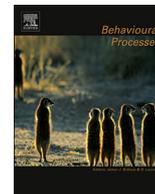




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Behavioral thermoregulation in avian embryos: Spectrum analysis of calls in warm and cold conditions



Jacopo P. Mortola

Department of Physiology, McGill University, room 1121, 3655 Sir William Osler promenade, Montreal, QC, H3G 1Y6 Canada

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ABSTRACT

During the last days of incubation vocalization is a form of communication between the avian embryo and the incubating parent. Commonly, embryonic calls increase when ambient temperature (T_a) deviates from the optimal range, but no information is available on whether the characteristics of the calls differ between warming and cooling. Rate of calls, power spectra (distribution of the call's energy among its frequency components) and spectrograms (time-frequency plots) were obtained in chicken embryos during the external pipping phase, in normothermia (38°C), during progressive cooling to $T_a = 27^\circ\text{C}$ (C) or progressive warming to $T_a = 43^\circ\text{C}$ (W) over a short (30 min) or a long (150 min) period. Over the T_a range investigated, the embryo's oxygen consumption did not change significantly from normothermia. Number of calls, average and peak amplitudes of the power spectrum increased as cold or heat increased, according to power functions with exponents significantly higher during warming than during cooling. The spectrum frequency at peak amplitudes did not vary with T_a . Number of calls and characteristics of the power spectra (average amplitude, peak amplitude and frequency at peak amplitude) at five degrees above normothermia (43°C) did not differ significantly from those at ten degrees below normothermia (27°C), whether the changes in T_a occurred rapidly or slowly. The incidence of spectrograms with characteristics of 'distress' (progressive decrease in frequency with time) at 43°C was similar to that at 27°C . It is concluded that the vocalization of the chicken embryo has a stereotyped sonogram independent of the T_a directional changes, while the T_a -sensitivity of vocalization (changes in number of calls and amplitude per $^\circ\text{C}$ change in T_a) is much higher when T_a rises than when T_a drops.

1. Introduction

All the ingredients necessary for the development and growth of the avian embryo are within the freshly laid egg, except for oxygen and heat. The adequate inflow of oxygen becomes a concern in species laying eggs at altitude (Monge and León-Velarde, 1991; Mortola, 2009). Differently, the control of temperature is of concern to all eggs. In addition to the daily and seasonal changes in ambient temperature (T_a), egg temperature rises with the progressive increase in embryo's metabolism (Mortola et al., 2015) and drops whenever the parent leaves the nest for foraging (Drent, 1970). Because in embryos the autonomic controls of heat production and loss are very limited (Mortola, 2009), the regulation of egg temperature is based largely on behavioral means. The parent controls the temperature of the clutch by repositioning the eggs within the nest and by adjusting their contact with its brood patch (Tøien et al., 1986). In addition, parents send T_a -related acoustic signals to the embryo, which then alters post-natal development in a temperature-dependent manner (Mariette and Buchanan, 2016; Mariette et al., 2018). For its part, the embryos communicate with the

parents through motion and vocalization (Vince, 1969).

Embryonic vocalization is a powerful communication tool, but its pre-requisite is the ability to generate adequate airflow. Therefore, mammalian fetuses cannot vocalize until the first aeration of the airways at birth. Differently, avian embryos can communicate vocally before birth because, throughout incubation, the volume of water lost through the eggshell is exchanged for air, with the formation of an air cell. Once the embryo has pierced through the air cell, pulmonary ventilation begins and, with it, the possibility of uttering calls. The embryonic pre-hatching calls have a long evolutionary history. Crocodile embryos generate calls to advertise the imminent hatching to the mother, presumably to synchronize hatching among siblings and lower the risks of predation (Vergne and Mathevon, 2008). Indeed, avian embryos of the precocial and of some altricial species investigated vocalize close to end-incubation, possibly to solicit parental care in preparation for hatching (Vince, 1969; Berlin and Clark, 1998).

In response to a drop in T_a some studies have indicated that avian embryos increase vocalization (e.g., Evans, 1986; Evans et al., 1994; Brua et al., 1996; Nichelmann and Tzschentke, 1997; Al Awam et al.,

E-mail address: jacopo.mortola@mcgill.ca.

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2011), while others have reported decreases or insignificant changes (Oppenheim and Levin, 1975; Gräns and Altimiras, 2007; Dawes, 1981; Berlin and Clark, 1998; Bugden and Evans, 1999, for additional references). In addition to species differences, several methodological and physiological factors can contribute to the variable results. Small amplitude calls can be difficult to separate from background noise, and the analytical methods used for the identification of the ‘call’ may contribute to quantitative and qualitative differences among studies. Further, differences in the degree of development may contribute to variations among studies, given that the degree of vocalization, like the autonomic response to cold, changes through the last phases of incubation (Berlin and Clark, 1998; Szdzyu et al., 2008). The degree of cold exposure and mode of the temperature change (oscillatory or steady, rapid or gradual) could have different impact on metabolic depression through the Q10 effect¹ (Mortola and Gaonac’h-Lovejoy, 2016; Ben-Ezra and Burness, 2017), with unpredictable results on vocalization. For the same variation in temperature, a slow rate of change could be tolerated better, through an ongoing process of adaptation, or worse, because of the longer duration of the exposure (Allen et al., 2016, and references therein).

Unlike cold, embryonic vocalization in the heat has received little attention. Egg overheating is a less common event than cooling but is more likely to be fatal. In fact, the threshold of heat tolerance is much closer to normothermia than that of cold tolerance (Webb, 1987). In the domestic chicken, a mild heat exposures (40 °C) did not increase the number of calls (Gräns and Altimiras, 2007), while a stronger heat stimulus (44–45 °C) increased vocalization in one study (Oppenheim and Levin, 1975) and was ineffective in another (Bugden and Evans, 1999). Berlin and Clark (1998) recorded a higher rate and duration of calls in internally-pipped Budgerigar embryos exposed to high Ta, and no differences in two embryos at a later developmental stage.

Beyond variations in the number and duration of calls with temperature, call type (from spectrogram) or frequency distribution may also vary with changes in Ta. For example, in the Red-capped Plover embryos, moderate hyperthermia increased the acoustic frequency, with no changes in rate or duration of the calls (Kostoglou et al., 2017). Whether embryos use different call types or call frequency at high versus low temperatures is unknown in any species.

The present work, in addition to the call number, examined the frequency distribution and modulation of the calls produced by chicken embryos at the external pipping stage, when temperature varied rapidly or slowly below and above normal incubation temperature. Because the incubation temperature is closer to the upper, than to the lower, critical temperatures the hypothesis was that the embryo’s vocalization activity (number of calls/minute) was higher in hot than in cold conditions and that the characteristics of the sound may differ between the two conditions.

2. Methods

Measurements were conducted on 22 embryos from freshly laid chicken eggs (*Gallus gallus*; White Plymouth Rock variety), purchased from a local supplier. The eggs were incubated at 37.5 °C and 60% relative humidity, monitored by a data logger inside the incubator, with 90-degree rotation four times per day. The onset of incubation was at around midday of embryonic day 0 (E0). Data were collected at the external pipping phase (EP) of the hatching process, which occurred between day 20 and 20.5 of incubation.

The egg was placed in a sealed 110 ml plastic container (“respirometer”), with two leads for the passage of air. The respirometer, almost entirely immersed into a circulating water bath, contained a

¹ Q10 (or Arrhenius factor) expresses the change in reaction velocity for a 10 °C change in temperature (T). Applied to metabolic rate (M), $Q10 = M_1/M_2^{10/(T_1-T_2)}$, where M_1 and M_2 are the metabolic rates at the corresponding T.

microphone and two thermocouples placed approximately at its center, close to the egg, for the measurement of ambient temperature (Ta).

2.1. Protocols

Two experiments were performed, to investigate the responses to either a fast (protocol 1) or a slow (protocol 2) change in temperature. In either protocol, the egg, with the embryo at the external pipping stage (EP), was placed in the respirometer set at normothermia (on average, 37.8 ± 0.1 °C) for one hour. During the following half hour, data were collected for two min every 10 min, and the average of these three recordings provided the normothermic values. Then, the setting of the water bath was adjusted to cause changes of the respirometer Ta in the cooling (C) or in the warming (W) direction, either rapidly (protocol 1, over 30 min) or slowly (protocol 2, over 150 min). In the case of protocol 1 Ta reached its final values of 43 °C (‘rapid warming’, RW) or 27 °C (‘rapid cooling’, RC) in ~30 min (Fig. 2, dashed lines). Then, normothermia was reestablished for 1 h, followed by, respectively, either RC or RW. This group of embryos (N = 10), therefore, experienced both cold and hot exposures, in alternate order among embryos. In the case of protocol 2, on two separate groups of embryos (N = 6 each), Ta changed at a rate approximately five times slower than it did in RW-RC embryos (Fig. 2, continuous lines). In one group (‘slow cooling’, SC), Ta decreased to ~36, 32 and 27 °C in, respectively, 30’, 90’ and 150’; in the other group (‘slow warming’, SW) at the same times Ta rose to ~40, 42 and 43 °C. In this case, owing to the length of the exposure, each embryo experienced only one challenge, either C or W.

2.2. Oxygen consumption

Oxygen consumption (\dot{V}_{O_2}) was measured by an open-flow methodology (Frappell et al., 1992) adapted to the chicken embryo (Menna and Mortola, 2003). A pump circulated air at the constant rate of 180 ml/min, under the control of a flowmeter, and delivered it into a CO₂ and O₂ gas analyzers (FoxBox, Sable Systems, NV, USA) arranged in series. Both a CO₂ scrubber (granular Soda Lime, Anachemia, Montreal, CA) and a water scrubber (CaSO₄, Indicating Drierite, Xenia, OH, USA) were placed in series at the inlet of the circuitry. The outflowing gas was monitored continuously, while the inflowing gas concentrations were checked every 10 min. Hence, \dot{V}_{O_2} at standard temperature, pressure and dry conditions (STPD) was equal to $\text{Flow} \cdot ([O_2]_{in} - [O_2]_{out}) / (1 - [O_2]_{in})$, to take into account the possibility of a respiratory quotient different from unity (Depocas and Hart, 1957; Mortola and Besterman, 2007).

2.3. Sound recording and analysis

A high-quality subminiature (6-mm diameter, 1 g weight) mono microphone for digital recording (Audio-Technica, Hong Kong, model AT9904) was placed inside the respirometer, 3 cm above the blunted end of the egg. According to the seller specifications, the microphone had a frequency response of 30 to 18,000 Hz. Mono-channel sound acquisition was at 44,100 Hz by use of Audacity® software (Boston, MA). Amplitude was measured in decibel (db), where db is the log₁₀ of the ratio between the recorded V_1 and the maximal amplitude V_0 [db = $20 \log_{10} (V_1/V_0)$]. To convert the negative db scale into a positive one, -100 was subtracted from each value. Therefore, db = 100 corresponded to the maximal sound amplitude; each halving of the sound dropped the amplitude value by 6 db, and 0 db was the minimal amplitude, taken as absolute silence.

For the analysis, first, the background noise was subtracted from the raw recording. Background noise was recorded a) separately, with an infertile egg in the respirometer in conditions identical to the experimental setting, and b) throughout the actual experiment during periods of no-calls. Whether recorded in a) or in b), background noise was

almost undetectable and its subtraction from the raw signal made no appreciable difference to the results. The analysis of the sounds did not require any filtering and covered the last two-minute epoch at each Ta of the study-protocols. It was based on an ad-hoc adaptation of the digital audio editor software (Audacity® version 2.0.3) to compute the number of calls, the power spectrum (frequency-energy distribution) and the spectrogram (time-frequency) of the sound over the whole 2-min period. A ‘call’ was defined as any sound with amplitude higher than 16 db, with at least 100 ms of silence between calls. These arbitrary criteria were chosen because best agreed to what most closely corresponded to the perception of a call by the human ear. A Fast-Fourier Transform (120 s at 44,100 Hz acquisition rate = $5292 \cdot 10^3$ samples) generated the spectrum, or frequency (Hz)-amplitude (db) relationship. Analysis of average amplitude, peak amplitude and frequency at peak amplitude considered the frequency range 1–6 KHz which, from the spectrograms (see Results) and previous data (Gottlieb and Vandenberg, 1968; Berlin and Clark, 1998), covers the bulk of the fundamental frequencies while excluding most of the higher frequency harmonics.

Based on spectrogram characteristics, Gottlieb and Vandenberg (1968) considered three types of embryonic calls. The calls associated with ‘distress’ or ‘alarm’ had frequencies that sharply decreased with time over a relatively large frequency range, while those associated with pleasure or contentment had ascending frequencies over a narrower range. The third type occurred most frequently during hatching; hence, it did not apply to the embryos of this study. Following the same criteria, the spectrograms of all calls were examined and classified either as ‘distress’ or ‘pleasure’, and their relative incidence at the various temperatures was computed.

To test whether the changes in air density that accompany the changes in air temperature could by themselves modify the sound characteristics, embryonic sounds were played back in the respirometer and recorded at different values of Ta. The power spectra at 22 °C were undistinguishable from those obtained at 46 °C (Fig. 1); this result excluded the possibility that changes in the physical properties of the air may have distorted the characteristics of the sounds recorded during cooling or warming.

2.4. Data presentation and statistical analysis

Values are presented as means \pm 1 SEM. Table 1 provides the sample sizes and some egg characteristics. The goal of the analysis was, first, to establish whether cooling or warming caused a difference in the parameter under consideration; then, if they did, whether the magnitude of the effect differed between warming and cooling. To this end, two approaches were used. First, data (number of calls and parameters

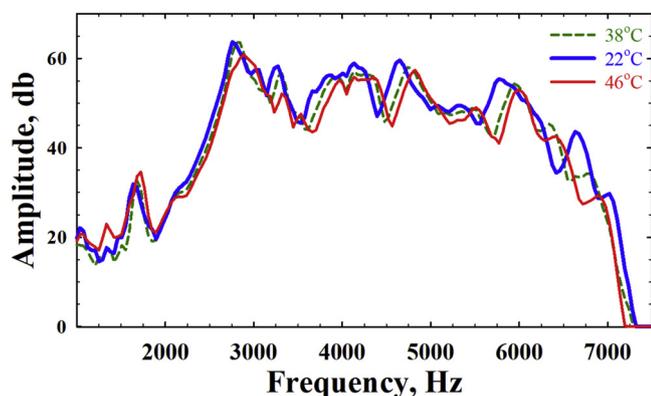


Fig. 1. Methodology: playback of the same 2-min sounds, with the respirometer maintained at three different ambient temperatures, 38 °C (dashed green line), 22 °C (blue thick line) and 46 °C (thinner red line). The power spectra of the recordings were almost undistinguishable from one another.

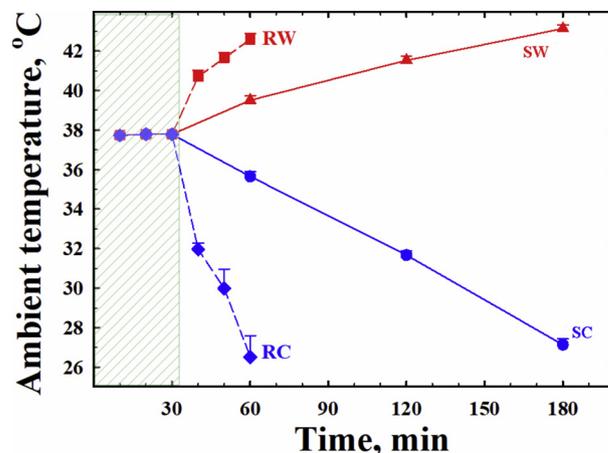


Fig. 2. Time profiles of ambient temperature (Ta) during slow cooling (SC, blue circles), slow warming (SW, red triangles), rapid cooling to 27 °C (RC, blue diamonds, dashed line) and rapid warming to 43 °C (RW, red squares, dashed line). Hatched area refers to the 30-min period in normothermia (Ta = 37–38 °C) preceding the change in Ta. Symbols are group means \pm 1 SEM (N = 6-to-10 per condition, Table 1).

of the spectrum) were plotted as function of Ta; then, a power function of the type $Y = aX^b$ fitted the cooling or warming portion of the graph. The statistical significance of the correlation coefficient of the function meant that the Ta change produced a significant difference from normothermia. When this was the case, the two functions (warming and cooling) were log-transformed into the linear $\log(Y) = \log a + b \log(X)$, where the slope b represented the exponent of the original function, and statistically compared by two-tailed t test. An additional analysis simply compared the raw values obtained at the most extremes Ta tested, 27 °C and 43 °C, that is, between a temperature drop in the cold almost twice as large as the temperature increase in the hot. This was done either by paired (RC vs RW) or by unpaired (SC vs SW) t test. Differences between sets of data, exponents b and the correlation coefficients of the power functions were considered statistically significant at $P < 0.05$.

3. Results

3.1. Normothermia

Table 2 presents the average values of \dot{V}_{O_2} , number of calls and sound characteristics during the normothermic phase of the protocols. All values related to vocalization had a substantial inter-embryo variability, as apparent by the 95% CI.

3.2. Oxygen consumption

Values of \dot{V}_{O_2} were slightly higher during slow changes in Ta (SW and SC) than during the rapid changes (RW and RC), but in neither case did they differ significantly from normothermia (Tables 3 and 4); the only exception was during the rapid changes in Ta, when \dot{V}_{O_2} at 43 °C was significantly higher than at 27 °C (Table 3). No significant correlation existed between Ta and \dot{V}_{O_2} whether during cooling or warming (Fig. 3, top panel).

3.3. Number of calls

Changes in Ta caused significant increases in the number of calls (Fig. 3, bottom panel). The slope of the log-transformed exponential during warming was 23.1, significantly higher than that (6.1) during cooling ($P < 0.01$). The number of calls did not differ significantly between 27 °C and 43 °C, in either case \sim 7 times higher than in

Table 1
Number of embryos and characteristics.

Protocol	N	Fresh egg weight, g	Days of incubation	Egg weight at study, g
Slow Cooling (SC)	6	61.9 ± 2.5	20.5 ± 0.2	53.2 ± 3.2
Slow Warming (SW)	6	63.7 ± 3.0	20.2 ± 0.2	53.8 ± 2.1
Rapid cooling (RC) and Rapid Warming (RW) ¹	10	60.5 ± 1.8	20.2 ± 0.2	52.3 ± 1.5
total	22	61.7 ± 1.3	20.5 ± 0.2	52.9 ± 1.2

Values are means ± 1 SEM. N, number of animals. ¹in alternate order among embryos.

Table 2
Normothermia (38 °C): absolute values.

Parameter	Mean ± SEM (N = 22)	95% CI
Ta (°C)	37.8 ± 0.1	37.9–37.7
\dot{V}_{O_2} (ml/min)	0.57 ± 0.02	0.60–0.54
Calls, number/min	1.4 ± 0.4	2.1–0.7
Average amplitude ^a (db)	13.0 ± 1.2	15.3–10.6
Peak amplitude (db)	32.5 ± 1.8	36.9–29.0
Frequency at peak amplitude (Hz)	2399 ± 138	2670–2128

Values (means ± 1 SEM) refer to 22 externally pipping embryos in normothermia (37–38 °C). 95% CI = 95% Confidence Intervals. Ta, ambient temperature. \dot{V}_{O_2} , oxygen consumption.

^a Computed over the 1–6 KHz frequency range; amplitude of 0 db corresponds to silence.

normothermia.

3.4. Power spectra

Fig. 4 shows the average power spectrum during RC and RW, while those during SC and SW are shown in Fig. 5. Visual inspection of the power spectrum curves showed that the amplitudes increased with progressive warming and cooling. However, the energy distribution throughout the frequency range (i.e., the curve shape) remained similar at all Ta, irrespective of the speed of the Ta changes. These qualitative results were confirmed statistically by the quantitative analysis, summarised in Table 3 (RC and RW) and Table 4 (SC and SW).

The average amplitude and the peak amplitude of the spectrum increased significantly above normothermia, both during warming and during cooling (Fig. 6). The slope of the log-transformed functions was, respectively, 6.5 (warming) and 1.7 (cooling) for the average amplitude and 3.2 (warming) and 1.1 (cooling) for the peak amplitude, in either case significantly higher during warming (respectively, P < 0.001 and P < 0.01). The frequency at peak amplitude did not correlate significantly with Ta, nor did it differ between 27 and 43 °C.

3.5. Spectrograms

The recordings of Fig. 7 are examples of spectrograms classified as

Table 3
Rapid changes in ambient temperatures: power spectrum characteristics.

Parameter	Cooling			Warming			P (27 °C versus 43 °C)
	32 °C	30 °C	27 °C	41 °C	42 °C	43 °C	
Ta (°C)	32.0 ± 0.3	30.0 ± 1.0	26.5 ± 1.1	40.7 ± 0.3	41.6 ± 0.3	42.6 ± 0.3	< 0.001
\dot{V}_{O_2} (ml/min)	0.53 ± 0.07	0.54 ± 0.07	0.53 ± 0.07	0.57 ± 0.04	0.61 ± 0.05	0.67 ± 0.05	< 0.01
Calls, number/min	3.1 ± 2.5	5.3 ± 3.4	14.1 ± 5.5	4.3 ± 3.9	7.1 ± 5.6	15.8 ± 7.8	ns
Average amplitude ^a (db)	16.3 ± 4.0	20.9 ± 4.6	25.0 ± 5.0	17.9 ± 4.4	19.5 ± 5.5	23.4 ± 5.3	ns
Peak amplitude (db)	36.8 ± 5.2	41.9 ± 5.3	50.7 ± 5.2	38.9 ± 4.7	38.0 ± 5.7	44.9 ± 5.6	ns
Frequency at peak amplitude (Hz)	3058 ± 407	3424 ± 362	3458 ± 373	2997 ± 384	2377 ± 288	3023 ± 315	ns

Values are means ± 1 SEM of 10 embryos at the ambient temperatures (Ta) indicated, each studied during cooling and warming temperatures, in alternate order. \dot{V}_{O_2} , oxygen consumption.

^a Computed over the 1–6 KHz frequency range; amplitude of 0 db corresponded to silence. P, level of significant difference between 43 and 27 °C (ns, not significant difference).

‘distress’ or ‘pleasure’ calls (Gottlieb and Vandenberg, 1968). Although most calls presented clearly recognizable patterns, a number of them had spectrograms either unclear or of intermediate pattern (12% in normothermia, 5% in cold and 6% in heat). ‘Pleasure’ calls represented almost the entirety of the calls at 36 °C and about two-thirds of the total at 38 °C, while they were a small minority in either cold (P < 0.01, paired t-test, from normothermia) or hot conditions (P < 0.01 from normothermia). As Ta decreased below, or increased above, normothermia the ‘distress-type’ calls became the large majority and much more frequent than in normothermia (P < 0.05, paired t-test, for either change in Ta). This distribution was somewhat clearer with slow, rather than with rapid, changes in Ta (Fig. 7, panel at right).

4. Discussion

In embryos the autonomic thermoregulatory responses initiate late in incubation and cannot be enough to maintain body temperature constant. Therefore, the control of egg temperature is left to the incubating parent while the embryo, for most of incubation, can only participate through changes in its motility pattern (Nechaeva, 2011; Du et al., 2011). With the onset of pulmonary ventilation, the possibility of vocalization gives the avian embryo a new tool of interaction with parents and siblings. Then, postnatally, as the efficiency of the autonomic thermoregulation improves, the reliance on vocally-induced parental help decreases (Bugden and Evans, 1997).

Vocalization is a powerful tool because the embryo’s demands can be heard from a distance, while detection of motion requires physical contact with the egg. Furthermore, the embryonic calls do not seem to increase the chances of being located by predators (Kostoglou et al., 2017). The results of the present study indicated that in response to changes in Ta chicken embryos increased the number of calls and their amplitude while keeping the frequency distribution of the spectrogram constant, and that the response to warming occurred far more readily than that to cooling.

4.1. Methodology

Vocalization can be quantified in many ways. The number of calls is probably the simplest and most frequently adopted approach (e.g.,

Table 4
Slow changes in ambient temperatures: power spectrum characteristics.

Parameter	Cooling			Warming			P (27 °C versus 43 °C)
	36 °C	32 °C	27 °C	40 °C	42 °C	43 °C	
Ta (°C)	35.7 ± 0.2	31.7 ± 0.2	27.1 ± 0.3	39.5 ± 0.2	41.5 ± 0.2	43.1 ± 0.2	< 0.001
\dot{V}_{O_2} (ml/min)	0.63 ± 0.06	0.66 ± 0.1	0.58 ± 0.08	0.62 ± 0.08	0.72 ± 0.07	0.78 ± 0.06	ns
Calls, number/min	3.3 ± 2.1	6.4 ± 3.9	14.4 ± 8.0	0.9 ± 0.6	10.4 ± 5.0	6.1 ± 3.6	ns
Average amplitude ^a (db)	12.5 ± 4.7	21.0 ± 5.9	18.8 ± 5.7	16.4 ± 5.8	34.7 ± 4.9	32.7 ± 7.4	ns
Peak amplitude (db)	32.4 ± 7.7	45.4 ± 7.4	42.4 ± 7.9	31.4 ± 7.9	55.6 ± 4.6	45.6 ± 6.9	ns
Frequency at peak amplitude (Hz)	2555 ± 294	2756 ± 236	2562 ± 330	2186 ± 597	3693 ± 334	3510 ± 278	ns

Values are means ± 1 SEM of 6 embryos during cooling and 6 embryos during warming, at the ambient temperatures (Ta) indicated. \dot{V}_{O_2} , oxygen consumption.

^a Computed over the 1–6 KHz frequency range; amplitude of 0 db corresponded to silence. P, level of significant difference between 43 and 27 °C (ns, not significant difference).

Berlin and Clark, 1998; Bugden and Evans, 1999; Toro-Velasquez and Mortola, 2014), even if what constitutes a ‘call’ requires somewhat arbitrary criteria of amplitude and time duration. Off-line computer analysis facilitates this approach, with results that may differ substantially from what is recognized by the human ear. An additional approach is the power spectrum and the spectrogram, which permit to visualize the structure of the sound and how energy distributes according to vibratory frequencies.

In the natural setting, cooling of the egg is common when the parent leaves the nest for foraging, or when early hatchlings push the unhatched eggs to one side. The risk of overheating occurs especially in those species with asynchronous hatching, when large broods pile over the late eggs, and in arid zones with hot climates (Mariette et al., 2018). The Ta changes chosen for the present experiments, although possibly beyond what most avian embryos may experience in natural conditions, were well tolerated and readily recovered from. Over these ranges, \dot{V}_{O_2} had no significant correlation with Ta; this would not have been the case in younger embryos, when the Q10 effect has a dominant role on \dot{V}_{O_2} , but is the case close to hatching, because of the onset of thermogenic mechanisms (Szdzyu et al., 2008; Ide et al., 2017).

Behavioral (e.g., calls) and autonomic (e.g., thermogenesis) control

of body temperature are expected to integrate each other with some degree of reciprocal compensation (Kingma, 2018). From the view point of the current experiments, the lack of correlation between \dot{V}_{O_2} and Ta should mean that the observed vocalization responses were not linked to the degree of thermogenesis. A closer look at the data during cooling (Fig. 3), however, shows that the values collected during the rapid change in Ta resulted in \dot{V}_{O_2} (top panel) and calls (bottom panel) below the fitting functions, and that the opposite was the case during the slow change in Ta. Hence, it is possible that the number of calls in response to changes in Ta was not totally independent of \dot{V}_{O_2} . Such eventuality did not seem to apply to the parameters of the frequency spectrum, the values of which were more spread across the best-fit lines (Fig. 6).

4.2. Normothermia

Mean values of \dot{V}_{O_2} in normothermia were as expected for embryos of this age (Mortola, 2009). A few calls (1–2/min) in normothermia are the norm (Bugden and Evans, 1999), at a rate that increases as hatching approaches (Vince, 1969; Evans, 1988). Possibly, calls in normothermia are directed to siblings to synchronize hatching and to parents to

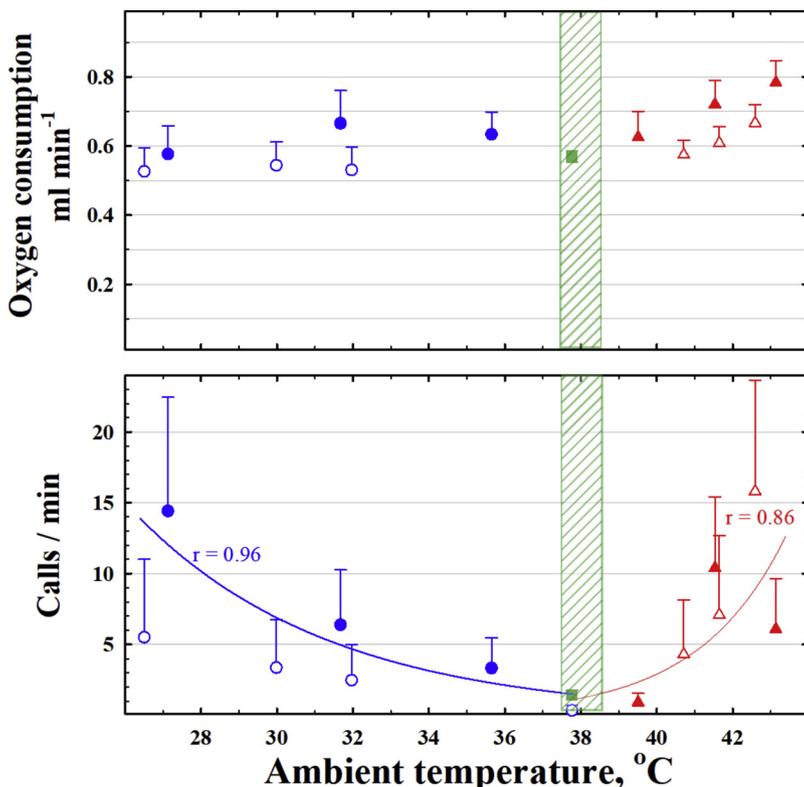


Fig. 3. Average values of oxygen consumption (top) and number of calls (bottom) at different ambient temperature (Ta) during cooling (blue circles) or warming (red triangles); bars are 1 SEM. The hatched area refers to normothermia. Open and filled symbols indicate, respectively, rapid and slow changes in Ta. The curves represent the best fit through the data points of the type $Y = aX^b$, of which r indicates the correlation coefficient. No significant correlation existed for Ta-oxygen consumption over the cold or hot range tested. N = 10 embryos, for the rapid temperature changes, repeated in cold and hot conditions; N = 6 for the slow cooling and N = 6 for the slow warming.

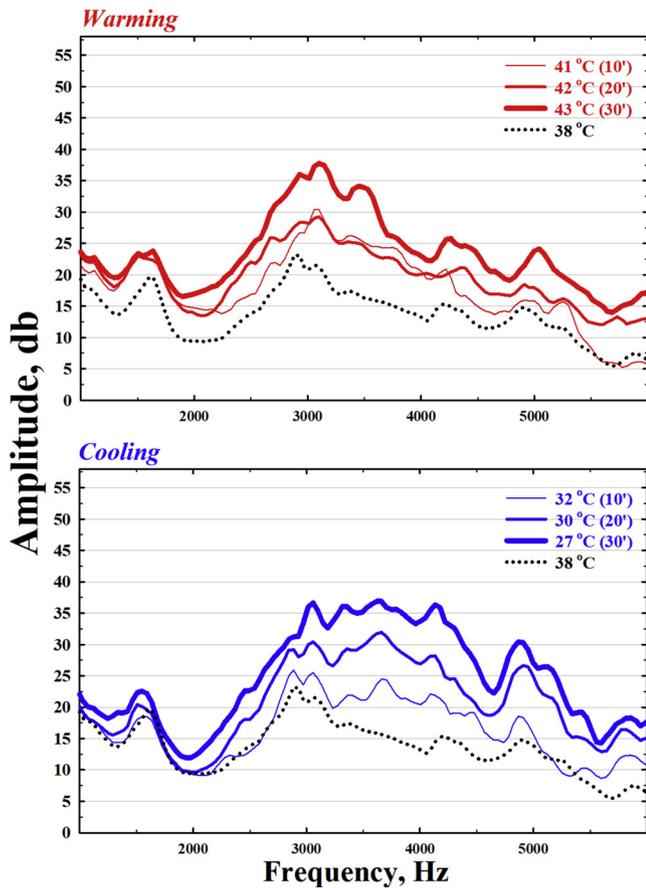


Fig. 4. Rapid changes in ambient temperature: average power spectrum of the sounds of embryos during warming (top, red lines) or cooling (bottom, blue lines). Progressively thicker lines indicate progressively greater departures from normothermia (dotted line, 38 °C). N = 10 embryos, each repeated in cold and hot conditions, in alternate order.

advertise the imminent birth and solicit help for the process (Vince, 1969).

Both the number and the amplitudes of the calls had large inter-embryo variability. Even small differences in the developmental stage may have contributed to the variability, since embryos increase the calling rate by the hour as they approach hatching (Gottlieb and Vandenberg, 1968; Evans et al., 1994; Berlin and Clark, 1998; Bugden and Evans, 1999). In addition, at E20 fluid clearance from the airways is still incomplete, and differences in airway fluid are likely to affect the power of vocalization.

4.3. Cooling and warming conditions

The current data indicated that the spectral characteristics of the calls, albeit slightly different from those in normothermia, were not specific regarding the directional changes in Ta. Likewise, the rate of ‘distress’ calls greatly increased in both C and W (Fig. 7). Hence, the parent cannot distinguish whether the embryo’s calls are meant to advertise a drop or an increase in temperature. The embryo’s stereotyped calls, therefore, solely meant to attract parental attention; then, it will be up to the parent to sort out their causative nature.

The rate and amplitudes of the embryo’s calls were adequately described by power functions, with significantly higher exponents during warming than during cooling. The fact that the data were described by power functions means that the differences in sensitivity between warming and cooling temperatures become more obvious the larger the stimulus. For example, from the fitting functions (Fig. 3) one can compute that a 1 °C increase or decrease in Ta will cause minute

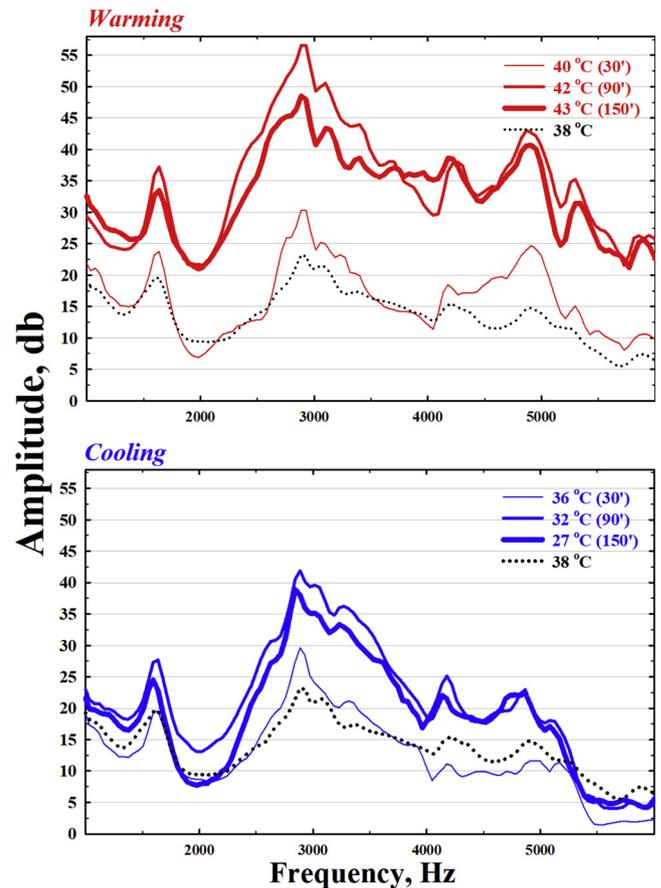


Fig. 5. Slow changes in ambient temperature: average power spectrum of the sounds of embryos during warming (top, red lines) or cooling (bottom, blue lines). Progressively thicker lines indicate progressively greater departures from normothermia (dotted line, 38 °C). N = 6 embryos exposed to cold and N = 6 embryos exposed to hot.

changes in the number of calls (respectively, 0.5 and 0.1 calls/min), probably behaviorally insignificant. However, a 4 °C increase or decrease in Ta will result in an increase of, respectively, 7 or only 2 calls/min. In fact, the vocalization at ten degrees below normothermia (27 °C) was similar to that measured at only five degrees above normothermia (43 °C). The present data cannot sort out what would be the minimum number of calls to attract parental attention, that is, to be behaviorally meaningful. If one assumes that the number of calls at each Ta is normally distributed, a significant difference from normothermia would occur when they deviate by two Standard Deviations (i.e., by > 95%). By these criteria, the calling threshold during cooling temperatures would be at 6 °C below normothermia (or 37.8–6 = 31.8 °C), while in the hot the threshold would be at 3.0 °C above normothermia, or 40.7 °C. This last value is comparable to the threshold (~41 °C) for thermal tachypnea, the predominant form of evaporative heat loss in chicken embryos close to hatching (Bícego and Mortola, 2017).

Despite changes in number and amplitude of the calls, the power spectrum retained a very similar frequency pattern at the various Ta. This agrees with what observed in broiler chicken embryos during cooling, which maintained the same rate and intensity of the calls as Ta dropped down to 30 °C (Gräns and Altimiras, 2007). Therefore, the call is constructed around a constant frequency spectrum, stereotyped and unchanged under various thermal stimuli. Previously, Berlin and Clark (1998), in discussing the calls of altricial Budgerigar’s embryos, commented that they typically consisted of a single frequency band (~5500 Hz), kept relatively constant both at low and high Ta.

There is very little information to hypothesize which mechanisms

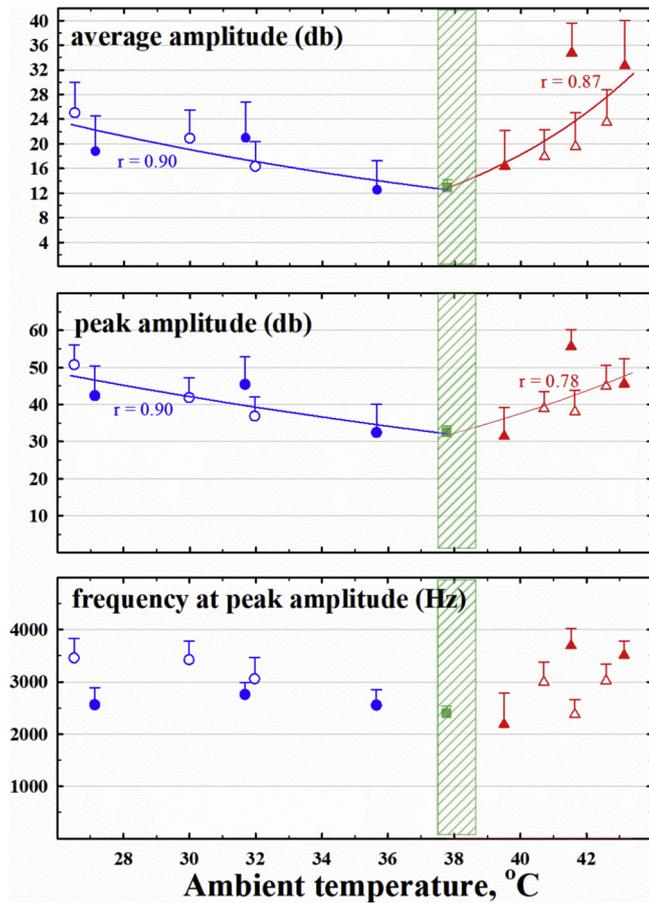


Fig. 6. From top to bottom, average amplitudes, peak amplitude and frequency at peak amplitude of the power spectrum at different ambient temperatures. Circles refer to cooling, triangles indicate warming; bars are 1 SEM. Open and filled symbols indicate, respectively, rapid and slow changes in Ta. The hatched area refers to normothermia. The curves represent the best fit through the data points of the type $Y = a \cdot X^b$, of which r indicates the correlation coefficient. No significant correlation existed for Ta-frequency over the cold or hot range tested. Rapid temperature change: $N = 10$ embryos, each repeated in cold and warm conditions, in alternate order. Slow temperature change: $N = 6$ embryos exposed to cold and $N = 6$ embryos exposed to hot.

could be at the basis of the much higher hot-, than cold-, sensitivity in embryos. This difference could originate at various sites, the concentration and sensory properties of skin receptors, their central contribution and afferent signal integration, cold- and hot-sensitivity of the pre-optic anterior hypothalamic neurons (Tzschentke and Basta, 2000) or the degree of habituation to cooling temperatures. It is possible only to speculate about the evolutionary pressures that favored this difference. An incubation at high Ta increases the rate of development and shortens the incubation time (Hepp et al., 2006), lowering the risk of predation. However, high incubation Ta decreases the safety margin against lethal egg temperatures (Reyna and Burggren, 2012). In fact, the lack of aerobic scope in chicken embryos, consistent with the current results of a minimal \dot{V}_{O_2} increase in the heat, has been interpreted as an indication that embryos operate at the highest \dot{V}_{O_2} compatible with survival (Ide et al., 2017). Even a very short period at 44 °C can be fatal (Oppenheim and Levin, 1975) and if prolonged for several hours the thermal tolerance can be just a couple of degrees above normothermia (Webb, 1987). Hence, notwithstanding the advantage of incubation close to the upper limits of the Ta tolerance, warnings about the risks of hyperthermia need to initiate much more readily than those about hypothermia.

4.4. Conclusions

Over a range of cooling and warming conditions, whether rapidly or slowly occurring, chicken embryos at end-incubation increased the number of calls and their amplitudes, while maintaining a stereotyped power spectrum. The call types emitted were similar between hot and cold temperatures but differed from those uttered at normothermia. Vocalization was unrelated to the degree of thermogenesis, and occurred much more readily during increases, than during decreases, in Ta. Most likely, the greater vocalization during warming reflects the fact that the incubation temperatures are close to the maximal tolerable limits.

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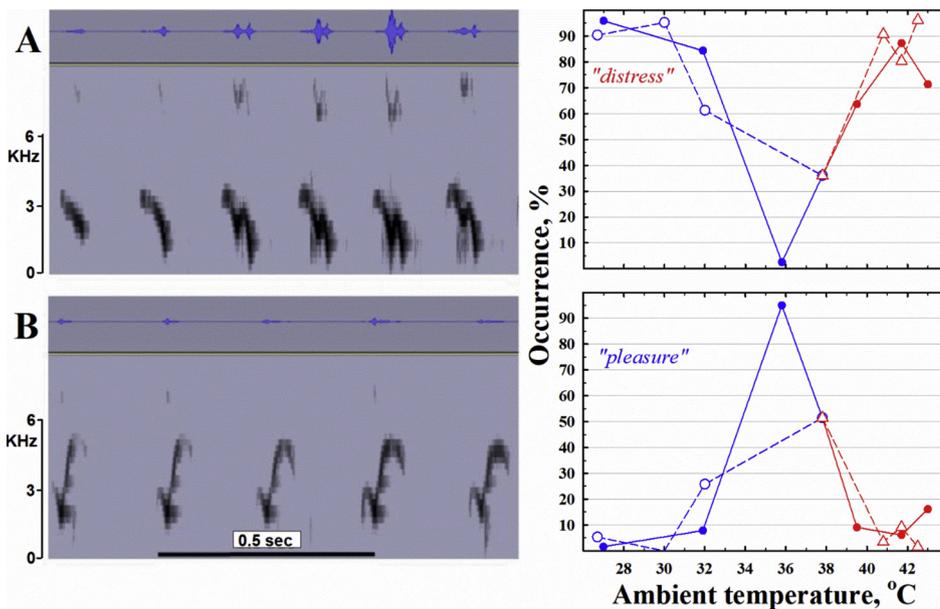


Fig. 7. Left. Exemplificative recordings of audiogram (upper recording) and corresponding spectrogram (lower panel) during hypothermia (A) and at rest in normothermia (B). These spectrograms fell into the categories of, respectively, ‘distress’ and ‘pleasure’ calls (Gottlieb and Vandenberg, 1968). The percentage occurrences of these two types of calls are indicated in the panels at right as function of the ambient temperatures. Circles refer to cooling, triangles indicate warming; open and filled symbols indicate, respectively, rapid and slow changes in Ta. The sum of ‘distress’ and ‘pleasure’ incidences did not always correspond to 100% because some spectrograms had an intermediate pattern and were not attributed to either category (Methods, 2.3.). Rapid temperature change: $N = 10$ embryos, each repeated in cold and hot conditions, in alternate order. Slow temperature change: $N = 6$ embryos exposed to cold and $N = 6$ embryos exposed to hot.

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