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Rates of oxygen uptake increase independently of changes in heart rate in late stages of development and at hatching in the green iguana, *Iguana iguana*

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ABSTRACT

Oxygen consumption (VO_2), heart rate (f_H), heart mass (M_h) and body mass (M_b) were measured during embryonic incubation and in hatchlings of green iguana (*Iguana iguana*). Mean f_H and VO_2 were unvarying in early stage embryos. VO_2 increased exponentially during the later stages of embryonic development, doubling by the end of incubation, while f_H was constant, resulting in a 2.7-fold increase in oxygen pulse. Compared to late stage embryos, the mean inactive level of VO_2 in hatchlings was 1.7 fold higher, while f_H was reduced by half resulting in a further 3.6 fold increase in oxygen pulse. There was an overall negative correlation between mean f_H and VO_2 when data from hatchlings was included. Thus, predicting metabolic rate as VO_2 from measurements of f_H is not possible in embryonic reptiles. Convective transport of oxygen to supply metabolism during embryonic incubation was more reliably indicated as an index of cardiac output (CO_i) derived from the product of f_H and M_h. However, a thorough analysis of factors determining rates of oxygen supply during development and eclosion in reptiles will require cannulation of blood vessels that proved impossible in the present study, to determine oxygen carrying capacity by the blood and arteriovenous oxygen content difference (A-V diff), plus patterns of blood flow.

1. Introduction

Very early in reptilian embryonic development, oxygen is delivered to the embryonic tissues by direct diffusion (Andrews, 2004) but as the embryo grows and differentiates diffusion becomes insufficient to supply oxygen across the increased distances to the tissues and it becomes dependent on convection of blood by the developing heart . Embryonic metabolic rates increase progressively during growth and development, and consequent increases in oxygen consumption (VO_2) of reptilian embryos has been reported (Dmi'el, 1970; Ackerman, 1981; Thompson, 1989; Whitehead and Seymour, 1990; Vleck and Hoyt, 1991; Aulie and Kanui, 1995; Birchard et al., 1995; Thompson and Stewart, 1997; Booth, 1998; Thompson and Russel, 1998, 1999; Booth et al., 2000). These processes are met by parallel development of and dependence on extra-embryonic tissue in the form of the chorioallantoic membrane (CAM) for respiratory gas exchange.

While embryonic metabolic patterns have been documented in different reptilian species, the accompanying cardiovascular adjustments that enable adequate supply of oxygen to the embryonic tissues are not fully understood. There have been some determinations of heart rate (f_H) during embryonic development in reptiles (see Taylor et al., 2014) that showed unchanging or even decreasing values during the final stages of incubation. Thus f_H is clearly failing to match increasing rates of VO_2 (Nechaeva et al., 2007).

In adult animals heart rate (f_H) has often been used as an index of metabolic rate. Several studies have proposed calibration equations for the relationship between f_H and VO_2 at rest and in different states, in order to predict metabolic rate, generally for free ranging animals (see Green, 2011). In many endothermic species (mammals and birds) f_H can be used to estimate VO_2 relatively reliably (Bevan et al., 1992; McPhee et al., 2003; Groscolas et al., 2010; Young et al., 2011; Currie et al., 2014). Nevertheless, in ectotherms that experience large fluctuations in body temperature, the influence of f_H on VO_2 can be confounded (Clark et al., 2006). For example, in adult reptiles f_H is a good predictor of VO_2 at different temperatures and exercise (Piercy et al., 2015) but not

List of abbreviations: A-V diff, arteriovenous oxygen content difference; CAM, chorioallantoic membrane; CaO_2, oxygen content in oxygenated blood; CvO_2, oxygen content in deoxygenated blood; CO_i, index of cardiac output; f_H, heart rate; M_b, body mass; M_h, heart mass; O_2 pulse, Oxygen pulse, mean oxygen uptake per heart beat of inactive animals; Q, cardiac output; VO_2, rate of oxygen consumption (expressed for individual embryos and hatchlings); Vs, calculated cardiac stroke volume.

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during rapid heating (Butler et al., 2002; Clark et al., 2006). However, a more thorough study of the relationship between f_H and VO_2 in embryonic reptiles has yet to be conducted.

The goal of our study was to determine the factors enabling the increase in oxygen uptake recorded during embryonic development and on subsequent hatching in the oviparous lizard *Iguana iguana*. Cannulation of blood vessels in the embryonic membranes of this species proved impossible so that the study was restricted to the relationship between VO_2 and f_H, measured separately using respirometry and a non-invasive monitoring device (Sartori et al., 2015a), in part to test the possibility that f_H could be a reliable indicator of VO_2. These data were used to calculate the average oxygen uptake per heart-beat, termed the 'oxygen pulse' plus regression analysis between VO_2, f_H, body mass and mass of the heart of iguanas at different points during embryonic incubation and in hatchlings.

2. Material and methods

2.1. Egg collection and incubation

Iguana eggs were supplied from the breeding programme conducted at the Jacarezário, University of São Paulo State, Rio Claro, UNESP (IBAMA 673766). All experimental procedures were approved by the Ethics Committee on Animal Care at the São Paulo State University (CEUA-UNESP no. 6597). Fertile eggs from different clutches were collected soon after being laid and placed in plastic trays (38 × 28.5 × 6.5 cm) containing water-saturated vermiculite. The trays were held in constant temperature incubators at 30 ± 0.5 °C throughout total incubation time of approximately 74 days and water content of the vermiculite and viability of eggs were checked in a daily basis. After hatching, animals were maintained at room temperature in plastic boxes (72 × 55 × 40 cm) provided with shelter and a light bulb for heating at a 12:12 h light-dark phase. Animals were fed with dark green leafy vegetables and fruits three times per week and had free access to water and shelter. A total of 51 eggs and 21 hatchlings were used in the study that took place over 2 breeding cycles in 2007 and in 2014.

2.2. Oxygen consumption (VO_2)

For measurement of embryonic oxygen consumption (VO_2) a total of 19 eggs were selected from two different clutches in 2007. Every 72 h, up to 6 eggs were put into cylindrical respirometry chambers of approximately 200 ml volume, attached to an intermittent flow respirometer (Sable Systems, Las Vegas, USA). In order to maintain a water saturated environment inside the chambers a piece of wet cotton wool containing approximately 1 ml of water was put at the bottom of the chamber. The chambers were maintained inside a climatic BOD (EL101/3 Eletrolab, SP, Brazil) at 30 °C (±0.5 °C) during the measurements.

The respirometry system was controlled via a computer interface (Datacan V, Sable Systems). A multiple flow controller (Multiplexer TR RM8, Sable Systems) operated an automatic switch between each of the six channels. Room air was pumped (SS-4 Sub-Sampler Pump, Sable Systems) through the system with airtight tubes. The multiplexer intercalated 50 min of an open phase, in which a continuous flow of air adjusted to 200 ml min^{-1} circulated through the chamber, with 10 min of a closed phase. During this phase the air from the selected chamber was dried with silica gel before being pumped from the chamber to the oxygen analyser (PA-10 O_2 Analyser, Sable Systems). Cycles of open-closed phases were repeated from 4 to 7 times. The sampling frequency was 1 s^{-1}.

For the measurement of the oxygen consumption of hatchlings in 2014 (N = 11, approx. 90 days old) we used the same protocol but used respirometry chambers made from PVC tubes with a volume of 110 ml. The hatchlings were fasted for four days and measured for a period up to 2–3 days, with occasional interruptions in the recordings for provision of water.

The values of oxygen percentages obtained were used to calculate the fractional decrease in oxygen of the chamber during the closed phase, and corrected for residual air volume and time, to provide VO_2 values in μl O_2 $animal^{-1}$ h^{-1} (Klein et al., 2006). Data are shown as mean values ± SEM. The pattern of VO_2 in embryos was related to eventual hatching (eclosion) times, and for calculations of O_2 pulse and correlation analysis VO_2 was grouped in 10% increments of incubation time between 10% and 90%. For hatchlings, the 6 first hours of measurement were excluded and inactive metabolic rates were calculated from stable values, excluding peaks related to sporadic bouts of activity.

Total volume of oxygen (VO_{2TOT}) consumed throughout the incubation period was calculated by the integral of the area under the VO_2 curve using SigmaPlot software. To calculate the energy required for total embryonic incubation we assumed that the main substrates used as fuel were lipids in which every ml of O_2 consumed corresponded to 19.64 J of energy (Thompson and Russel, 1999).

2.3. Heart rate (f_H)

Eggs were selected from eight clutches in 2014 at the estimated percentage incubation times of 10%, 30%, 50%, 70% and 90%, based on oviposition date and the recorded mean of 74 ± 1 days of incubation at 30 °C. Each egg was weighed and candled to select a region for the insertion of a thermocouple, in order to record egg temperature during the f_H measurements. A small piece of latex membrane was attached by cyanoacrylate glue to the eggshell over the selected region and the shell was then punctured beneath the latex patch using a 26G needle and an implantable tissue thermocouple (T-type thermocouple probe, AD Instruments) was inserted by 5 mm into the amniotic fluid. Despite the expertise of an author (e.g. Crossley et al., 2003) it proved impossible to cannulate CAM vessels in iguana embryos so that f_H could not be monitored as blood pressure and blood samples could not be withdrawn for analysis. Accordingly, f_H was recorded using a digital egg monitor (Buddy System, Avitronics, UK), a non-invasive heart rate recorder that detects and amplifies, via infrared sensors, the signal of a heart beat within the egg, as described for birds and reptiles (Lierz et al., 2006; Du et al., 2009; Sartori et al., 2015a). Each egg was enclosed in moistened gauze and placed in an egg monitor that was in turn placed within a portable incubator (Caltech EIP-010, PE, Brazil) set at 30 °C (±0.5 °C). The signal outputs from the egg monitor and from the thermocouple (via a T-type Pod, AD Instruments, Sydney, Australia) were connected to a signal acquisition system (PowerLab, AD Instruments). f_H was determined at the point when the egg had warmed to 30 °C, as recorded by the thermocouple, which occurred at an average time of 32 ± 6 min (Sartori et al., 2015a).

For measurement of post embryonic f_H 10 three-month old hatchlings were instrumented with two lengths of Teflon insulated silver wire (A-M Systems, WA, USA), inserted subcutaneously either side of the mid-thoracic wall and secured to the skin with cyanocrilate glue. The hatchlings were enclosed in 50 ml Falcon tubes, with holes at both ends that allowed the anterior portion of the head and the tail to sit outside of the tube. These wire leads were externalized through a slot cut through the wall of the Falcon tube and connected to an ECG amplifier (Animal Bio Amp, ADInstruments). Signals of the ECG were recorded using LabChart data aquisition software (LabChart 7, ADInstruments). Because movements of the animal produced interference in the recordings and transiently increased f_H from basal values, we choose to collect data overnight, in order to obtain a true inactive value of f_H and larger segments of undisturbed recordings.

2.4. Masses and allometric relations

After completion of the f_H measurements embryos and hatchlings were killed by isoflurane vaporization and weighed. The heart was

Table 1
Egg mass, yolk-free body mass (M_b) of embryos and mass of hatchlings, heart mass (M_h), heart rate (f_H), rate of oxygen consumption (VO_2) of embryos from 10 to 90% incubation and hatchling iguanas. Values are mean ± SEM, N is indicated in parenthesis. Mass-specific VO_2 was calculated from mean VO_2 and M_b, oxygen pulse (O_2 pulse) from mean data for f_H and VO_2 and an index of cardiac output (CO_i) from the product of M_h and f_H.

Age	Egg mass (g)	M_b (g)	M_h (mg)	f_H (beats min^{-1})	VO_2 (μl O_2 $animal^{-1}$ h^{-1})	Mass-specific VO_2 (μl O_2 g^{-1} h^{-1})	O_2 pulse (μl O_2 $beat^{-1}$)	CO_i
10%	16.1 ± 0.6 (6) [a]	0.13 ± 0.01 (6) [a]	1.1 + 0.1 (5) [a]	111 ± 4 (6) [a]	321 ± 23 (13) [a]	2469	4.8×10^{-2}	124.3
30%	22.7 ± 1.6 (6) [b]	0.60 ± 0.05 (6) [ab]	2.1 ± 0.1 (6) [ab]	89 ± 2 (8) [ab]	330 ± 45 (8) [a]	550	6.1×10^{-2}	186.9
50%	24.9 ± 0.8 (6) [b]	1.31 ± 0.04 (6) [ab]	3.2 ± 0.6 (5) [ab]	88 ± 1 (6) [ab]	324 ± 39 (13) [a]	247	6.1×10^{-2}	281.6
70%	31.6 ± 1.1 (6) [c]	2.99 ± 0.13 (6) [ab]	6.8 ± 0.3 (6) [ab]	89 ± 2 (6) [ab]	272 ± 24 (14) [a]	91	5.0×10^{-2}	605.2
90%	33.2 ± 0.8 (6) [c]	7.20 ± 0.41 (6) [b]	19.0 ± 1.2 (6) [b]	90 ± 5 (6) [ab]	693 ± 49 (14) [b]	96	12.8×10^{-2}	1710.0
Hatchl	-	13.90 ± 0.36 (8) [bc]	38.3 ± 2.0 (8) [bc]	42 ± 1(8) [b]	1166 ± 46 (11) [c]	84	46.3×10^{-2}	1596.0

Superscript letters refer to significant statistical differences between age groups within the variable measured.

dissected out under a stereomicroscope (Zeiss Stemi 2000 C, Germany) and weighed on an electronic balance to the nearest 0.0001 g. Relations between heart (M_h) and body mass (M_b), f_H, VO_2 and incubation time were determined using least-square or non-linear regressions.

2.5. Oxygen pulse and index of cardiac output

Oxygen pulse is defined as the average volume of oxygen consumed by the animal's tissues (expressed as μl per animal) during each heart beat. This was calculated for each developmental stage (10–90% incubation and hatchlings) using the mean oxygen consumption values obtained for embryos in the whole egg, and for hatchlings as whole animals (μl O_2 min^{-1}) and dividing this value by the mean heart rate (beats min^{-1}) for each stage. An index of cardiac output (CO_i) was determined by the product of mean f_H and mean M_h, using heart mass as an index for stroke volume (Birchard and Reiber, 1996).

2.6. Statistical analysis

We conducted a two-way ANOVA analysis to detect differences in embryonic VO_2 between the two clutches used. As they were statistically similar, data was combined and analysed with one-way ANOVA. A Student Newman Keuls *post hoc* test was used to detect differences between incubation periods.

Egg mass, M_b, M_h, f_H, and VO_2 for the selected percentages of the incubation period were analysed by one-way Anova with the *post hoc* test of Student Newman Keuls to determine the difference between the different stages of the ontogeny. When data failed assumptions of normality or homoscedasticity we used Anova on Ranks and Dunn's *post hoc* test.

Least-squares linear regression analysis was used to calculate allometric equations to describe ontogenetic scaling relationships between masses, incubation time, VO_2 and f_H. When data failed the premisses of the test, we logarithmically transformed the data or used non-linear regressions until normality or homoscedasticity were not violated and the best fit was found, based on the correlation coefficients (R^2). All tests were performed with SigmaPlot v.11 software. Significance level was attributed at 95% probability (P of 0.05).

3. Results

3.1. Growth and allometry

Table 1 shows mean data for egg mass, body mass (M_b), heart mass (M_h), f_H, VO_2 both for whole eggs and hatchlings (μl O_2 $animal^{-1}$ h^{-1}) and mass-specific values (μl O_2 g^{-1} h^{-1}), and O_2 pulse plus CO_i for all ontogenetic periods studied. The mass of embryos (M_b) increased markedly early in development with a 22 times increment between 10% and 70% incubation. Over the same period mass of the heart increased 6 times. However, in both cases these changes only became significant at 90% of incubation time, when both M_b and M_h had increased a further 2–3 times from the 70% level. When comparing to initial values (10%) the close to hatching values (90% incubation) correspond to an increase of 55 and 16 times respectively for M_b and M_h. 3-month old hatchling M_b and M_h both doubled from the 90% level. A linear plot of the relationship between individual values of M_h and the M_b of yolk-

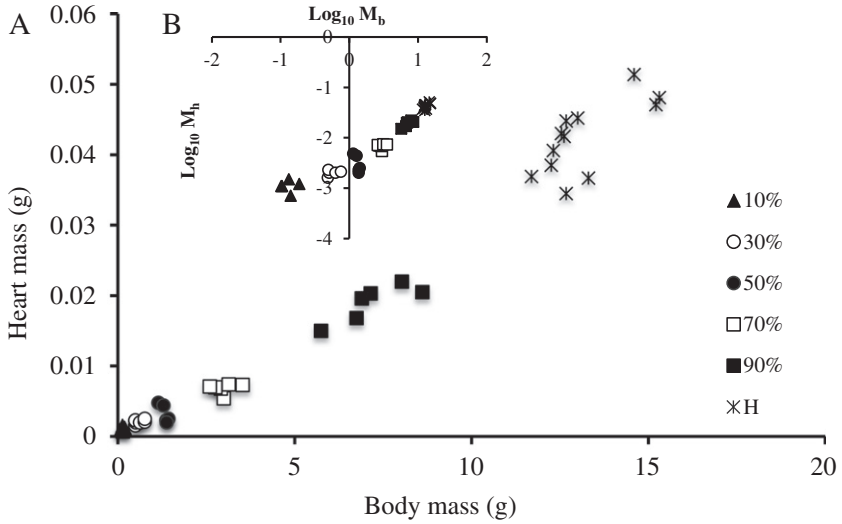

Fig. 1. A) Regression plot from body mass (M_b) vs. heart mass (M_h); B) A double logarithmic plot is included as an insert to illustrate the non-linear nature of the progressive change in heart mass with body mass during development. 10% is represented by filled triangles; 30% open circles, 50% closed circles, 70% open squares, 90% closed squares and hatchlings by asterisks.

Table 2
Regression analysis of the relationships between body mass (M_b), heart mass (M_h), heart rate (f_H), oxygen consumption (VO_2) during the different ontogenetic periods. When variables were not measured in the same animal mean values were used. Transformation of data was performed when necessary.

	R^2	P	Regression equation
log VO_2 embryo vs Inc time (t, in days) *	0.70	<0.001	$\log VO_2 = (0.0109\ t) - (0.000412\ t^2) + (0.00000525\ t^3) + 2.4$
log VO_2 vs log f_H **	0.67	0.05	$\log f_H = -0.5 \log VO_2 + 3.2$
log Mb vs log Mh **	0.93	<0.001	$\log M_h = -0.8 \log M_b + 2.4$
VO_2 vs M_b	0.94	0.001	$VO_2 = -64\ M_b + 238$
log VO_2 vs log M_h **	0.71	0.03	$\log VO_2 = 0.36 \log M_h + 3.45$
f_H vs M_b	0.76	0.02	$f_H = -3.9\ M_b + 101.6$
f_H vs M_h	0.82	0.01	$f_H = -1243.5\ M_h + 100.6$

* Indicates polynomial regression.
** Indicates a non-linear relationship.

free embryos and of hatchlings revealed that the overall increase in M_h showed an apparent proportional relationship with M_b (Fig. 1A). However, a double-log plot of these data (Fig. 1B) to illustrate the separation of data obtained from early stage embryos, revealed a non-linear relationship with a relatively high degree of correlation (Fig. 1B; Table 2).

3.2. Oxygen uptake (VO_2)

Oxygen consumption of embryonic iguanas was approximately 300 μl O_2 egg^{-1} h^{-1} throughout early and mid-incubation time. At the embryonic period close to hatching VO_2 increased, doubling to a mean value of 693 ± 49 μl O_2 egg^{-1} h^{-1} at 90% incubation (Table 1; Fig. 2), which was significantly above the value recorded at 10% incubation. The embryonic mean VO_2 curve best fitted a cubic polynomial regression ($R^2 = 0.7$, $F = 45.17$; $P < 0.0001$; Table 2). However, mass-specific VO_2 progressively decreased throughout the incubation period so that at 90% each gram of tissue consumed 26 times less oxygen than 10% embryos (Table 1). Based on the VO_2 data plot illustrated by Fig. 2, the total mean volume of oxygen (VO_{2TOT}) consumed throughout incubation, from the time close to oviposition to approximately 24 h prior to hatching, was 654 ml, corresponding to a mean expenditure of 12.84 kJ of energy per egg. Dividing this value by the mean yolk-free body mass of close to hatching embryos it corresponds to 0.92 kJ per gram of embryo, which represents the total cost of producing a newly hatched animal.

In hatchlings, VO_2 was highly variable with daily cycle peaks that occurred between 09:00 h and 22:00 h (Fig. 3), apparently reflecting changes in activity levels with a circadian rhythm (Tosini and Menaker, 1995; Klein et al., 2006). During these peaks of activity VO_2 reached values from 1300–7500 μl O_2 $animal^{-1}$ h^{-1}. Excluding these peaks gave a routine VO_2 for inactive hatchlings close to 1200 μl O_2 $animal^{-1}$ h^{-1} at 30 °C (Table 1; Fig. 3), which was significantly higher than in late stage embryos, being 4 times higher than the rate in 70% embryos and 2 times higher than in 90% embryos. Mass-specific VO_2 of hatchlings was reduced by 12% from that recorded from 90% embryos (Table 1).

3.3. Heart rate (f_H)

Mean f_H was unchanged in embryonic iguanas, varying around 90 beats min^{-1} throughout development. The mean f_H of hatchlings of 42 ± 1 beats min^{-1} was calculated based on stable values recorded overnight. During the day it was possible to detect periods of activity that related to peaks in VO_2 and affected the f_H recordings from hatchlings, with f_H reaching values close to 80 beats min^{-1}. These elevated rates were discounted in the calculation of mean settled values. Despite the stable rates recorded from hatchlings being half the rate in embryos from all stages, the mean values differed significantly only from the initial embryonic f_H at 10% incubation due to the highly variable data obtained (Table 1; Fig. 4B).

3.4. Oxygen pulse and index of cardiac output

For calculation of O_2 pulse we divided the mean stable VO_2 (Table 1; Fig. 4A, transformed to μl O_2 $animal^{-1}$ min^{-1}) by the mean stable f_H (Table 1; Fig. 4B), from embryos at the range of % incubation times and from hatchlings. As mean heart rate was relatively constant during embryonic development the calculated values for O_2 pulse (Fig. 4C) resembled the pattern of variation in VO_2, with O_2 pulse increasing 2.6-fold from 70% to 90% incubation. Hatchlings showed much higher values

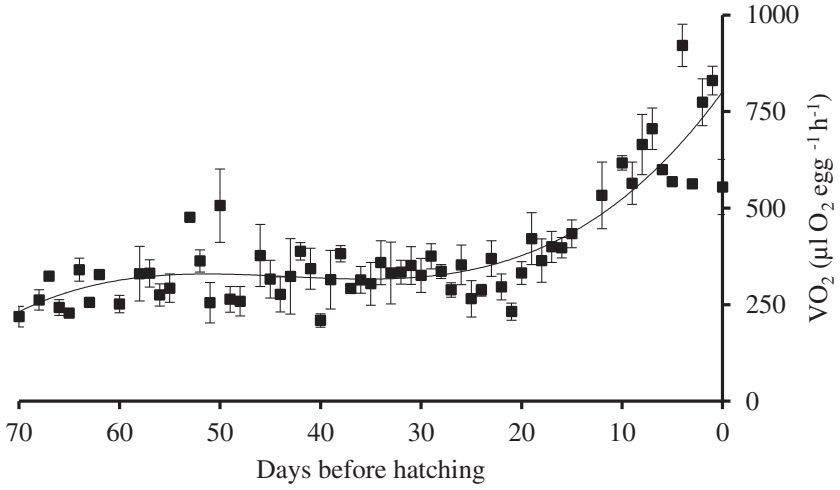

Fig. 2. Profile of oxygen consumption (VO_2, mean rates ± SEM) recorded from iguana embryos throughout the incubation period at 30 °C. Embryos of two different clutches were combined by plotting data against eclosion dates. Data are provided in μl O_2 egg^{-1} h^{-1}.

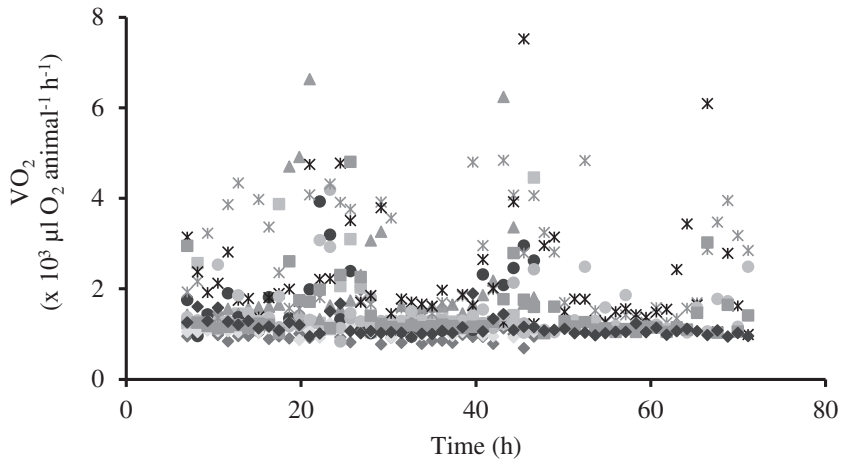

Fig. 3. Profile of oxygen consumption (VO_2) recorded from individual hatchling iguanas showing the marked circadian rhythm of metabolism. Data are provided as µl O_2 hatchling^{-1} h^{-1}.

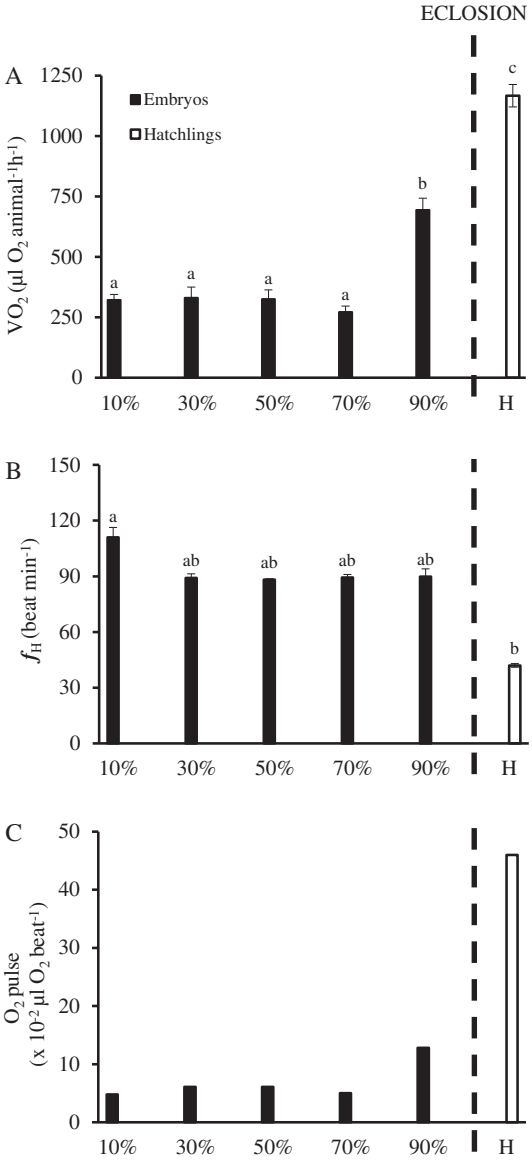

Fig. 4. Mean rates (±SEM) of A) heart rate (f_H, beats min^{-1}); B) oxygen consumption (VO_2, µl O_2 egg^{-1} h^{-1} and µl O_2 hatchling^{-1} h^{-1}); and C) calculated oxygen pulse (O_2 pulse, µl O_2 beat^{-1}) of iguana eggs at 10, 30, 50, 70, 90% of incubation (closed bars) and after hatching (H; open bars). Dashed line represents eclosion time.

of O_2 pulse, which increased 10-fold from the value at 10% incubation. This increase was related to their reduced f_H and increased (4 times) VO_2 (Table 1). CO_i increased progressively from 10 to 90% of incubation due to the progressive increase in mass of the heart, measured in mg (Table 1). Values increased 14-fold from 124 to 1710. The major increases were from 50% to 70% then to 90% incubation, with each stage accompanied by a doubling in heart mass. Hatchlings showed an index of 1596, a value similar to but 6% lower than the late 90% embryos, attributable to a halving in f_H despite a further doubling in heart mass (Table 1).

3.5. Regressions

Throughout development, mean VO_2 and f_H were correlated with mean M_b and mean M_h, which both increased progressively (Fig. 1). Table 2 shows the regression equations for the relationships between VO_2, f_H and mass. The relationship between mean VO_2 and mean f_H in the different periods of incubation and after hatching showed a barely significant, non-linear, negative correlation (Table 2; Fig. 5) with the major influence being the reduction in f_H following eclosion (Fig. 4). These changes underlie the values for oxygen pulse.

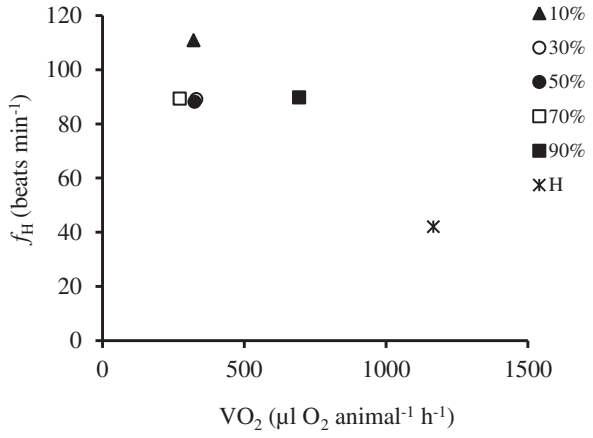

Fig. 5. Regression plot from embryos at 10–90% incubation and hatchling iguanas of mean VO_2 (µl animal^{-1} h^{-1}) against mean f_H (beats min^{-1}). This reveals a negative, non-linear relationship. 10% is represented by filled triangles; 30% open circles, 50% closed circles, 70% open squares, 90% closed squares and hatchlings by asterisks.

4. Discussion

The total energetic costs of producing a hatchling are influenced by the mass of the egg, so comparisons among amniotes are complex due to a large variation in egg sizes (Ar et al., 2004). Nevertheless, costs of producing a gram of iguana hatchling were within the range reported for other squamate embryos (Dmi'el, 1970). The final 30% of the embryonic VO_2 curve seems to be an important and critical part of incubation, as metabolic rate parallels the exponential curve for increasing M_b, indicating a rapid and expensive phase of growth during this period. According to the model proposed for avian embryonic metabolism (Hoyt, 1987; Vleck and Vleck, 1987) there is a link between the exponential increase in M_b and the exponential increase in metabolism due to a continuous increase in the costs of growth (incorporating new tissue) and costs of maintenance (proportional to the actual body mass). The result is that the final growth phase represents the most energetically expensive portion of the developmental process (Birchard and Reiber, 1995).

The factors that contribute to both the VO_2 and f_H pattern in embryonic reptiles, such a diverse and polyphyletic group, are not completely elucidated and may be phylogenetic, developmental or environmental (Thompson, 1989). Some species, especially squamates, present a relatively stable heart rate, as showed for the snake *Lamprophis fuliginous* (Crossley and Burggren, 2009) and the lizards *Bassiana duperreyi* (Radder and Shine, 2006; Du et al., 2010), *Pogona henrylawsonii* (Crossley and Burggren, 2009), plus the iguana in our present study. In iguanas, an excitatory tone, probably due to high levels of circulating catecholamines, influenced f_H throughout the entire incubation period, whereas an inhibitory parasympathetic tone only became apparent immediately before hatching, increasing further after eclosion (Sartori et al., 2015b). A role for circulating catecholamines plus the delayed onset of parasympathetic control during embryonic development has been described in a number of reptiles (Sartori et al., 2015b; Taylor et al., 2014). An apparent exception is the turtle *Chelydra serpentina* that showed onset of parasympathetic tone at around 75% of the incubation period (Alvine et al., 2013) and exhibited a significantly decreased f_H at the end of incubation.

One aim of the present study was to test the utility of using f_H as an indicator of metabolic rate. In iguana embryos from 10 to 70% incubation mean values for f_H and VO_2 were unchanging so that f_H reliably reflected aerobic metabolic rate. However, the 2-fold increase in VO_2 at 90% incubation was accompanied by an unchanging heart rate, resulting in a 3-fold increase in O_2 pulse so this relationship was not maintained and a negative correlation between f_H and VO_2 was established when the marked reduction in f_H in hatchlings was considered. Clearly, VO_2 can vary independently of f_H, depending on the incubation stage or after hatching. Based on the relationship we found between the f_H and VO_2 of green iguanas we can conclude that during the incubation period of reptiles f_H is not an appropriate predictor of VO_2 and should not be used as an index without further measurements to assess the relationship of these variables during the complete development of the species. However, several studies have used measurements of f_H as an indicator of metabolic rate in embryonic reptiles. Aubret (2013) used f_H data as an indication of resting metabolic rates in embryos of the snakes *Natrix maura* and *Natrix natrix*, while Zhang et al. (2016), in their study of the effects of light on embryos and hatchlings of four species of lizards affirmed their belief that embryonic heart rates are "an important index of metabolism", basing this on the work of Du et al. (2010). The promulgation of this belief could result in future researchers mistakenly using f_H as an indicator of VO_2 without assessing if this relationship is true for all stages of embryonic development.

The relationship between the convective properties of the cardiovascular system and oxygen consumption (VO_2) is determined by the Fick Principle, in which the amount of oxygen consumed by an organism can be calculated from the equation: $VO_2 = (CaO_2 - CvO_2) \cdot f_H \cdot Vs$, where $CaO_2 - CvO_2$ is the difference in oxygen concentration between arterial and mixed venous blood (termed A-V diff and also known as tissue oxygen extraction); f_H is the heart rate and Vs is the stroke volume, the volume of blood ejected from the heart during a single beat.

Based on the Fick Principle, the functional equivalent of O_2 pulse will be determined by the product of Vs and the A-V diff. Therefore changes in ventricular volume and blood oxygen carrying capacity together with changes in oxygen extraction, could account for the higher rates of VO_2 in relation to f_H during the later periods of embryonic development (Tazawa and Whittow, 1994; Burggren and Pinder, 1991; Birchard and Reiber, 1996; Crossley and Altimiras, 2000). So far, embryonic Vs has not been measured directly but heart mass is often used as a correlated index for its prediction (Burggren and Pinder, 1991; Birchard and Reiber, 1996; Nechaeva et al., 2007). In the iguana heart mass in embryos and hatchlings represents 0.2 to 0.3% of the body mass, approximately half of the 0.6% proportion typically found in adult mammals (Schmidt-Nielsen, 1984). Heart mass increased 16 fold during incubation and following hatching it doubled. M_b increased 55 times during embryonic development. The apparent discrepancy in growth of heart mass relative to body mass may relate to the reduction in mass-specific rates of oxygen consumption as the embryo and hatchling grows, reducing the requirement for oxygen delivery per unit mass.

As the increase in M_h must reflect an increase in Vs then the product of M_h and f_H can be used as an index of cardiac output (CO_i), and consequently as an estimation of the delivery of oxygen to satisfy metabolic demand. Thus the increasing values of CO_i obtained during embryonic development will increase the amount of O_2 delivered to the growing embryo, despite the unchanging embryonic f_H. Hatchlings showed a marked increase in cardiac mass that was accompanied by a reduction in f_H. The consequent value for CO_i was similar to that derived for 90% embryos so that their markedly increased VO_2 and oxygen pulse are likely to relate to changes in blood oxygen carrying capacity and affinity.

Converting O_2 pulse for whole hatchling in μl to values of ml O_2 g^{-1} $beat^{-1}$, resulted in a value of 3.3×10^{-5}, which falls in the range of values obtained for inactive individuals from other lizard species between of 3×10^{-5} and 4×10^{-5} ml O_2 g^{-1} $beat^{-1}$ (Bennet, 1972). Hatching represents the transition from respiratory gas exchange over the vascularized extra-embryonic CAM, which is in parallel to other systems, to exchange over the lungs, which are in series with the other systems (Birchard and Reiber, 1996). This transition can increase the gas exchange capacity (Burgreen and Warburton, 1994, Aulie and Kanui, 1995) due to the large surface area of the alveoli of the lung and their directed perfusion with deoxygenated blood that can deliver a relatively high diffusion capacity for oxygen. Adult iguanas experiencing increased oxygen demand due to increasing temperatures or intrinsic changes such as exercise or feeding must depend on increases in f_H and A-V diff to sustain aerobic metabolism (Tucker, 1966).

Mechanisms that match oxygen supply and demand during embryonic development in reptiles need to be further explored. In addition to f_H and VO_2 measurements, studies should include: difference in oxygen content from arteries and veins (A-V diff) of the CAM, CAM blood flow, stroke volume, hematocrit, hemoglobin concentration, embryonic blood shunting and oxygen affinity of the embryonic hemoglobin. This broader approach was not possible in the present study due to the acute technical difficulty of cannulating blood vessels and future work will concentrate on species enabling this approach.

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References

Ackerman, R.A., 1981. Oxygen consumption by sea turtle (Chelonia, Caretta) eggs during development. Physiol. Zool. 54 (3), 316–324.

Alvine, T., Rhen, T., Crossley II, D.A., 2013. Temperature-dependent sex determination modulates cardiovascular maturation in embryonic snapping turtles *Chelydra serpentina*. J. Exp. Biol. 216, 751–758.

Andrews, R.M., 2004. Patterns of embryonic reptiles. In: Deeming Reptilian, D.C. (Ed.), Incubation: Environment, Evolution and Behaviour. Nottingham University Press, Nottingham, pp. 75–102.

Ar, A., Belinsky, A., Dmi'el, R., Ackerman, R.A., 2004. Energy provision and utilization. In: Deeming, D.C. (Ed.), Reptilian Incubation: Environment, Evolution and Behaviour. Nottingham University Press, Nottingham.

Aubret, F., 2013. Heart rates increase after hatching in two species of natricine snakes. Sci. Rep. 3, 3384.

Aulie, A., Kanui, T.I., 1995. Oxygen consumption of eggs and hatchlings of the Nile crocodile (*Crocodylus niloticus*). Comp. Biochem. Physiol. A 1l2 (1), 99–102.

Bennet, A.F., 1972. The effect of activity on oxygen consumption,oxygen debt, and heart rate in the lizards *Varanus gouldii* and *Sauromalus hispidus*. J. Comp. Physiol. 79, 259–280.

Bevan, R.M., Keijer, E., Butler, P.J., 1992. A method for controlling the feeding behaviour of aquatic birds: heart rate and oxygen consumption during dives of different duration. J. Exp. Biol. 162, 91–106.

Birchard, G.F., Reiber, C.L., 1995. Growth, metabolism, and chorioallantoic vascular density of developing snapping turtles (*Chelydra serpentina*): influence of temperature. Physiol. Zool. 68 (5), 799–811.

Birchard, G.F., Reiber, C.L., 1996. Heart rate during development in the turtle embryo: effect of temperature. J. Comp. Physiol. B. 166, 461–466.

Birchard, G.F., Walsh, T., Rosscoe, R., Reiber, C.L., 1995. Oxygen uptake by Komodo dragon (*Varanus komodoensis*) eggs: the energetics of prolonged development in a reptile. Physiol. Zool. 68 (4), 622–633.

Booth, D.T., 1998. Effects of incubation temperature on the energetics of embryonic development and hatchling morphology in the Brisbane river turtle *Emydura signata*. J. Comp. Physiol. B. 168 (5), 399–404.

Booth, D.T., Thompson, M.B., Herring, S., 2000. How incubation temperature influences the physiology and growth of embryonic lizards. J. Comp. Physiol. B. 170 (4), 269–276.

Burggren, W.W., Pinder, A.W., 1991. Ontogeny of cardiovascular and respiratory physiology in lower vertebrates. Annu. Rev. Physiol. 53, 107–135.

Butler, P.J., Frappell, P.B., Wang, T., Wikelski, M., 2002. The relationship between heart rate and rate of oxygen consumption in Galapagos marine iguanas (*Amblyrhynchus cristatus*) at two different temperatures. J. Exp. Biol. 205, 1917–1924.

Clark, T.D., Butler, P.J., Frappell, P.B., 2006. Factors influencing the prediction of metabolic rate in a reptile. Funct. Ecol. 20, 105–113.

Crossley II, D.A., Altimiras, J., 2000. Ontogeny of cholinergic and adrenergic cardiovascular regulation in the domestic chicken (*Gallus gallus*). Am. J. Physiol. 279, 1091–1098.

Crossley II, D.A., Burggren, W.W., 2009. Development of cardiac form and function in ectothermic sauropsids. J. Morphol. 270, 1400–1412.

Crossley II, D.A., Hicks, J.W., Altimiras, J., 2003. Ontogeny of baroreflex regulation in the American alligator *Alligator mississippiensis*. J. Exp. Biol. 206, 2895–2902.

Currie, S.E., Körtner, G., Geiser, F., 2014. Heart rate as a predictor of metabolic rate in heterothermic bats. J. Exp. Biol. 217, 1519–1524.

Dmi'el, R., 1970. Growth and metabolism in snake embryos. J. Embryol. Exp. Morpholog. 23 (3), 761–772.

Du, W.-G., Radder, R.S., Sun, B., Shine, R., 2009. Determinants of incubation period: do reptilian embryos hatch after a fixed total number of heart beats? J. Exp. Biol. 212, 1302–1306.

Du, W.-G., Ye, H., Zhao, B., Warner, D.A., Shine, R., 2010. Thermal acclimation of heart rates in reptilian embryos. PLoS ONE 5, e15308.

Green, J.A., 2011. The heart rate method for estimating metabolic rate: review and recommendations. Comp. Biochem. Physiol. A 158, 287–304.

Groscolas, R., Viera, V., Guerin, N., Handrich, Y., Côté, S.D., 2010. Heart rate as a predictor of energy expenditure in undisturbed fasting and incubating penguins. J. Exp. Biol. 213, 153–160.

Hoyt, D.F., 1987. A new model of avian embryonic metabolism. J. Exp. Zool. 1987 (1), 127–138.

Klein, W., Perry, S.F., Abe, A.S., Andrade, D.V., 2006. Metabolic response to feeding in *Tupinambis merianae*: circadian rhythm and a possible respiratory constraint. Physiol. Biochem. Zool. 79 (3), 593–601.

Lierz, M., Gooss, O., Hafez, H.M., 2006. Non-invasive heart rate measurement using a digital egg monitor in chicken and turkey embryos. J. Avian Med. Surg. 20 (3), 141–146.

McPhee, J.M., Rosen, D.A.S., Andrews, R.D., Trites, A.W., 2003. Predicting metabolic rate from heart rate in juvenile Steller sea lions *Eumetopias jubatus*. J. Exp. Biol. 206, 1941–1951.

Nechaeva, M.V., Vladimirova, I.G., Alekseeva, T.A., 2007. Oxygen consumption as related to the development of the extraembryonic membranes and cardiovascular system in the European pond turtle (*Emys orbicularis*) embryogenesis. Comp. Biochem. Physiol. A 148, 599–610.

Piercy, J., Rogers, K., Reichert, M., Andrade, D.V., Abe, A.S., Tattersall, G.J., Milsom, W.K., 2015. The relationship between body temperature, heart rate, breathing rate, and rate of oxygen consumption, in the tegu lizard (*Tupinambis merianae*) at various levels of activity. J. Comp. Physiol. B. 185, 891–903.

Radder, R., Shine, R., 2006. Thermally induced torpor in fullterm lizard embryos synchronizes hatching with ambient conditions. Biol. Lett. 2, 415–416.

Sartori, M.R., Taylor, E.W., Abe, A.S., Crossley II, D.A., 2015a. An appraisal of the use of an infrared digital monitoring system for long-term measurement of heart rate in reptilian embryos. Comp. Biochem. Physiol. A 188, 17–21.

Sartori, M.R., Leite, C.A.C., Abe, A.S., Crossley, D.A., Taylor, E.W., 2015b. The progressive onset of cholinergic and adrenergic control of heart rate during development in the green iguana, *Iguana iguana*. Comp. Biochem. Physiol. A 188, 1–8.

Schmidt-Nielsen, K., 1984. Scaling: Why is Animal Size so Important? Cambridge University Press, New York

Taylor, E.W., Leite, C.A.C., Sartori, M.R., Wang, T., Abe, A.S., Crossley II, D.A., 2014. The phylogeny and ontogeny of autonomic control of the heart and cardiorespiratory interactions in vertebrates. J. Exp. Biol. 217, 690–703.

Tazawa, H., Whittow, G.C., 1994. Embryonic heart rate and oxygen pulse in two procellariiform seabirds, *Diomedea immutabilis* and *Puffinus pacificus*. J. Comp. Physiol. B. 163, 642–648.

Thompson, M.B., 1989. Patterns of metabolism in embryonic reptiles. Respir. Physiol. 76, 243–256.

Thompson, M.B., Russel, K.J., 1998. Metabolic cost of development in one of the world's smallest lizard eggs: implications for physiological advantages of the amniote egg. Copeia 1998 (4), 1016–1020.

Thompson, M.B., Russel, K.J., 1999. Embryonic energetics in eggs of two species of australian skink, *Morethia boulengeri* and *Morethia adelaidensis*. J. Herpetol. 33 (2), 291–297.

Thompson, M.B., Stewart, J.R., 1997. Embryonic metabolism and growth in lizards of the genus Eumeces. Comp. Biochem. Physiol. A 118 (3), 647–654.

Tosini, G., Menaker, M., 1995. Circadian rhythm of body temperature in an ectotherm (*Iguana iguana*). J. Biol. Rhythm. 10 (3), 248–255.

Tucker, V.A., 1966. Oxygen transport by the circulatory system of the green iguana, *Iguana iguana* at different body temperatures. J. Exp. Biol. 44, 77–92.

Vleck, C.M., Hoyt, D.F., 1991. Metabolism and energetics of reptilian and avian embryos. In: Deeming, D.C., Ferguson, M.W.J. (Eds.), Egg Incubation: Its Effects on Embryonic Development in Birds and Reptiles. Cambridge University Press, Cambridge.

Vleck, C.M., Vleck, D., 1987. Metabolism and energetics of avian embryos. J. Exp. Zool. Suppl. 1, 111–125.

Burgreen, W.W., Warburton, S.J., 1994. Patterns of form and function in developing hearts: contribution from nom-mammalian vertebrates. Cardioscience 5 (3), 183–191.

Whitehead, P.J., Seymour, R.S., 1990. Patterns of metabolic rate in embryonic crocodilians *Crocodylus johnstoni* and *Crocodylus porosus*. Physiol. Zool. 63, 334–352.

Young, B.L., Rosen, D.A.S., Hindle, A.G., Haulena, M., Trites, A.W., 2011. Dive behaviour impacts the ability of heart rate to predict oxygen consumption in Steller sea lions (*Eumetopias jubatus*) foraging at depth. J. Exp. Biol. 214, 2267–2275.

Zhang, Y.-P., Li, S.-R., Ping, J., Li, S.-W., Zhou, H.-B., Sun, B.-J., Du, W.-G., 2016. The effects of light exposure during incubation on embryonic development and hatchling traits in lizards. Sci. Rep. 6:38527. http://dx.doi.org/10.1038/srep38527.