable differences in amplitude across frequency bins using the methods outlined above. We generated positive controls from the pre-playback period for each of the four 2 min exposures (i.e., 42, 45, 54, and 65 dB) to reduce any changes in background noise that might have occurred between subsequent exposures. The pre-playback signals for live embryos were also used to characterize base-line egg vibrations, i.e., without intentional stimulation, using qualitative comparisons with vibrational signals from the eggs of dead embryos.

Statistical analysis.

To determine whether the intensity of embryonic vibrations varies in response to heat calls, we compared the proportion of eggs showing a change or no change in the amplitude of their vibrations between groups (i.e., post-playback, positive control, and negative control groups) using Fisher's exact tests. We performed separate tests for each frequency bin within each volume treatment. We then performed pairwise Fisher's exact tests, using the Benjamini-Hochberg method (Benjamini & Hochberg, 1995) to correct for multiple-hypothesis testing, to identify frequency ranges where the proportion of eggs changing in vibration intensity post-playback was significantly different to both the positive and negative controls. We then qualitatively compared the affected frequency ranges between volume treatments (42, 45, 54, and 65 dBA SPL).

Results

Characterizing egg vibrations.

Table 1. Average number and standard deviation (SD) of peaks in amplitude within each 5 Hz frequency bin for unstimulated eggs.

Frequency bin (Hz)	Average	SD
0-5	0.05	0.23
5-10	1.43	3.80
10-15	2.32	2.86
15-20	2.76	2.30
20-25	1.16	2.19
25-30	2.03	2.68
30-35	0.73	1.43
35-40	1.27	3.07
40-45	2.86	2.95
45-50	9.95	6.43
50-55	3.49	4.21
55-60	3.14	2.90
60-65	1.59	2.51
65-70	2.19	4.14
70-75	1.54	2.71
75-80	0.14	0.35
80-85	0.08	0.28
85-90	0.03	0.16
90-95	0.00	0.00
95-100	0.00	0.00
100-105	0.05	0.23
105-110	0.03	0.16
100-115	0.08	0.36
115-120	0.46	1.24

Overall, we found that zebra finch eggs have complex vibrational signals, with individual eggs showing multiple peaks (i.e., frequencies at which the amplitude falls $\pm 2SD$ above the mean) in vibrations across the frequency range (0 to 120 Hz) analyzed here. On average, we detected 37.4 ± 10.8 peaks in an egg's base-line vibrations; however, the frequencies of these peaks varied widely between eggs (Table 1; Figure S1). As a result, vibration amplitude varied greatly within each 5 Hz frequency bin, with the

greatest variation occurring at frequencies below 75 Hz (Figure 4a). Despite this variability, there was one distinct peak within the 45 to 50 Hz bin in all the eggs sampled prior to exposure to heat calls. When we analyzed the first and last 10 s of the pre-playback signal, this peak appeared to be retained overtime (Figure 4b). Moreover, this peak did not overlap with vibrations detected from the surface of the egg for dead embryos (negative control), which showed one peak at 30 Hz when averaged across eggs (Figure 4a).

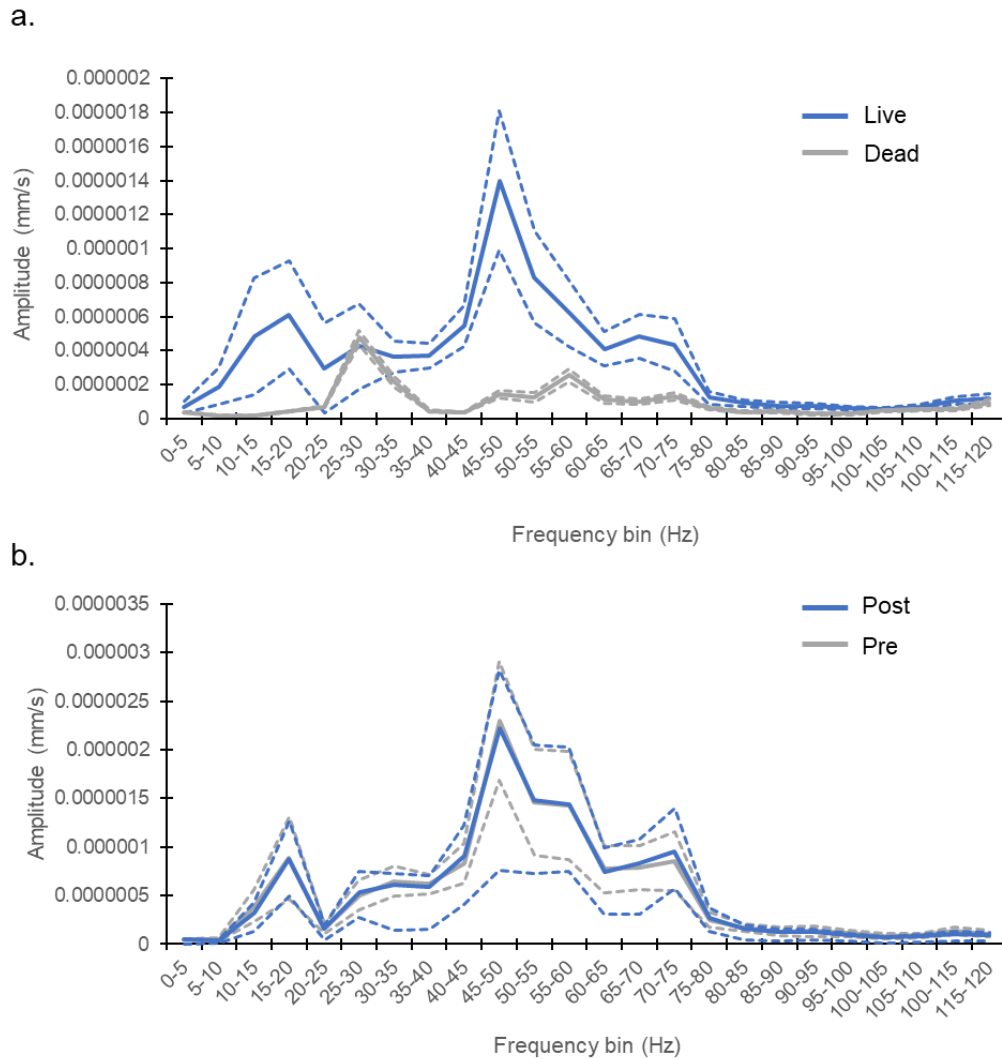


Figure 4. Average amplitude of egg vibrations for live and dead zebra finch embryos. The vibrations of live ($n = 37$) and dead ($n = 16$) zebra finch eggs were recorded and fast-Fourier transformed (FFT) to obtain the amplitude of vibrations across all resolvable frequencies (0 to 120 Hz). The amplitude of vibrations was then averaged at intervals of 5 Hz to generate frequency bins. Data are shown as the averaged FFT for (a) live and dead eggs for the entire 20 s prior to playback and (b) live eggs for the first and last 10 s of the pre-playback period. Dashed lines indicate 95% confidence intervals.

Effect of heat calls on egg vibrations.

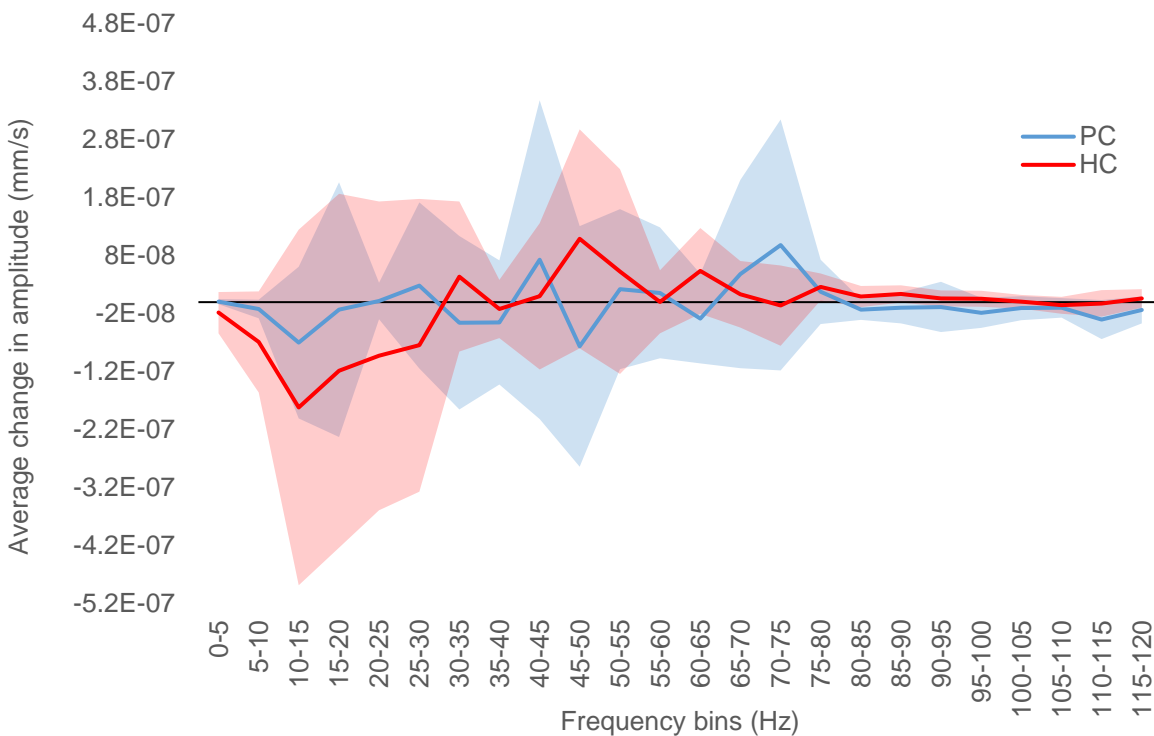


Figure 5. Average change in amplitude of egg vibrations before and after exposure to heat calls. The vibrations of live zebra finch eggs were recorded before and after exposure to playbacks; for the positive control (PC, n = 16), change in vibration amplitude was obtained by subtracting fast Fourier transforms (FFT) for the first and last 10 s of the pre-playback period; change in vibration amplitude after heat call exposure was obtained by subtracting the FFTs of pre- and post-playback signals for each egg (HC, n = 37). Colored areas indicate 95% confidence intervals for PC (blue) and HC (red) groups.

We found that exposure to heat calls at 45 dB affected wide variation in the amplitude of egg vibrations across frequencies (Figure 5; Supplementary Figure 2). While vibrations above 80 Hz showed little to no significant changes in amplitude after the heat call was played, vibrations at frequencies <80 Hz showed both increases and decreases in amplitude. Since many of these frequencies may be of similar biological significance, we more coarsely evaluated changes in amplitude in 5 Hz frequency bins. Although we observed a general trend of vibrations between 0 to 30 Hz decreasing in amplitude and

vibrations between 40 to 65 Hz increasing in amplitude, the confidence interval still suggests wide variation in responses between embryos (Figure 5).

Table 2. Proportion of eggs with significant changes in vibration amplitude across frequency ranges prior to playback (positive control) and after heat call exposure (post vs pre-playback). The frequency ranges where a majority (>50%) of eggs changed in amplitude are indicated in bold.

Frequency range (Hz)	Positive control	Heat calls
0-5	0.0%	10.8%
5-10	0.0%	21.6%
10-15	30.0%	64.9%
15-20	85.0%	83.8%
20-25	5.0%	35.1%
25-30	35.0%	51.4%
30-35	65.0%	43.2%
35-40	50.0%	48.6%
40-45	60.0%	67.6%
45-50	100.0%	97.3%
50-55	85.0%	73.0%
55-60	50.0%	78.4%
60-65	40.0%	59.5%
65-70	50.0%	48.6%
70-75	45.0%	62.2%
75-80	10.0%	18.9%
80-85	0.0%	8.1%
85-90	0.0%	2.7%
90-95	0.0%	0.0%
95-100	0.0%	0.0%
100-105	0.0%	5.4%
105-110	0.0%	5.4%
110-115	0.0%	13.5%
115-120	0.0%	21.6%

Given the wide variation in the amplitude of embryonic vibrations in response to heat calls, we decided to assess the frequency ranges in which the majority of eggs showed one or more notable changes in vibration amplitude. After exposure to heat calls, we found that vibrations in nine out of 24 (37.5%) frequency bins showed a notable change in amplitude for the majority of eggs (>50%; Table 2), in contrast 5 out of 24 (20.83%) frequency bins showed notable changes in amplitude during the pre-

playback positive control period. Thus, observed changes in vibration amplitude across most of the frequency bins (15/24 or 62.5%) appeared to be driven by <50% of the eggs sampled. However, we could identify only one frequency range (10-15 Hz) at which the proportion of eggs changing was significantly associated with exposure to heat calls, rather than any background noise that was measured in the negative controls (two-tailed Fisher’s exact test: $df = 2$, $p < 0.05$, $N = 57$; Figure 6, Table 3).

Table 3. *P*-values for Fisher’s exact test pairwise comparisons across frequency bins. All *p*-values represent adjusted values after applying the Benjamini-Hochberg correction for pairwise comparisons: heat call-exposed eggs (HC), negative controls (NC), positive controls (PC). We indicated *p*-values (< 0.05) in bold.

Frequency range (Hz)	HC vs. NC	HC vs. PC	PC vs. NC
0-5	0.13	0.12	1.00
5-10	0.13	0.12	1.00
10-15	<0.001	0.02	0.02
15-20	<0.001	1.00	<0.001
20-25	0.54	0.04	0.22
25-30	<0.001	0.28	<0.001
30-35	<0.001	0.17	0.016
35-40	0.0123	1.00	0.014
40-45	<0.001	0.58	<0.001
45-50	0.05	1.00	0.05
50-55	0.09	0.35	0.04
55-60	0.25	0.06	0.03
60-65	0.23	0.27	0.73
65-70	0.77	1.00	0.75
70-75	0.53	0.40	0.29
75-80	0.01	0.47	0.02
80-85	0.82	0.82	1.00
85-90	0.78	1.00	0.78
90-95	1.00	1.00	1.00
95-100	0.67	1.00	0.67
100-105	1.00	1.00	1.00
105-110	1.00	0.80	0.80
110-115	1.00	0.29	0.29
115-120	1.00	0.04	0.08

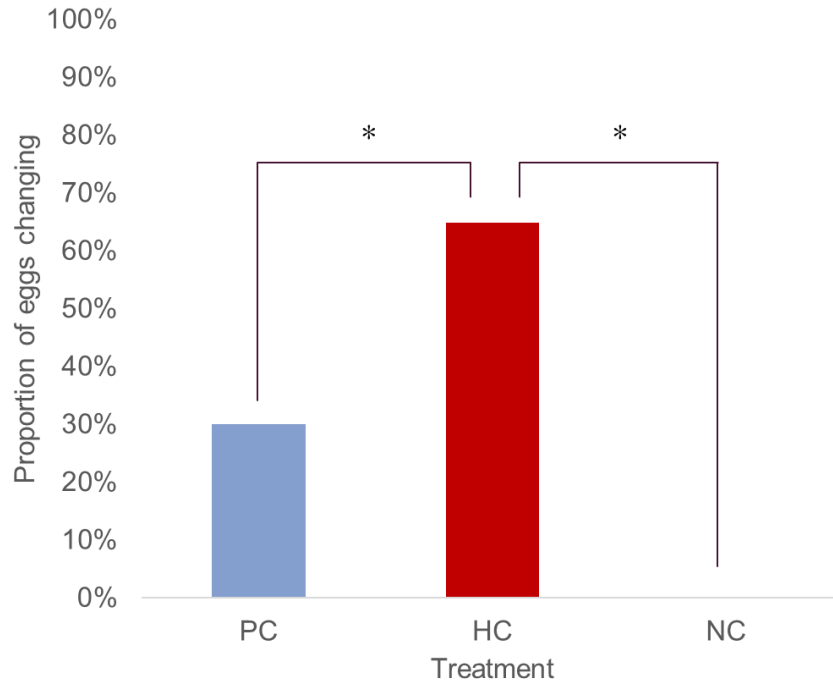


Figure 6. Exposure to heat calls increases the likelihood that egg vibrations will change in intensity over time. The vibrations of live zebra finch eggs were recorded before (PC, positive control; $n = 20$) and after (HC, heat call-exposed; $n = 37$) exposure to heat calls at 45 dB SPL. Dead eggs were used as a negative control (NC, negative control; $n = 16$). Bars indicate the percentage of eggs that showed significant variation in vibration amplitude in the 10 to 15 Hz range across treatments. Asterisks (*) indicate significant differences between groups (Fisher's exact test, $df = 2$, $p < 0.05$).

Effect of playback amplitude on egg vibrations.

To determine whether the amplitude (loudness) of the playbacks affected how eggs' vibrations changed in response to heat calls, we qualitatively compared the frequencies ranges that showed a significant change in amplitude across the four volume treatments (42, 45, 54 and 65 dB SPL). All volumes of the playbacks except for the lowest (i.e., 42 dB) affected an increase in the number of eggs changing compared to both controls in the 10 to 15 Hz range (Fisher's exact test, $df = 2$, $p < 0.05$; Figure 7, Supplementary Table 3-5). Although the number of eggs changing in response to 42 dB heat calls was significantly greater than in the negative control group (i.e., dead eggs) at this range of

frequencies, there was no difference between the exposed group and the positive control (Fisher's exact test, $df = 2$, $p = 0.16$).

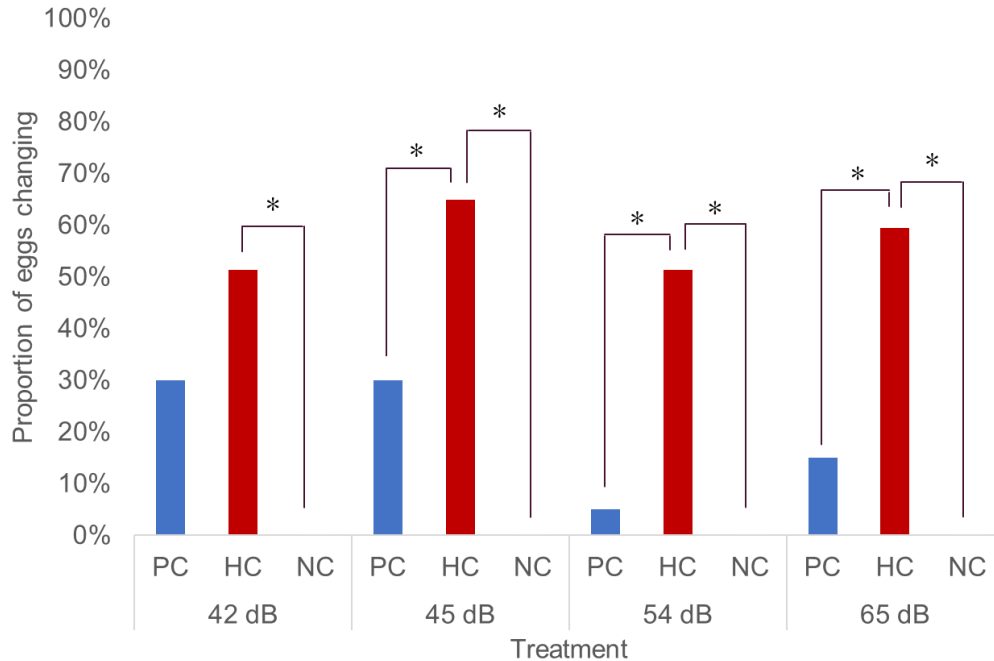


Figure 7. Heat calls played at low amplitudes reduced the effect of exposure on the intensity of egg vibrations. The vibrations of live zebra finch eggs were recorded before (PC, positive control; $n = 20$) and after (HC, heat call-exposed; $n = 37$) exposure to heat calls at four different volumes (42, 45, 54, and 65 dB SPL). Dead eggs were used as a negative control (NC, negative control; $n = 16$). Bars indicate the percentage of eggs that showed significant variation in vibration amplitude in the 10 to 15 Hz range across treatment groups. Significant differences (Fisher's exact test, $df = 2$, $p < 0.05$) between groups within each volume treatment are indicated by asterisks (*).

Discussion.

The effect of heat calls on egg vibrations.

We detected changes in the intensity of vibrations coming from eggs that contained live zebra finch embryos following exposure to conspecific heat calls. Specifically, heat calls affected a two-fold increase in the proportion of eggs that exhibited a change in the

intensity of 10 to 15 Hz vibrations. Although it is difficult to determine the exact processes that affect vibrations within this range of frequencies and amplitudes, this signal is likely generated by the movement and/or contraction of live embryonic tissues. This conclusion is supported by the observation that variation in vibration intensity was minimized in dead embryos, suggesting that the movements of living tissues within the egg are affecting this signal. Thus, changes in the intensity of 10 to 15 Hz vibrations may indicate a reliable and immediate response from embryos to sound, supporting our initial hypothesis. Although previous work has shown that embryos are capable of perceiving sound (Höchel et al., 2002; Impekoven, 1976; Mariette & Buchanan, 2016; Rivera et al., 2018), this is one of few studies to show that altricial bird embryos are capable of short-term responses to acoustic stimuli.

Most importantly, our results indicate a potential signal that could be received by parents or siblings in the nest. For example, although fairy-wren embryos have been shown to lower their heart rate in response to conspecific calls, there is little evidence to suggest that change in heart rate could transmit information from one individual to another in the nest (Colombelli-Négrel et al., 2014). Here, however, we identify changes in vibrations at the egg's surface that could be detected by various mechanoreceptors found in the feathers, skin, and/or beaks of other birds (Ziolkowski et al., 2022). Of course, while we cannot determine whether this signal represents intentional communication or a byproduct of some physiological or behavioral change from the embryo, this should not be considered a prerequisite for establishing bidirectional communication. Indeed, communication occurs when either intentional or incidental signals produced by one organism (the sender) are perceived and acted upon by

another organism (the receiver) (Bradbury & Vehrencamp, 1998). Thus, to determine whether egg vibrations in the 10 to 15 Hz range can support the potential for bidirectional information flow between parent birds and their developing embryos, it is more important that we establish whether parents and/or siblings (i.e., embryos inside other eggs in the nest) respond to changes in the intensity of vibrations in this frequency range. Given that previous research has identified the potential for information transfer via vibrations between the eggs of a precocial bird species (Yellow-legged gulls: Noguera & Velando, 2019), we believe that this could be a productive direction for research in zebra finch and other altricial birds.

While we have linked changes in egg vibration intensity within the 10 to 15 Hz frequency range to exposure to heat calls, it is crucial to acknowledge that other changes in vibration intensity occurred at different frequencies in live eggs prior to the onset of playbacks. This would suggest that vibrations at the egg's surface are highly variable over time, generating a more complex signal that could interfere with the detection of vibrations in the 10 to 15 Hz range by parents or siblings in the nest. Variation in the amplitude of vibrations at other frequencies are likely the result of other factors we could not control, such as slight changes in temperature, humidity, and/or background noise that could affect embryonic movement (Hope et al., 2022; Sheldon et al., 2018). The observed sensitivity of embryos to even slight changes in environmental conditions occurring in this controlled environment could therefore undermine the use of such a subtle form of communication in wild populations.

Moreover, even if zebra finch are capable of distinguishing egg vibrations across this range of frequencies, the likelihood of masking by external sources of noise and

vibration is high. Anthropogenic noise has long been known to interfere with the detection and transmission of animal signals, and the substrate vibrations human activities affect likely extend far beyond their source (Barber et al., 2011; Brumm & Slabbekoorn, 2005; Gil et al., 2015; Kern & Radford, 2016). These vibrations, particularly at the low frequencies generated by vehicles and other man-made structures (Berglund et al., 1996), could seriously constrain vibratory communication in the nest. Furthermore, since we found that low volumes (42 dB SPL) diminished the effect of heat call exposure on egg vibrations, background noise could also affect signal production. While egg vibrations in the 10-15 Hz range may be important for prenatal communication in zebra finches, both their production and transmission could be affected by anthropogenic disturbance.

The effect of playback volume on egg vibrations.

We found that the volume of heat calls does not appear to affect the likelihood of the observed response above a certain threshold (42 dB SPL), supporting the use of 65 dB SPL heat calls (approximately 20 dB higher than the recorded average for heat calls in the field) in former playback studies (Mariette & Buchanan, 2016). Although these results do not support our prediction that increasingly loud heat calls would affect the intensity and character of the embryos response, consistency in response may be important if heat calls vary in amplitude within and between individuals. Unfortunately, however, we cannot make generalizations about responses to heat calls since we have used the heat calls from only one individual, but future studies should explore how variation in heat calls affect the vibration profile of exposed eggs. Furthermore, the lack of a response observed at the lowest amplitude of the playback could reduce the

responsiveness of eggs to parental calls in the presence of vibroacoustic interference; therefore, it may be important to see how responses to increasingly loud heat calls changes in the context of noise.

A generalized egg vibration profile in unstimulated embryos.

Beyond revealing an immediate response of zebra finch embryos to sound, our results also suggest a generalized vibration profile of eggs, which could shed light on the behavior and development of this species. Although we did not observe similarities in the amplitude of vibrations across many frequencies, there was a consistent peak in vibration amplitude at 40 to 45 Hz. This peak was not observed in dead embryos, suggesting that a specific movement or contraction of embryonic tissue is affecting vibrations in this frequency range. If this behavior occurs reliably at this stage of development, it may be possible to track how egg vibrations change over time and in response to different environmental factors. Directly establishing the relationship between the observed peak in vibration amplitude and specific physiological or behavioral changes in the egg could elucidate the functional significance of this pattern in the context of zebra finch embryonic development.

Vibrations in the context of development: Mechanisms and future directions.

Without direct observation of the embryo, we are unable to determine the behavioral or physiological mechanisms that are likely affecting vibrations at the egg's surface.

However, several studies have reported changes in the amniotic contractions, motility, and heart rate of bird embryos in response to acoustic stimulation, all of which could feasibly affect these vibrations (Colombelli-Négrel et al., 2014; Höchel et al., 2002;

Impekoven, 1976; Jackson & Rubel, 1978). Decreases in heart rate specifically have been considered a physiological indicator of attention in embryos (Rivera et al., 2018), and it may follow that a reduction in embryonic activity in general would also indicate perception of sound. We did note a consistent reduction in the intensity of 10 to 15 Hz vibrations in eggs, which could be attributed to a reduction in embryonic tissue contractions or movements. Thus, it is possible that the change in the amplitude of 10 to 15 Hz vibrations reflects a typical response to sound.

Although we suspect that changes in vibration intensity occurring in the 10 to 15 Hz range should also relate to processes affecting adaptation to acute thermal stress, our experimental design limits the conclusions we can make about responses to heat calls rather than to sound exposure in general. While we attempted to investigate how changes to the temporal and spectral characteristics of heat calls affected the vibration patterns we measured at the egg's surface, the presence of background noise throughout our experimental trials made it impossible to distinguish how embryos were directly responding to the playbacks. Thus, in order to link the observed changes in vibration to the acquisition of heat tolerant traits and behaviors, it is imperative that we continue to examine vibratory responses to other calls or similar sounds. Outside of their potential function in communication, changes in the vibration patterns of embryos in response to heat calls could affect physiological changes that provide long-term benefits to the developing embryo. Indeed, recent research in zebra finch found that prenatal exposure to heat calls affected an increase in mitochondrial efficiency, which can affect an organism's developmental trajectory and determine its thermal limits. Although these findings were suggested to indicate a direct sensitivity of mitochondria to

heat calls, it is possible that changes in embryonic movement or vibrations could be mediating these changes in mitochondrial function. Acute and chronic muscle movements are known to affect mitochondrial adaptations, such as increases in mitochondrial density, respiration, and general function (reviewed in Huertas et al., 2019). Therefore, heat calls could feasibly stimulate changes in embryonic movement and/or vibration that could affect changes in mitochondrial function.

While our results cannot support a direct causal relationship between changes in vibration intensity and heat calls specifically, we have identified a generalized vibration signal that has potential biological significance. Previous research conducted in chicken embryos under a dissecting microscope revealed rhythmic contractions of the amnion, which could feasibly generate the consistent peak in vibration observed between 40 and 45 Hz surface (Nechaeva et al., 2004; Wu et al., 2001). Although these contractions appeared to occur at a much slower rate than we measured using the LDV, it is possible that embryonic movements generate resonant frequencies that cannot be observed by eye. Studies in humans, for example, have found that isometric contractions and tremors of certain muscle tissue can produce peaks in vibrations at 10, 20, and 40 Hz (McAuley et al., 1997). While bird and human muscles cannot be directly compared, the cellular processes involved in embryonic muscle development are similar between mammals and birds (Maier, 1997). Although bird skeletal muscles tend to have a higher proportion of fast-twitch fibers than humans, this difference could explain such rapid contractions occurring during embryonic development (Reiser et al., 1982). Of course, we can continue to speculate about the origins of the complex vibrational signal recorded here, but it is evident that further research is necessary to fully understand it. It

should be noted, too, that even with an understanding of the processes affecting these vibrations, the physical properties of the egg may distort what is recorded at its surface. Nevertheless, since surface vibrations are the ultimate consequence of embryonic movement and what the parent or siblings in the nest are most likely to detect, this gap in our understanding should not undermine the significance of our findings in the context of communication.

Additional considerations.

Despite the significance of these findings, there are several variables to consider that may affect the breadth and relevance of our interpretations. Most importantly, we cannot rule out the possibility that the observed vibrational response is specific to the heat call that we used in our playback experiments. Although we do not know the extent of intra- or inter-individual variation in heat call characteristics, the identity of the calling individual may affect both the character and intensity of the response. For example, repeated exposure to the calls of the colony throughout development may affect the likelihood for an embryo to respond to the heat calls from an unfamiliar bird. Our playbacks, however, were derived from one unrelated, wild zebra finch. Since the incubating eggs only received attenuated background colony noise prior to the heat call exposure, our findings may thus underestimate the extent of embryonic perception and response to heat calls. Future studies should therefore investigate how egg vibrations are affected by the identity of the individual producing the heat calls and, moreover, whether repeated exposures to these calls over time affects the likelihood for response.

Although the focus of our study was on the immediate response of embryos to heat calls, it may be important to understand whether this response is sustained over time. If the observed changes in vibration intensity act as an important pre-requisite to the development of enhanced mitochondrial function and other such adaptations to heat, it is unlikely that one exposure is sufficient to affect long-term fitness. In order to connect short-term vibrational changes to long-term, postnatal responses (Mariette, 2016), future studies should therefore assess how different levels of exposures to heat calls (i.e., one to multiple instances) affects both types of responses. Conversely, it may also be important to investigate vibrational responses to heat calls at shorter time scales, since the signals measured here reflect an averaged response over a 20 s period. If vibrations function largely in communication, rapid changes in this signal (i.e., in fractions of a second) may provide important signals to parents that would be obscured in longer recordings.

We chose to assess changes in vibration intensity in frequency bins of 5 Hz to reduce the computational resources required for data processing and, without precedent, we had little data to inform our methodologies. However, it is conceivable that a change in bin size could affect the consistency of the results between eggs and/or reveal changes in amplitude that were not accounted for with the methods used here. Further data processing at smaller or larger bin sizes may elucidate these differences; however, we believe that a more informed approach may be most effective. If we can determine what embryonic changes are affecting vibrations between 10 to 15 Hz, we may be able to measure the amount of variation that is typical of this type of movement or response. For example, some work (Mariette, unpublished data) has suggested that

hatchlings can “shiver” in response to heat calls, which could feasibly generate vibrations in the 10 to 15 Hz range. Measurement using LDV could identify the range of vibration frequencies these shivers produce, which could help justify an appropriate bin size for comparing responses between individuals. However, if the vibrations produced in the 10 to 15 Hz range are intended for communication with parents and/or siblings in the nest, it may be necessary to better understand the sensitivity of zebra finch to vibratory stimuli to determine appropriate bin size. Given that the detection of low-frequency sounds is generally poor in birds (Zeyl et al., 2020), it may be unlikely that small differences in vibration frequencies (i.e., < 5 Hz) are perceptible or relevant to the receiving individual. However, the presence of mechanoreceptors documented in several avian species (e.g., Herbst corpuscles; reviewed in Ziolkowski et al., 2022) should likely increase the threshold for detection via non-auditory pathways.

Lastly, while we took several steps to reduce competing vibrations, i.e., sound-proofing materials and using a negative control, we still observed wide variation in vibrations across several frequency ranges. The laser appeared to be incredibly sensitive to various mechanical vibrations and noises occurring outside the aviary (e.g., large vehicle traffic and air-conditioning units of neighboring buildings), suggesting that some of the difference observed between trials can be attributed to background noise. Ideally, these experiments could be conducted in a sound-proof room that could eliminate background noise and vibrations all together. Previous studies using LDV have also applied reflective tape to the substrate being recorded, which when applied to the egg’s surface, could increase the laser’s sensitivity to changes in vibrations (Endo, Takanashi, Mukai, & Numata, 2019). It is also important to note that some of the

variation we observed in the amplitude of vibrations could be affected by the random or idiosyncratic movements of individual eggs in response to other factors. For example, it's possible that some of the variation in vibration intensity observed between eggs can be attributed to minor differences in the speed and stage of the embryo's development. Given that significant changes can occur to the embryo and its surrounding tissues in a matter of hours (Murray et al., 2013), these temporal differences could contribute to the observed variability in vibration frequency and intensity. Given our limited capacity to record multiple eggs at the same time, some eggs were measured several hours prior to eggs considered to be at the same developmental stage. A larger sample size and informed adjustments to our experimental design could therefore greatly improve the reliability and applicability of our findings.

Conclusion.

Here, we used playback experiments and laser Doppler vibrometry to investigate the hypothesis that prenatal exposure to parental heat calls affects detectable changes in the intensity (or amplitude) of egg vibrations. To our knowledge, this study provides the first experimental evidence that altricial bird embryos are capable of immediately responding to their acoustic environment, as we found significant changes in the intensity of egg vibrations at certain frequencies after exposure to heat calls.

Furthermore, we found that the volume of heat calls does not appear to affect the likelihood of this response above a certain threshold (42 dB SPL), supporting the use of 65 dB SPL heat calls (approximately 20 dB higher than the recorded average for heat calls in the field) in former playback studies (Mariette, 2016). Although it is unclear whether the observed changes in vibration intensity are a direct attempt at

communication from the embryo or an indirect consequence of movement and other physiological changes, these results highlight the potential for laser Doppler vibrometry (LDV) to become an important non-invasive method for gauging embryonic perception and response to external stimuli. Of course, while anthropogenic noise could belie their function in communication, more research is necessary to determine whether egg vibrations could directly affect important and, potentially, adaptive physiological changes occurring during embryonic development.

Supplementary Tables and Figures.

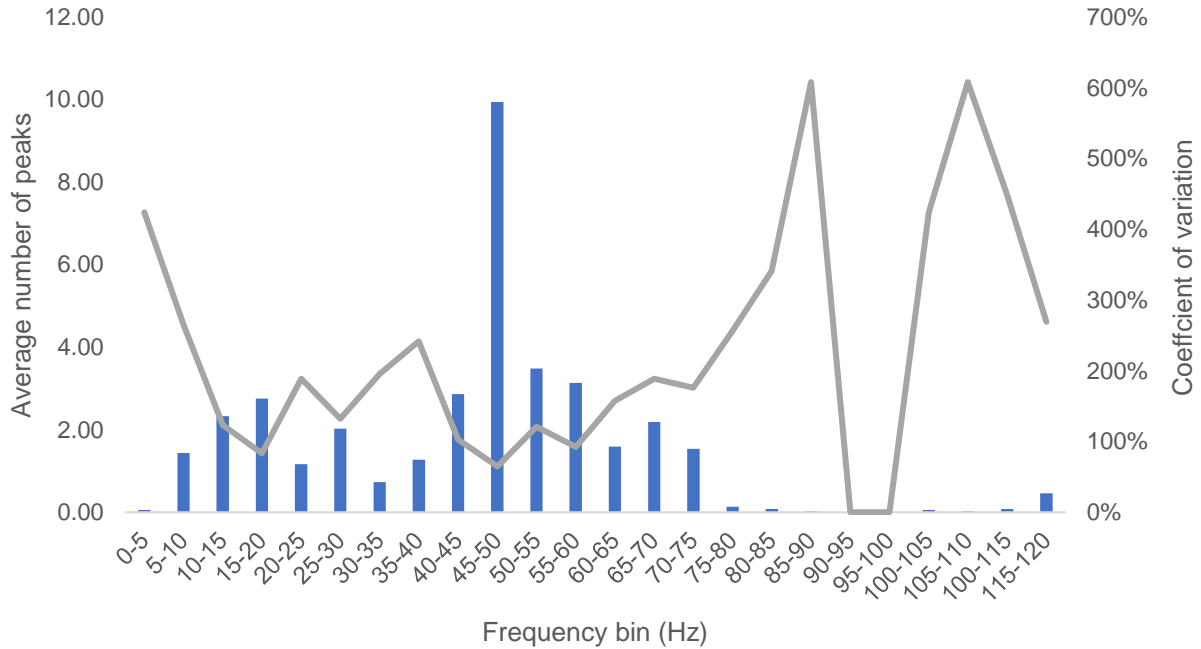


Figure S1. Average number of peaks in vibration amplitude across frequency bins for live zebra finch eggs prior to heat call exposure. The vibrations of live eggs ($n = 37$) were recorded and fast-Fourier transformed (FFT) to obtain the amplitude of vibrations across all resolvable frequencies (0 to 120 Hz). Bars indicate the average number of peaks in egg vibration amplitude within 5 Hz frequency bins; grey line indicates the coefficient of variation for counts within each bin.

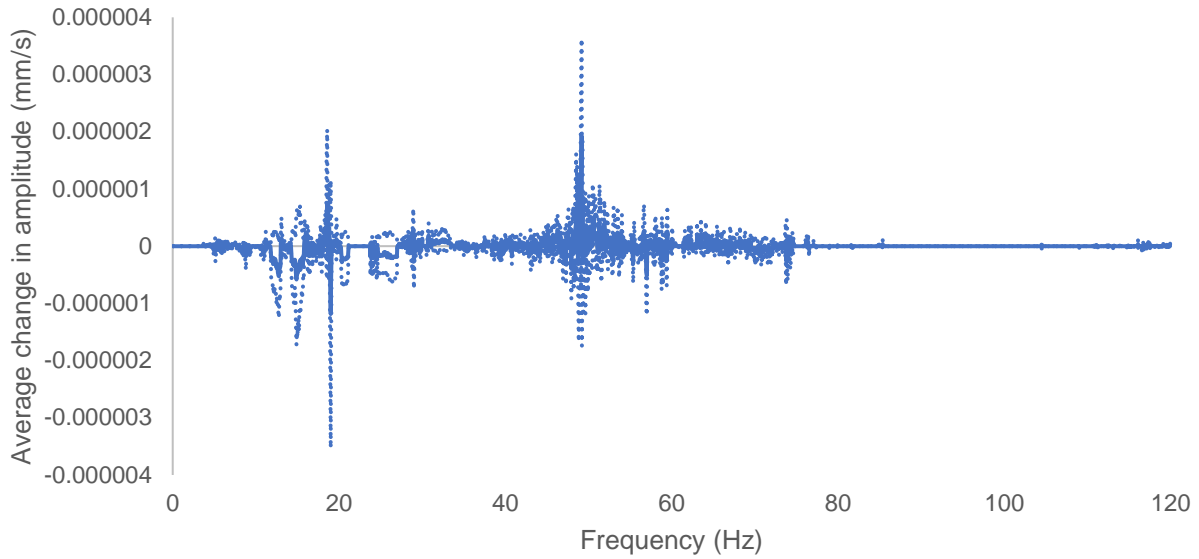


Figure S2. Average change in amplitude of egg vibrations after exposure to heat calls. The vibrations of live zebra finch eggs ($n = 37$) were recorded before and after exposure to playbacks and the change in vibration amplitude for each egg was obtained by subtracting the post- and pre-playback fast Fourier transforms (FFT). Data are shown as the average change in amplitude at all resolvable frequencies ($dF = 0.1$, 0 to 120 Hz) with dotted lines indicating 95% confidence intervals.

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