

RESEARCH ARTICLE

# Altered embryonic development in northern bobwhite quail (*Colinus virginianus*) induced by pre-incubation oscillatory thermal stresses mimicking global warming predictions

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**Citation:** Reyna KS, Burggren WW (2017) Altered embryonic development in northern bobwhite quail (*Colinus virginianus*) induced by pre-incubation oscillatory thermal stresses mimicking global warming predictions. PLoS ONE 12(9): e0184670. <https://doi.org/10.1371/journal.pone.0184670>

**Editor:** Arda Yildirim, Gaziosmanpasa Universitesi, TURKEY

**Received:** March 31, 2017

**Accepted:** August 28, 2017

**Published:** September 19, 2017

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**Data Availability Statement:** All relevant data are within the paper. Data can also be found online: [doi:10.5061/dryad.mb622](https://doi.org/10.5061/dryad.mb622)

**Funding:** This research was funded by National Science Foundation grant IOS 1543301 to W. Burggren. The funder had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Abstract

Global warming is likely to alter reproductive success of ground-nesting birds that lay eggs normally left unattended for days or even weeks before actual parental incubation, especially in already warm climates. The native North American bobwhite quail (*Colinus virginianus*) is such a species, and pre-incubation quail eggs may experience temperatures  $\geq 45^{\circ}\text{C}$ . Yet, almost nothing is known about embryonic survival after such high pre-incubation temperatures. Freshly laid bobwhite quail eggs were exposed during a 12 day pre-incubation period to one of five thermal regimes: low oscillating temperatures ( $25\text{--}40^{\circ}\text{C}$ , mean =  $28.9^{\circ}\text{C}$ ), high oscillating temperatures ( $30\text{--}45^{\circ}\text{C}$ , mean =  $33.9^{\circ}\text{C}$ ), low constant temperatures ( $28.85^{\circ}\text{C}$ ), high constant temperatures (mean =  $33.9^{\circ}\text{C}$ ), or commercially employed pre-incubation temperatures ( $20^{\circ}\text{C}$ ). After treatment, eggs were then incubated at a standard  $37.5^{\circ}\text{C}$  to determine subsequent effects on embryonic development rate, survival, water loss, hatching, and embryonic oxygen consumption. Both quantity of heating degree hours during pre-incubation and specific thermal regime (oscillating vs. non-oscillating) profoundly affected important aspects of embryo survival and indices of development and growth. Pre-incubation quail eggs showed a remarkable tolerance to constant high temperatures (up to  $45^{\circ}\text{C}$ ), surviving for  $4.5 \pm 0.3$  days of subsequent incubation, but high oscillating pre-incubation temperature increased embryo survival (mean survival  $12.2 \pm 1.8$  days) and led to more rapid development than high constant temperature (maximum  $38.5^{\circ}\text{C}$ ), even though both groups experienced the same total heating degree-hours. Oxygen consumption was  $\sim 200\text{--}300 \mu\text{l O}_2 \cdot \text{egg} \cdot \text{min}^{-1}$  at hatching in all groups, and was not affected by pre-incubation conditions. Oscillating temperatures, which are the norm for pre-incubation quail eggs in their natural habitat, thus enhanced survival at higher temperatures. However, a  $5^{\circ}\text{C}$  increase in pre-incubation temperature, which equates to the predicted long-term increases of  $5^{\circ}\text{C}$  or more, nonetheless reduced hatching rate by approximately 50%. Thus, while pre-incubation bobwhite eggs may be resilient to moderate oscillating temperature increases, global warming will likely severely impact wild bobwhite quail populations, especially in their strongholds in southern latitudes.

## Introduction

Temperature influences the rates of virtually all developmental processes in bird embryos, including metabolism, development, and growth [1–3]. Thus, it is not surprising that, within a zone of thermal tolerance, higher mean temperatures result in higher avian embryonic growth rates than lower mean temperatures [4–13], albeit with some potentially negative effects on hatchability. Many birds—especially domesticated species—maintain the incubation temperature of their eggs relatively constant and close to optimal temperature (typically 35–38°C) through behavioral and physiological manipulations [14]. As a result, most studies evaluating temperature effects on avian development use a constant incubation temperature equal to mean daily-temperatures of a particular species' natural environment [14].

For economically important species such as the domestic chicken (*Gallus gallus*) or turkey (*Meleagris gallopavo*), for example, constant temperatures during incubation represent the natural incubation conditions and certainly the commercial industry standard. Consequently, relatively few studies have used cyclic thermal protocols to evaluate hatchability or post-natal growth—e.g., [15]. However, constant (non-cyclic) incubation temperatures do not fully represent the naturally encountered daily thermal fluctuations that eggs of many precocial, non-domesticated birds. Some species may lay multiple eggs in a clutch (e.g., ducks, gallinaceous birds, and ratites), depositing eggs in their nest at a rate of one egg per day. In such species, true incubation by one or more parents does not begin until the penultimate or ultimate egg is laid (see [2] for review, [16]). Thus, for many precocial birds there is an extended *pre-incubation* period—the focal period of this study—where eggs that are laid first are unattended for the entire pre-incubation period and thus lack the thermal protection and stabilization provided by an incubating parent.

Pre-incubation egg temperatures closely follow what can be large diurnal thermal fluctuations of the environment [17]. The magnitude of the diurnal thermal fluctuations varies locally, seasonally, and from year to year, being for example typically greater during drought years than non-drought years. Pre-incubation egg temperatures can paradoxically reach actual incubation temperatures, which could begin development of the fertilized egg. In fact, potentially lethal temperatures have been measured in pre-incubation eggs found in ground-nests of precocial birds. For example, internal egg temperatures of pre-incubated northern bobwhite eggs (*Colinus virginianus*) in their nest but not yet being tended, followed ambient thermal fluctuations in non-drought years and often exceeded 40°C during peak thermal intensity [12, 17, 18]. In fact, bobwhite nest temperatures in drought conditions regularly peaked at 45°C and were recorded as high as 60°C [19]. Bobwhite eggs in ground nests could be severely impacted by further increases in temperature variability and magnitude [7]. Indeed, the impacts of climate change and local habitat warming, especially on the thermal biology of species including birds and their development, only continue to grow—for an entry into the voluminous and expanding literature see [20–24].

The objective of this study was thus to determine if pre-incubation is actually an unrecognized critical period for subsequent avian morphological and physiological development that can be revealed by experimental thermal protocols (e.g., [23]). Specifically, we investigated if exposure to diurnally oscillating temperatures or a constant temperature of equal heating degree-hours (the product of temperature (°C) X time (hours)) during pre-incubation affects subsequent morphological and physiological development of northern bobwhite embryos during their actual incubation period, the duration of incubation, the hatchability of eggs, and the hatching synchrony of eggs. We hypothesized that bobwhite embryos exposed to oscillating temperature treatments would exhibit a differential rate of development, metabolism, and hatching compared to those exposed to a constant temperature of the same mean value, and

that an increased heat load would negatively affect development, metabolism and hatching success.

### Materials and methods

Fertilized northern bobwhite eggs were collected from captive-reared breeding pairs of flight ready birds at Lake Cumberland Game Bird Farm (Mill Springs, KY, USA). Eggs were packaged and shipped to the University of North Texas (Denton, USA) on the day of collection. Eggs arrived on-site within 2 business days with a written record of the date and time of egg collection. Upon arrival, bobwhite eggs were randomly divided into 5 groups of 15 eggs, placed on plastic egg trays blunt end up, and given a unique identifying number with an indelible marker. Each egg was then weighed to the nearest 0.01 g with a digital scale (Ohaus Explorer Pro, Pinebrook, NJ, USA) and placed into the assigned pre-incubation treatment.

Lake Cumberland Game Bird Farm was approved for egg production by the United States Department of Agriculture (USDA) and certified by the USDA National Poultry Improvement Plan. This research was approved by the University of North Texas Institutional Animal Use Care Committee, protocol # 0808.

### Pre-incubation protocol

Each of the 5 egg groups (N = 15 eggs per group) were assigned to a thermal chamber (G.Q.F. 1583 Hova-Bator with circulated air) with a unique experimental thermal protocol of oscillating or constant temperatures for a 12-d pre-incubation period. The groups consisted of a low and high oscillating temperature regime (LT<sub>Osc</sub>, HT<sub>Osc</sub>), a low and high constant temperature regime (LT<sub>Const</sub>, HT<sub>Const</sub>), and a commercial regime (T<sub>Comm</sub>) that was standard in the commercial poultry production industry [25]. The specifics of these incubation regimes are given in Table 1.

Peak temperatures of the low and high groups were selected based on thermal studies which described temperatures peaking at ≥40°C in non-drought years and ≥45°C in simulated drought years [17, 19], in Texas. The experimental temperature regime for each group (low and high) ensured that the temperature of the constant treatment was equivalent to the mean of the oscillating treatment, and heating degree-hours were equivalent within the low and high groups. A heating degree-hour was defined as 1°C above physiological zero (25°C for *Colinus virginianus*) [26] for 1 hour, such that 26°C for 1 h = 1 heating degree-hour, and 27°C for 1 h = 2 heating degree-hours. Thus, the mean temperature for the LT<sub>Osc</sub> group was equal to that of the LT<sub>Const</sub> group (28.85°C) and mean temperature for the HT<sub>Osc</sub> group was equal to that of the HT<sub>Const</sub> group (33.85°C). Further, each low group (LT<sub>Osc</sub> and LT<sub>Const</sub>) received 92.4 heating degree-hours per day, and each high group (HT<sub>Osc</sub> and HT<sub>Const</sub>) received 212.4 heating degree-hours per day, for the entire pre-incubation. The commercial regime (T<sub>Comm</sub>; 20°C) received no heating degree-hours per day, since pre-incubation temperatures did not reach

**Table 1. Diel thermal regimes for oscillating and constant treatments for eggs of northern bobwhites during a 12-d pre-incubation period.**

Time of Day	Oscillating Thermal Regime		Constant Thermal Regime		
	Low Temp (LT <sub>Osc</sub> )	High Temp (HT <sub>Osc</sub> )	LowTemp (LT <sub>Const</sub> )	High Temp (HT <sub>Const</sub> )	Commercial Temp (T <sub>Comm</sub> )
0000–0759	25.00°C	30.00°C	28.85°C	33.85°C	20.00°C
0800–1059	30.00°C	35.00°C	28.85°C	33.85°C	20.00°C
1100–1359	35.00°C	40.00°C	28.85°C	33.85°C	20.00°C
1400–1659	40.00°C	45.00°C	28.85°C	33.85°C	20.00°C
1700–2359	25.00°C	30.00°C	28.85°C	33.85°C	20.00°C

<https://doi.org/10.1371/journal.pone.0184670.t001>

physiological zero. Oscillating chambers were kept at base temperatures of 25°C (LT<sub>Osc</sub>) and 30°C (HT<sub>Osc</sub>) from midnight (0000h) to 0800h and were increased by 5°C at 0800h, 1100h, and 1400h. At 1700h, chamber temperatures were returned to their base temperature. These diurnal fluctuations were carried out each day of the 12-d pre-incubation period. The constant temperature chambers were kept at the intended temperatures throughout the entire 12-day pre-incubation period (Table 1). All thermal chambers maintained a relative humidity (RH) of 60% to minimize compounding factors with temperature treatments. Eggs were not turned during this pre-incubation period.

### Assessment of development during pre-incubation

To establish a baseline of pre-incubation development for egg groups subjected to each pre-incubation thermal regime, and to investigate whether various pre-incubation thermal groups affected subsequent embryonic development, eggs from each group were randomly selected for analysis (23 eggs per thermal regime group were selected over the duration of the study). Following the pre-incubation protocol, on the morning of the 13<sup>th</sup> day of incubation, selected eggs were weighed to the nearest 0.01 g to determine the amount of water loss during pre-incubation. Further, eggs were opened and if embryos were present, they were separated from the egg (yolk-free), weighed to the nearest 0.01 g, aged, and staged according to morphological indicators of development [27, 28].

### Incubation protocols

After the pre-incubation period, on the morning of the 13<sup>th</sup> day, eggs from each group were removed from the thermal chamber, weighed to the nearest 0.01 g, and immediately placed into the incubator (G.Q.F. 1502 Sportsman incubator, G.Q.F. Manufacturing Co., Savannah, GA, USA). Incubator temperature was maintained at a constant 37.5±0.5°C with a RH of 60%. Eggs were turned automatically every 3 h for the first 19 days of the 23 day incubation [1]. On day 20 (D20) of incubation, eggs were weighed to the nearest 0.01 g and placed in the hatching chamber of the same incubator and egg turning was stopped [1]. Hatching was determined when eggs were star-pipped, defined as an externally pipped egg where the embryo created a small hole in the shell (approximately 3 mm<sup>2</sup>) to initiate hatching [24]. This definition of hatching was used to compensate for any artificial hatching difficulties. Upon star-pipping, the duration of incubation, degree of hatching synchrony, and percentage of eggs hatched was recorded. The stage at the time of death was recorded for eggs that did not hatch. A subset of incubating eggs was allowed to go to hatching, and the hatchlings were subsequently maintained for one day at 37.5±0.5°C with a RH of 60%, for oxygen consumption measurements (see below).

### Oxygen consumption

During incubation, on incubation days 10, 12, 14, 16, 18, 20, 22, and on 1-d post hatch, VO<sub>2</sub> of the embryos *in ovo* was recorded via flow-through respirometry as an indicator of development and timing of physiological processes (e.g., internal pipping). On these selected days, 6 eggs from an individual group were removed and immediately placed into individual metabolic chambers located in a modified incubator (37.5±0.5°C) as part of the flow-through respirometry system.

The respirometry system was operated by pumping compressed normoxic air (159.22 mmHg) into an airflow manifold (MF-8 Sable Systems International, Las Vegas, NV, USA) which split the airflow into 7 channels and set the rate air flow through the system at 100±1 ml·min<sup>-1</sup> [28]. The seven Nalgene air tubes then went into the incubator in a sealed opening

and connected to copper coils of the same diameter, to warm the air to incubation temperature. Air then flowed through Nalgene tubes connected the copper coils, then to the 7 metabolic chambers (modified 2.5 oz sterilized Gerber baby food jar) simultaneously. Chamber 1 was kept empty (the “blank”) and used as a reference for normoxic air, while chambers 2–7 contained eggs. From each chamber, air flowed to a multiplexor (Sable Systems International, Las Vegas, NV, USA) that regulated the sequence of airflow from the chambers to a column of Drierite and soda lime, which removed water vapor and carbon dioxide, respectively. A subsample pump then pulled a low-pressure air sample of each chamber, sequentially through the oxygen analyzer (FC-1B, Sable Systems International, Las Vegas, NV, USA). Each chamber was sampled at 5 sec consecutive intervals for 15 min. Oxygen measurements from the analyzer were sent to the computer via a Sable Systems Universal Interface II and processed by system software (Data acquisition system 2.0 and Datacan V, Sable Systems International, Las Vegas, NV, USA).  $VO_2$  was measured as the difference in oxygen concentration between the normoxic air flowing into the blank chamber (chamber 1) and the expired air flowing out of each treatment chamber (chambers 2–7).

## Statistical analyses

All data was tested with a Shapiro–Wilks normality test [29] and Hartley’s Fmax test [29] before specific statistical analyses were performed. An independent t-test [29] was used to compare oscillating to constant temperature egg groups. An ANOVA [29] was used to identify the relationship between groups for all experiments. A Kaplan–Meier survival analysis [30] was used to compare survival rates and time to mortality among groups. Significance between groups was determined with a Student–Newman–Keuls (SNK) multiple range *post hoc* test [29]. All statistical tests were conducted using SigmaStat 3.5 software (Systat Software Inc. San Jose, CA), or manually [29, 31]. Statistical decisions were made with a 0.05 level of probability. All data are reported as mean±S.E. unless otherwise indicated.

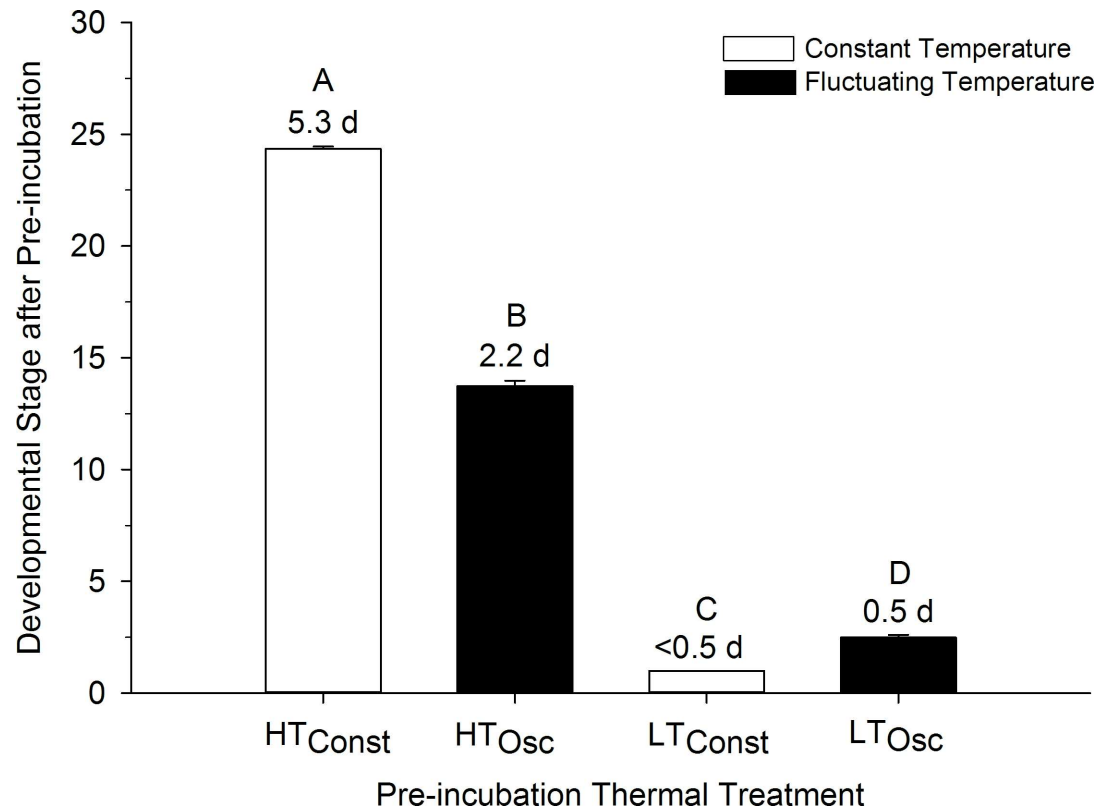
## Results

### Pre-incubation development

For the subsample of embryos extracted directly after the 12-d pre-incubation period, mean stages of development were different among pre-incubation treatments (ANOVA,  $P < 0.001$ ). Oscillating and constant groups significantly differed from each other (Fig 1) and developmental stage of all groups were statically unique ( $P < 0.05$ ). During pre-incubation, eggs of the high-constant temperature ( $H_{\text{Const}}$ ) group were the most developed, advancing to a mean developmental stage of  $21.4 \pm 0.2$ , equivalent to 5.3 incubation days at  $37.5^\circ\text{C}$  [27, 28]. Eggs exposed to high-oscillating temperatures ( $HT_{\text{Osc}}$ ) developed to a mean stage of  $13.7 \pm 0.2$ , equivalent to 2.2 incubation days at  $37.5^\circ\text{C}$ . Low-oscillating ( $LT_{\text{Osc}}$ ) embryos developed to a mean stage of only  $2.5 \pm 0.1$ , equivalent to 0.5 incubation days at  $37.5^\circ\text{C}$ , while pre-incubation development was lowest in low-constant ( $LT_{\text{Const}}$ ) eggs, which progressed to a mean stage of just  $1.1 \pm 0.1$ , or  $< 0.5$  incubation days at  $37.5^\circ\text{C}$  (Fig 1). Commercial pre-incubation eggs ( $T_{\text{Comm}}$ ) had no pre-incubation development.

### Survival curves hatching success

Mortality occurred prior to hatching in all groups including the commercial group, as anticipated. The survival rate and stage of development at the time of death was significantly different between groups (Log rank survival analysis;  $P < 0.001$ ). A Holm–Sidak Pairwise MCP statistically grouped the treatments receiving the lowest amounts of heating degree-hours



**Fig 1. Mean developmental stages of northern bobwhite embryos after exposure to specific thermal groups during a 12-d pre-incubation period.** Numbers above bars show days of incubation equivalent to the indicated developmental stages. Letters indicate statistical groupings. n = 23 eggs per group. Commercially incubated eggs did not have any pre-incubation development.

<https://doi.org/10.1371/journal.pone.0184670.g001>

(LT<sub>Osc</sub>, LT<sub>Const</sub>, and T<sub>Comm</sub>) into one group. These groups had a statistically higher survival rate compared to the high heating degree-hour groups (HT<sub>Osc</sub> and H<sub>Const</sub>;  $P < 0.001$ , Fig 2). Mean survival time for LT<sub>Const</sub> eggs was  $22.2 \pm 1.4$  days. The low oscillating group had a mean survival time of  $22.1 \pm 0.7$  days, and this was not statistically different from the LT<sub>Const</sub> group ( $P = 0.25$ ). Mean survival for HT<sub>Osc</sub> eggs was  $12.2 \pm 1.8$  days which was significantly different from H<sub>Const</sub> eggs ( $4.47 \pm 0.3$  days), none of which hatched during any trials (Fig 2).

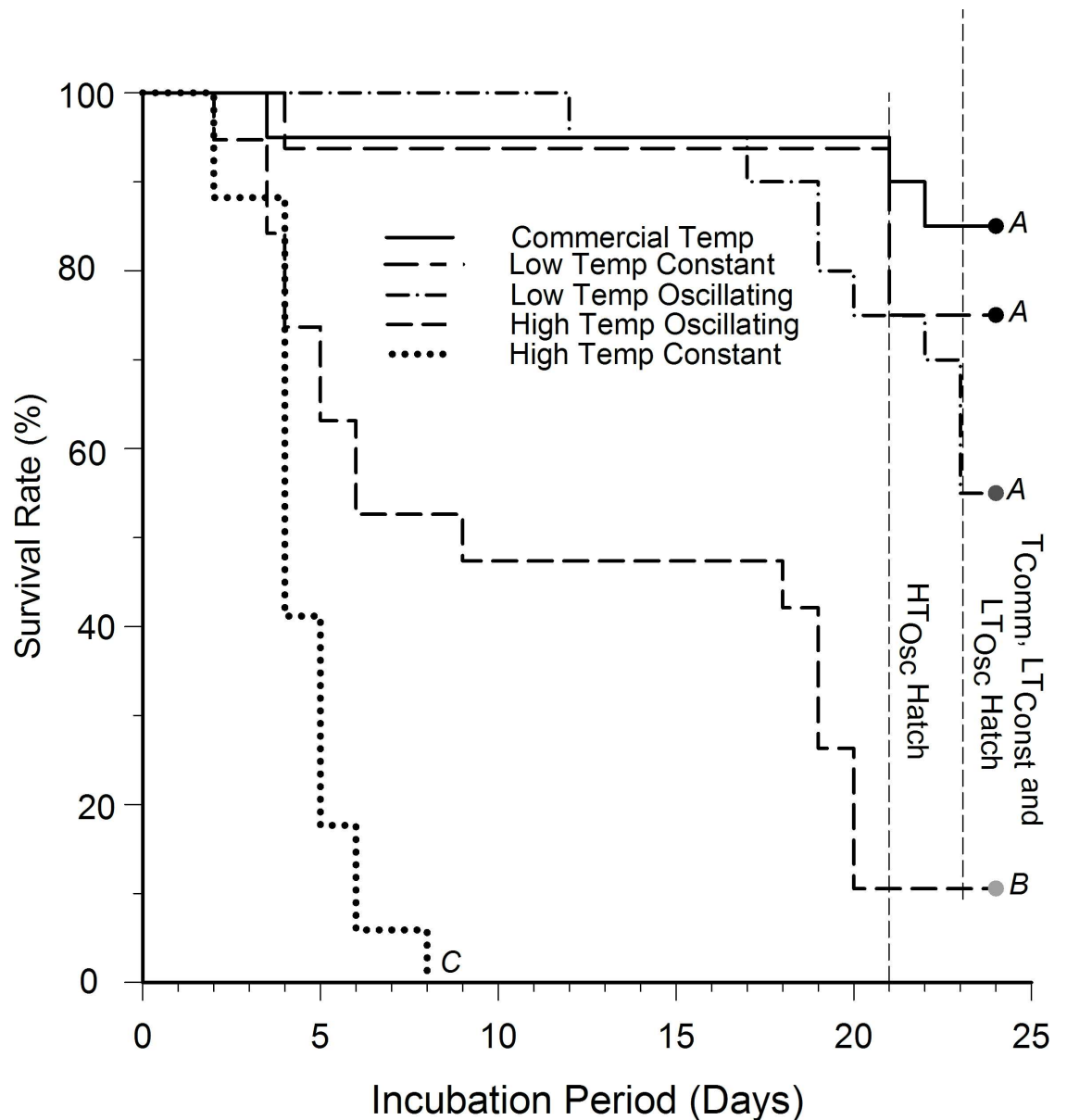
Commercial eggs hatched at a significantly higher rate ( $80.8 \pm 3.6\%$ ) than all treatment groups (Fig 3; ANOVA,  $P < 0.001$ ). Hatching success of the LT<sub>Const</sub> treatment ( $63.3 \pm 6.7\%$ ) was not significantly different from the LT<sub>Osc</sub> treatment ( $53.5 \pm 6.4\%$ ;  $P = 0.24$ ). HT<sub>Osc</sub> hatch rates ( $6.0 \pm 2.1\%$ ) did not statistically differ ( $P = 0.53$ ) from H<sub>Const</sub> hatch rates (0.0%). Hatch rates for LT<sub>Osc</sub> was significantly higher than for the HT<sub>Osc</sub> groups ( $P < 0.001$ ).

Pre-incubation thermal treatment not only affected survival and hatching success, but also the timing of specific hatching events—internal pipping, external pipping, and hatching. All of these developmental landmarks were significantly different between commercial and treatment groups (Fig 4; ANOVA,  $P < 0.001$ ). HT<sub>Osc</sub> eggs internally pipped earlier ( $D18.6 \pm 0.2$ ) than LT<sub>Const</sub> eggs ( $D19.7 \pm 0.2$ ), LT<sub>Osc</sub> eggs ( $D20.5 \pm 0.2$ ), and T<sub>Comm</sub> eggs ( $D20.6 \pm 0.2$ ). Similarly, HT<sub>Osc</sub> eggs externally pipped earlier ( $D20.0 \pm 0.0$ ) than LT<sub>Const</sub> eggs ( $D21.5 \pm 0.3$ ), LT<sub>Osc</sub> eggs ( $D21.5 \pm 0.2$ ), and T<sub>Comm</sub> eggs ( $D21.7 \pm 0.2$ ). HT<sub>Osc</sub> eggs also hatched earlier ( $D21.0 \pm 0.0$ ) than T<sub>Comm</sub> eggs ( $D23.0 \pm 0.0$ ) and LT<sub>Osc</sub> eggs ( $D23.5 \pm 0.2$ ), while LT<sub>Const</sub> eggs took the longest to hatch of treatment groups ( $D23.8 \pm 0.1$ ).

Additionally, the duration from internal pip to hatching was significantly different between treatment groups ( $P < 0.001$ ) (Fig 4). The hatching duration was not significantly different between  $LT_{Const}$  ( $3.7 \pm 0.4$  days) and  $LT_{Osc}$  ( $3.2 \pm 0.1$  days) treatments ( $P = 0.16$ ), but  $HT_{Osc}$  had a shorter duration of hatching events than  $LT_{Osc}$  ( $P < 0.001$ ). The duration between internal pip and hatch was the shortest for  $HT_{Osc}$  ( $2.4 \pm 0.2$  days) and  $T_{Comm}$  eggs ( $2.4 \pm 0.2$  days). All eggs in a given group hatched essentially synchronously—i.e., within 24 h of each other.

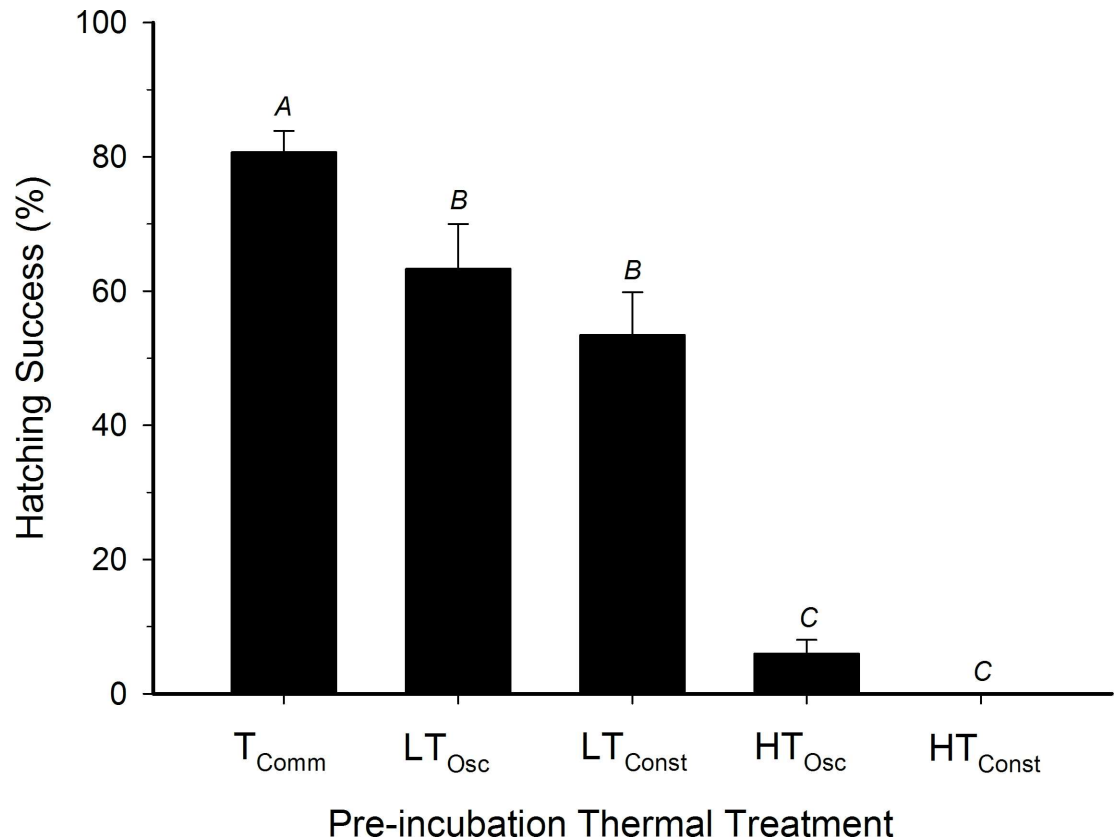
### Oxygen consumption

Oxygen consumption ( $VO_2$ ) increased significantly as a function of development (Fig 5).  $VO_2$  in  $LT_{Const}$  and  $LT_{Osc}$  groups was similar in overall pattern throughout incubation, but absolute



**Fig 2. Kaplan-Meier survival curves showing survival rate and time to mortality of northern bobwhite embryos exposed to different thermal protocols during a 12-day pre-incubation period.** Stair-step declines show time of mortality.  $n = 45$  eggs per treatment group. Letters indicate statistical groupings with a  $P < 0.05$  level of significance.

<https://doi.org/10.1371/journal.pone.0184670.g002>



**Fig 3. Hatching success of northern bobwhite embryos exposed to different thermal protocols during a 12 day pre-incubation period.**  $n = 45$  eggs per group. Letters indicate statistical groupings.

<https://doi.org/10.1371/journal.pone.0184670.g003>

values differed significantly ( $P < 0.05$ ) on D10, D15, D18, D20, and D21 of incubation (Fig 5A). HT<sub>Osc</sub> embryos had a significantly higher  $VO_2$  than H<sub>Const</sub> embryos on D10 but not on D15, however H<sub>Const</sub> embryos did not survive past day 15 of incubation, so no further comparisons were possible (Fig 5B). Hatchling  $VO_2$  was higher than at any point in embryonic development in all groups, but there were no significant differences between treatments in hatchlings.

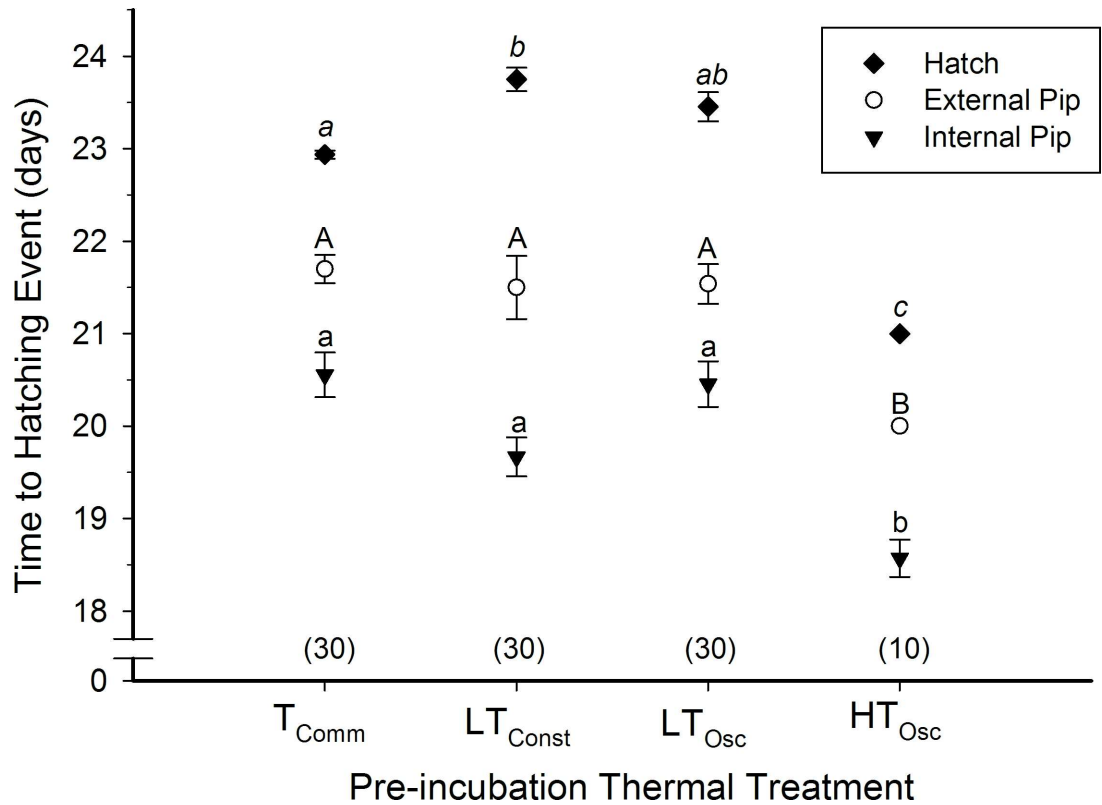
To further aid in  $VO_2$  comparison, semi-log plots were created (Fig 6). Slopes relating  $VO_2$  to development were not significantly different between LT<sub>Const</sub> and LT<sub>Osc</sub> pre-incubation treatments (Fig 6A;  $t$  test for slopes,  $P > 0.50$ ). Slopes relating  $VO_2$  to development were significantly different between LT<sub>Osc</sub> and HT<sub>Osc</sub> pre-incubation treatments (Fig 6B;  $t$ -test for slopes,  $0.02 > P > 0.001$ ). As noted above, H<sub>Const</sub> eggs did not survive past day 15 of incubation, so no oxygen consumption comparisons were made with this treatment group.

### Egg mass and water loss

Mean egg weight loss among thermal groups during the pre-incubation period was higher in LT<sub>Osc</sub> and HT<sub>Osc</sub> egg groups than in all other pre-incubation groups ( $P < 0.001$ ), with a percentage loss of  $4.9 \pm 0.2\%$  and  $3.9 \pm 0.5\%$  respectively. There was no difference between HT<sub>Const</sub> eggs ( $2.8 \pm 0.5\%$ ) and LT<sub>Const</sub> eggs ( $2.6 \pm 0.1\%$ ) and between LT<sub>Const</sub> eggs and T<sub>Comm</sub> eggs ( $2.14 \pm 0.04\%$ ;  $P > 0.05$ ) (Fig 7).

Unlike pre-incubation, no significant difference ( $P = 0.88$ ) in the percentage of fresh egg weight loss during incubation was detected between HT<sub>Osc</sub> and H<sub>Const</sub> ( $P = 0.05$ ) or LT<sub>Osc</sub> and





**Fig 4. Time to internal and external pipping and hatching for northern bobwhite eggs exposed to T<sub>Comm</sub>, LT<sub>Const</sub>, LT<sub>Osc</sub>, and HT<sub>Osc</sub> thermal treatments during a 12 day pre-incubation period.** Sample size in parentheses. Events are statistically grouped (0.05 level of significance) with lower-case, italicized, and capital lettering.

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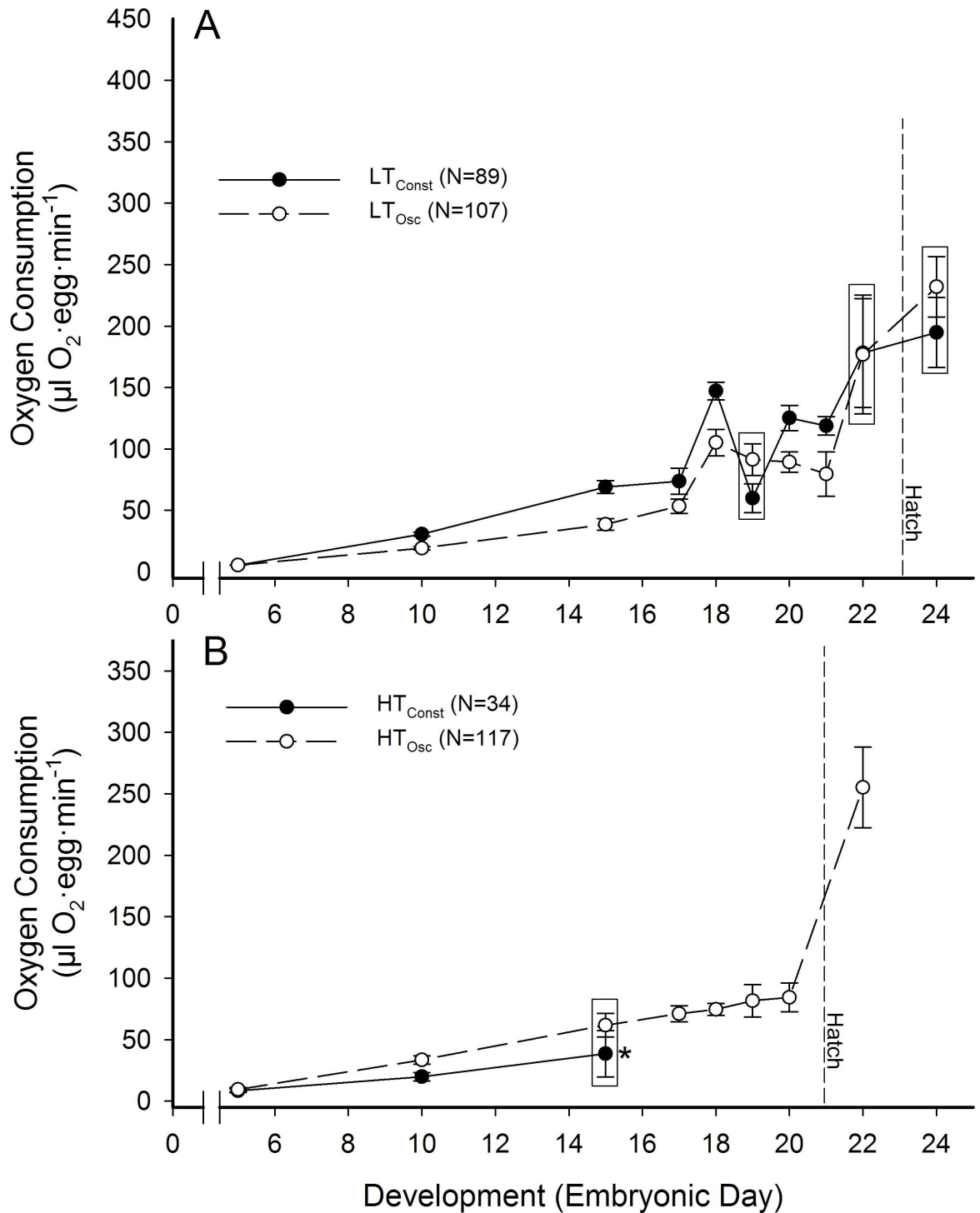
LT<sub>Const</sub> (P = 0.88) groups. However, there was a significant (P < 0.05) difference in egg weight loss between T<sub>Comm</sub> eggs (11.5 ± 0.3%) and H<sub>Const</sub> eggs (14.9 ± 1.5%) during incubation (Fig 7).

Combining the pre-incubation and incubation periods, all treatment groups lost a greater percentage of fresh egg mass than T<sub>Comm</sub> eggs (ANOVA, P < 0.05). The mean percentage of fresh egg mass lost by T<sub>Comm</sub> eggs was 11.5 ± 0.3%, lower than H<sub>Const</sub> (17.7 ± 1.9%), HT<sub>Osc</sub> (14.9 ± 1.5%), LT<sub>Const</sub> (15.9 ± 0.6%), and LT<sub>Osc</sub> eggs (18.3 ± 0.7%). No difference was detected in percentage of fresh egg mass lost between HT<sub>Osc</sub> and H<sub>Const</sub> (P = 0.28), or LT<sub>Osc</sub> and LT<sub>Const</sub> (P = 0.12) (Fig 7).

## Discussion

### Pre-incubation modification of subsequent embryonic development

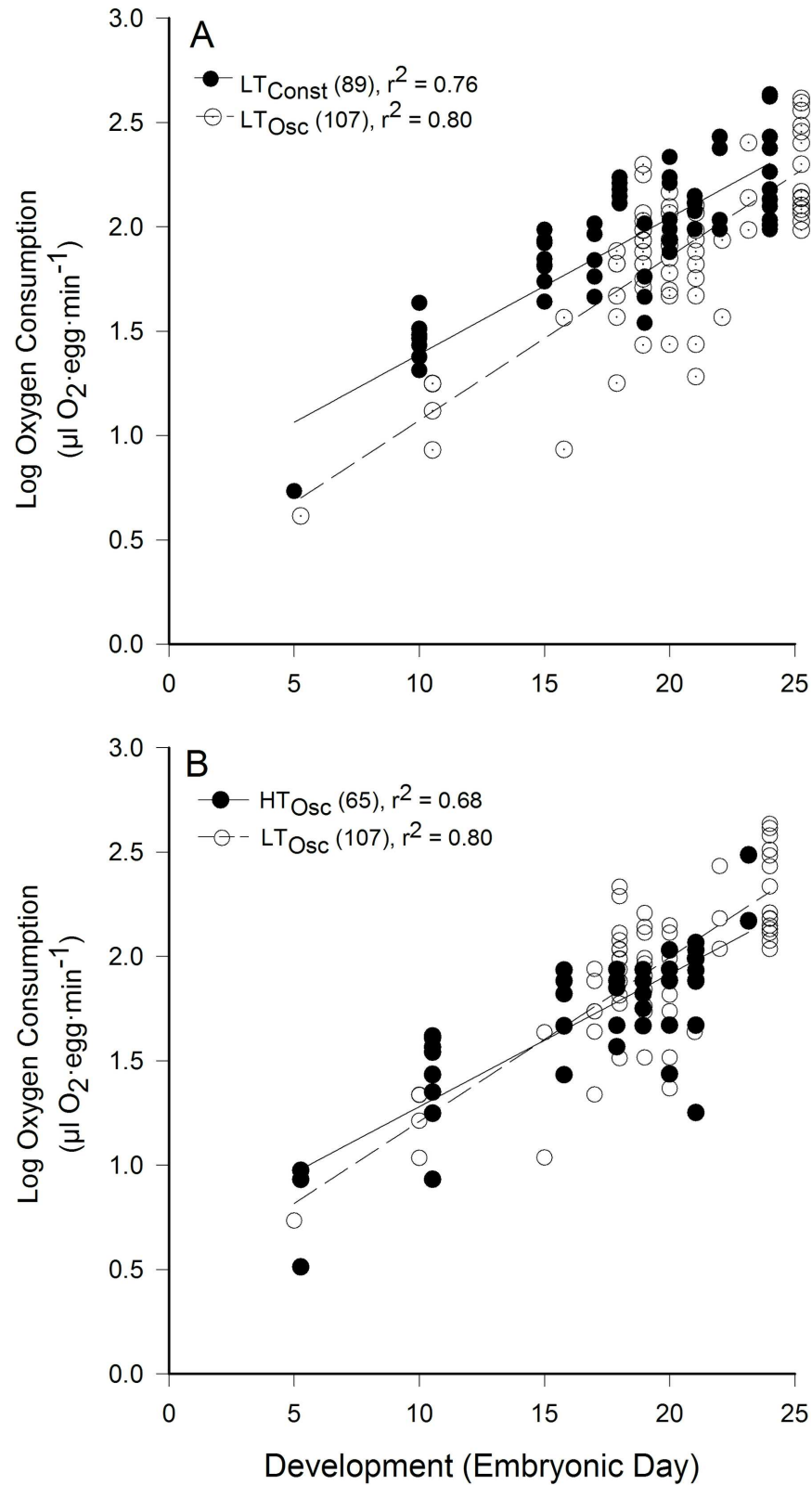
This study shows that quail embryos actually begin to develop during pre-incubation when eggs are exposed to temperatures above “physiological zero”. As expected, more development was obtained with more heating degree-hours and less development occurred with fewer heating degree-hours, even when slightly above physiological zero. Eggs exposed to high heat loads (HT<sub>Osc</sub> and H<sub>Const</sub>) and low heat loads (LT<sub>Osc</sub> and LT<sub>Const</sub>) had equal quantities of heating degree-hours within groups, but they all exhibited differential pre-incubation development and growth rates. Groups exposed to a constant temperature had the largest variation between high and low groups, with a difference of ~5 days of development between H<sub>Const</sub> and LT<sub>Const</sub>.



**Fig 5. Oxygen consumption of northern bobwhite embryos as a function of development and pre-incubation thermal groups.** Statistically similar means are grouped within boxes. \* No  $\text{H}_{\text{Const}}$  eggs survived past day 15.

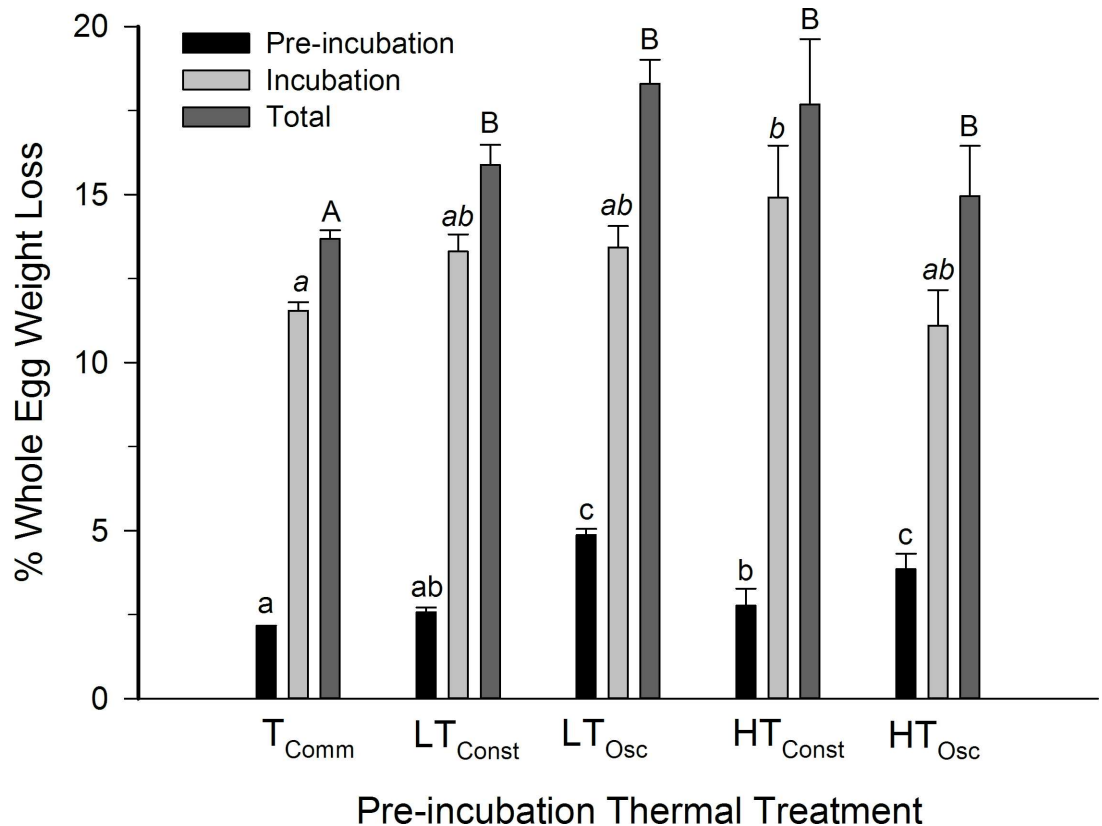
<https://doi.org/10.1371/journal.pone.0184670.g005>

even though each group received the same amount of heating degree-hours (Fig 1). The difference in groups with oscillating temperature was much less, with about 1.7 days of development difference between  $\text{HT}_{\text{Osc}}$  and  $\text{LT}_{\text{Osc}}$ . Thus, at least for quail development, the quantity of heat



**Fig 6. Regressions relating oxygen consumption to development.** A) No significant differences existed between the slope of the lines for  $LT_{Const}$  and  $LT_{Osc}$  (ANOVA;  $P > 0.50$ ). B) The slope of the line for relating  $LT_{Osc}$  was significantly higher than for  $HT_{Osc}$  ( $0.02 > P > 0.001$ ). Coefficient of determination ( $r^2$ ) and n values are shown.

<https://doi.org/10.1371/journal.pone.0184670.g006>



**Fig 7. Mean percentage of fresh egg weight loss for northern bobwhite eggs measured after exposure to oscillating and constant thermal treatment for a 12 day pre-incubation period.** n = 45 eggs per group. Letters (lower-case, italicized, and capital) indicate statistical groupings within each treatment.

<https://doi.org/10.1371/journal.pone.0184670.g007>

(e.g., number of heating degree-hours) is not the only driver of development and that the nature of the heat (oscillating or constant) is also a major factor.

Recently, intragenerational epigenetic modification of embryonic development has been induced by the length of pre-incubation storage in chicken embryos [32]. These changes were not the result of high temperatures, since the eggs were stored well below physiological zero. Whether an oscillating temperature at cool temperatures with no likelihood of beginning embryonic development would actually alter embryonic development remains to be investigated.

### Survival and hatching assessment

The thermal protocol used during the pre-incubation period in *Colinus virginianus* had a profound effect on subsequent embryonic survival. While high pre-incubation temperatures severely affected subsequent embryonic survival, it is interesting to note that an equivalently high oscillating temperature, while leading to only poor survival, nonetheless was more successful in producing viable embryos than a constant high temperature.

Observations of the effect of pre-incubation temperature and other conditions on subsequent embryonic survival have mostly derived from interest in poultry production or basic experimentation [32–38]. Such studies on the pre-incubation period have almost invariably considered steady-state storage conditions, rather than oscillations in environmental parameters, such as the oscillations in temperature employed in the current study. Few studies on oscillating temperatures exist for any aspect of avian egg storage—the few such studies have

been directed towards inducing temperature oscillations once formal incubation has begun [15].

Embryonic survival and hatching success was not statistically different between constant and oscillating groups. However,  $H_{\text{Const}}$  embryos did not hatch, dying at ~4 to 15 days of incubation (Fig 2). The death of  $H_{\text{Const}}$  embryos is noteworthy, since eggs were kept for 12 days at 33.85°C, well within the natural incubating range of 30–35°C measured in nature for this species [17]. While the objective of this study was not to determine the cause of death in eggs at constant high temperature, we speculate that constant temperatures near incubation temperature require eggs to be turned daily for survival. Indeed, studies have shown that, while turning of quail eggs is not required when eggs are stored at 12.8°C, turning is required during incubation at temperatures between 35–38°C [1, 39, 40]. High mortality was not experienced in  $LT_{\text{Const}}$  groups (28.85°C) presumably because the mass of the embryo was that at <0.5 day of age (i.e. a much smaller embryo), and thus was not affected by the absence of turning.

Bobwhite quail eggs exposed to high oscillating temperatures internally pipped, externally pipped, and hatched two days earlier than all other groups, ultimately showing a reduced incubation period (Fig 4). This should not be surprising since the high oscillating temperature protocol ( $HT_{\text{Osc}}$ ) during pre-incubation enabled approximately 2 additional days of development than in  $LT_{\text{Osc}}$  and  $LT_{\text{Const}}$  groups. Both  $LT_{\text{Osc}}$  and  $LT_{\text{Const}}$  eggs, which developed approximately 0.5 days during the pre-incubation period, exhibited an extended incubation time of 0.5 d (Fig 4).  $HT_{\text{Osc}}$  eggs not only started development earlier, but also showed overall reduced incubation duration of  $HT_{\text{Osc}}$  eggs. This may be an adaptation to the thermally stressful environments inhabited by northern bobwhite quail, where both high temperature and temperature fluctuation are common and would affect the eggs during their pre-incubation period.

Collectively, our results suggest that oscillating temperatures act differently on developing organisms than constant temperatures of equal heating degree-hours. The impact of these differences is increased as heat loads are increased, i.e., higher heating degree-hours result in more variation in development in embryos exposed to constant and oscillating temperatures. Further, within oscillating groups, higher oscillating heat loads caused an increase in the rate of development during pre-incubation, a decrease in the rate of development during incubation, and had more of a negative effect on hatching.

## Hatching synchrony

While the current study did not focus on hatching synchronization, it is interesting to note that, irrespective of thermal regime, all quail eggs hatched within 24h of each other—i.e. “synchronously” [41]. Northern bobwhites do not begin incubation until the last egg is laid [42]. Consequently, all eggs receive equal amounts of incubation, resulting in synchronous hatching. This hatching adaptation seems beneficial by allowing the incubating parent to quickly transition behavior from incubating sessile eggs to brooding motile hatchlings [41, 43]. Importantly, limited studies have investigated hatching synchrony in warmer climates where nest temperatures fluctuate during pre-incubation and can often exceed physiological zero (the temperature above which embryonic development begins). In warmer climates, those eggs laid early could actually begin to develop prior to incubation, resulting in asynchronous development at the onset of incubation and asynchronous hatching at the other end of embryonic development. In this case, eggs might hatch asynchronously unless a synchronizing mechanism takes place [6, 44–46]. Hatching synchrony has been observed in the laboratory among quail eggs that differed in developmental stages by 2 days [46], but not by up to 5 days, which is the probable result of northern bobwhites in warm climates given our experimental findings on stage of development in high temperature groups at the onset of incubation. Interestingly,

eggs must remain in physical contact with each other for synchronous hatching to occur, especially in the last 20% of incubation when pipping occurs [46, 47]. Regardless of relatedness or order of laying, viable bobwhite eggs of the same age hatch synchronously if touching [48]. When isolated, their time of hatch is spread over a longer period [45]. Bobwhite eggs accelerated hatching when placed in contact with more-advanced eggs (24 h advanced in development) and delayed hatching when placed in contact with more-retarded eggs (24 h retarded in development) [45], suggesting that both an acceleration and delay of hatching time occur between earlier laid and later laid eggs in the wild.

## Oxygen consumption and development

Oxygen consumption during avian embryonic development has been very well documented, and the reader is directed to recent discussions of that literature [16, 49–51]. Many factors influence avian embryonic  $VO_2$ , most notably body mass, oxygen availability, and environmental temperature. Yet, in precocial birds, there is a representative pattern of  $VO_2$  change during development in which  $VO_2$  typically increases until approximately 80% of incubation time. Thereafter, rate of  $VO_2$  change plateaus until the embryo internally pips into the air cell.  $VO_2$  increases once more, followed by final increase at the time the chick pips the shell and hatches [47].

The oxygen consumption pattern of change in the current study (Fig 5) was generally that described for precocial birds [47], and the pattern is consistent with reports of developing northern bobwhite quail [52] as well as the Coturnix quail, *Coturnix coturnix* [41], a precocial bird with similar sized eggs. Importantly, in our study the thermal protocol during pre-incubation significantly affected  $VO_2$ , which was different between  $LT_{Const}$  and  $LT_{Osc}$  thermal groups. The slopes of the normalized  $VO_2$  data were not different between  $LT_{Const}$  and  $LT_{Osc}$  treatments (Fig 6A), indicating no overall differences in rate of development to match the elevation of development induced by higher temperature.

There were significant differences in  $VO_2$  between  $HT_{Osc}$  and  $LT_{Osc}$  groups logically due to  $HT_{Osc}$  embryos having developed more during pre-incubation (Fig 1), thus requiring more oxygen on any given day during incubation. However, the lower slope of  $VO_2$  for  $HT_{Osc}$  eggs during incubation showed that  $HT_{Osc}$  eggs developed at a slower rate during incubation than  $LT_{Osc}$  eggs after a higher rate of development during pre-incubation (Fig 6B). Additionally,  $HT_{Osc}$  eggs did not exhibit the same oscillating  $VO_2$  patterns in the last 20% of incubation as  $LT_{Osc}$  and  $LT_{Const}$  eggs. Rather,  $VO_2$  of  $HT_{Osc}$  eggs stayed relatively constant during the pipping process (Fig 5B). Why and how  $VO_2$  during incubation is affected by pre-incubation thermal protocols awaits further investigation.

## Implications for natural bobwhite quail populations

The intention of comparing  $LT_{Osc}$  (simulated non-drought temperatures) and  $HT_{Osc}$  (simulated drought temperatures) groups was to better understand the differences between drought and non-drought conditions as well as the potential impacts of global warming on avian development generally, and quail populations specifically. In this context, one of the most interesting findings is the significant difference in hatch rates between high-oscillating ( $HT_{Osc}$ ,  $6.0 \pm 2.1\%$ ) and low-oscillating ( $LT_{Osc}$ ,  $53.5 \pm 6.4\%$ ) groups (Fig 2). The  $HT_{Osc}$  thermal group was pre-incubated at a temperature  $5.0^\circ\text{C}$  higher than the  $LT_{Osc}$  group, a temperature increase coincident with the predicted increase in global average temperature of  $5 \pm 1^\circ\text{C}$  in the United States by the period 2071–2100 relative to the period 1961–1990 [53]. Our results show that a  $5^\circ\text{C}$  increase in pre-incubation temperature of just 12 days reduces hatching rate of northern bobwhites by approximately one half. Reductions in the percentage of juvenile bobwhite quail

at different latitudes [54] or during drought [55, 56] could, according to our findings, be caused by increased heat loads during the 12 day pre-incubation period. Eggs in ground nests could be severely impacted by further increases in temperature variability and magnitude as global warming continues. Thus, our study indicates that the dynamic nature and magnitude of diel temperature should be considered when evaluating the biological response to predicted temperatures of global warming.

## Acknowledgments

We thank D. Reyna, G. Bowling, and H. Valls for providing logistical support with egg incubation, and E. Dzialowski for providing training on measuring oxygen consumption.

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