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Incubation temperature and energy expenditure during development in loggerhead sea turtle embryos

Karen A. Reid^{a,*}, Dimitris Margaritoulis^b, John R. Speakman^a^a Institute of Biological and Environmental Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen, AB24 2TZ, Scotland^b ARCHELON, the Sea Turtle Protection Society of Greece, Solomou 57, GR-10432 Athens, Greece

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ABSTRACT

The choice of a suitable nest habitat by oviparous reptiles that deposit eggs into a nest and provide no subsequent parental care is likely to play a major role in the survival of the offspring. In particular variations in nest temperature may influence the rate at which embryos utilise their yolk energy.

The effects of nest temperature on total energy use are however complex. High temperatures may advance development and shorten the time to hatching, thereby reducing energy use, but they also stimulate metabolic rate increasing energy use. The net effect of temperature on total energy demands is therefore uncertain.

Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were measured by open-flow respirometry during the incubation of loggerhead sea turtle eggs at three temperatures (27.6, 30.0 and 31.8 °C).

At each temperature, VO_2 and VCO_2 showed a peak followed by a decline to hatching. Incubation temperature was negatively related to incubation duration and positively related to the maximum metabolic rate of the embryos. Peak VO_2 was 74.8 ml/egg/day at 27.6 °C, 91.9 ml/egg/day at 30.0 °C, and 97.9 ml/egg/day at 31.8 °C. Peak VO_2 occurred closer to hatching in eggs incubated at higher temperatures.

Total energy expenditure was greatest at the lowest incubation temperature and lowest at the highest temperature. Total VO_2 and VCO_2 were 1777 ml/egg and 1226 ml/egg, respectively, at 27.6 °C, 1680 ml/egg and 1235 ml/egg at 30.0 °C, and 1613 ml/egg and 1191 ml/egg at 31.8 °C. Using the actual RQ values, this corresponds to a cost of development of 34,963 J/egg at 27.6 °C, 33,403 J/egg at 30.0 °C, and 32,107 J/egg at 31.8 °C.

At all temperatures, the calculated respiratory quotient values did not suggest that yolk substrates were oxidised proportionately, but more likely indicated their sequential use.

Nest temperatures may play a key role in energy use, with cooler temperature nests increasing the overall energy demands placed on developing embryos.

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1. Introduction

In eutherian mammals, developing embryos behave as part of the maternal body and have similar metabolic rates (Kleiber, 1987). In birds, embryonic metabolism is determined partly by incubation temperature, regulated by the attending parent (Vleck and Hoyt, 1991). In contrast, the embryos of oviparous reptiles undertake variable proportions of development outside the maternal body, frequently unattended (Shine, 1991). Developmental rates are thus strongly influenced by conditions experienced at the nest site (Deeming and Ferguson, 1991; Packard, 1991; Vleck and Hoyt, 1991).

Theoretical models describing the energetic costs of embryonic development divide energy consumption into two components, the cost of new tissue synthesis and the cost of maintaining existing embryonic tissue (Vleck et al., 1980). These costs are fuelled by the production of adenosine triphosphate (ATP), obtained in reptiles through the oxida-

tion of yolk substrates during the course of respiration. Energy expenditure can therefore be quantified through the measurement of oxygen consumption (VO_2) and carbon dioxide production (VCO_2). The calorific value of 1 l of consumed oxygen can be calculated through the use of energy equivalent conversion factors, the values of which reflect the nature of the substrate oxidised. Although standard values may be used (e.g. Booth and Astill, 2001), it is also possible to calculate the conversion factors from respiratory quotients (RQ), determined by the ratio of VCO_2/VO_2 . From the RQ, the oxidation substrate (carbohydrate, protein or fat) can also be inferred (Schmidt-Nielsen, 1977). Assuming a standard or average energy equivalent presumes that yolk components are metabolised proportionately throughout incubation (Gettinger et al., 1984), which may not be true for all reptiles (Thompson and Stewart, 1997).

Variation in the incubation duration of developing reptiles is primarily determined by temperature (Deeming and Ferguson, 1991; Godfrey and Mrosovsky, 2001). In embryos of the loggerhead sea turtle *Caretta caretta*, a 1 °C rise in constant incubation temperature approximates to a reduction in incubation duration of about 8.5 days

* Corresponding author. Tel.: +44 7944193759.

E-mail address: karenanreid@hotmail.com (K.A. Reid).

in natural nests (Mrosovsky, 1980). Within the optimal range, the positive effects of temperature on rate constants mean embryonic development proceeds more quickly at higher temperatures, resulting in shorter incubation durations and higher rates of embryonic metabolism, due to higher biosynthesis and maintenance costs (Vleck and Hoyt, 1991). Conversely, embryos incubating at lower temperatures experience extended incubation durations with lower levels of metabolism. In the green turtle *Chelonia mydas*, the outcome of this trade-off between the rate of metabolism and the duration of incubation is that embryos incubating at different temperatures expend similar amounts of energy during development (Booth and Astill, 2001). However, in birds, longer incubation durations are associated with higher costs of development (Vleck and Hoyt, 1991), and in freshwater turtles, higher costs are incurred at incubation temperatures close to the range limits (Booth, 1998a). If energetic costs of development are higher, the size of the energy store available to support the newly emerged hatchling may be reduced. In sea turtles, this may be detrimental due to the high metabolic demands associated with hatching, emergence and dispersal (Kraemer and Bennett, 1981; Baldwin et al., 1989; Wyneken and Salmon, 1992; Clusella Trullas et al., 2006).

Three different patterns of oxygen consumption have been described during embryonic development in reptiles. Snakes typically show an exponential increase (Dmi'el, 1970), lizards show either a sigmoidal or a peaked pattern (Vleck and Hoyt, 1991; Birchard et al., 1995; Thompson and Stewart, 1997; Booth et al., 2000), and crocodylians show a peaked pattern (Whitehead and Seymour, 1990). Previous studies have drawn attention to the fact that both green turtles from Heron Island and leatherback sea turtles *Dermochelys coriacea* exhibit a peaked pattern of embryonic metabolism, where VO_2 first increases but then declines towards hatching (Booth and Astill, 2001; Thompson, 1993). This pattern also occurs in some freshwater turtles (Gettinger et al., 1984; Webb et al., 1986). However, Atlantic loggerhead turtles do not exhibit such a pronounced peak (Ackerman, 1981a), with oxygen consumption following a sigmoidal pattern demonstrated by a reduction in the rate of increase in VO_2 close to hatching. It is therefore possible that inter-specific differences in these patterns exist.

Understanding the micro-environmental factors which influence hatching success and hatchling survival can further our knowledge of the habitat requirements for successful sea turtle reproduction. From an evolutionary perspective, the effects of temperature on the sea turtle embryogenesis are of particular interest, as a number of phenotypic traits with the potential to influence reproductive fitness may be influenced by temperature, including hatchling sex determination (Yntema and Mrosovsky, 1982) and body size (Glen et al., 2003). During this study, we characterised the patterns of embryonic metabolism for loggerhead sea turtles from the Mediterranean nesting area of Kyparissia Bay, Greece (37.15°N, 21.40°E). Here incubation durations of the loggerhead sea turtle *Caretta caretta* show considerable variation (range 43–67 days; Margaritoulis et al., 2003), associated with variability in ambient temperatures (Rees and Margaritoulis, 2004). By artificially incubating eggs at three temperatures within the range typical of this population, we aimed to determine the effects of temperature on metabolic rate, and to consider whether biologically significant differences in the energetic costs of embryogenesis occurred.

2. Material and methods

2.1. Egg incubation

Eggs were incubated at three temperatures; 27.6 °C, 30.0 °C and 31.8 °C. Each incubator contained 3 boxes (hereafter referred to as Box 1, 2 or 3) of 10 eggs, thus enabling replication of measurements within each temperature treatment.

During the 2002 nesting season, 60 eggs were collected from a clutch laid on 9th August, and 30 eggs from a second clutch laid on

11th August. Eggs were removed from the nests at around 10 am on the morning after they were laid (Permit No. 105402/4148, Greek Ministry of Agriculture), and transported in a foam-lined box by air-conditioned car to the incubators. Within each incubator there were 3 separate boxes of 10 eggs. Each temperature treatment contained 2 boxes of 10 eggs from the first clutch and 1 box of 10 eggs from the second clutch.

The basic method of egg incubation was similar to Method 2 detailed in McLean et al. (1983). Eggs were brushed free of sand and weighed to the nearest 0.01 g on an electronic balance (PT 150, Sartorius, Gottingen, Germany). Ten eggs were set in each tupperware box (dimensions 350 × 250 × 100 mm) on a double layer of 10 mm thick foam. The box lids contained 20 ventilation holes (2 mm diameter) and boxes and foam had been previously cleaned with distilled water. The eggs were then packed with vermiculite (Silvapearl horticultural grade), which had been pre-soaked in distilled water and squeezed to remove excess water until a moist and crumbly texture was achieved. The top third of the eggs were left uncovered.

Incubators were constructed using 3 water baths (dimensions 310 × 620 × 290 mm, Grant Instruments, Cambridge, UK). A glass fish-tank (dimensions 260 × 480 × 350 mm) was fixed to each of the water bath bases using silicon. The fish-tanks were fitted with lids containing a ventilation hole (2 cm²) at one end. To minimise the probability of tank movement, weighted wooden platforms were erected over the lids. Water was added to the water baths to a depth of around 200 mm, and water temperatures were controlled using aquarium heaters (Rena Cal 50W, Rena France, Anney Cedex, France). A homogenous temperature was maintained through continual water circulation using aquarium filters (2006 internal filter, Eheim, Germany). The incubation temperature of the fish-tanks, and hence the egg boxes they contained, was therefore determined by the water temperature in the water bath surrounding the tank. By the second day of incubation white spot development was apparent on either the top or the side of the majority of eggs, indicating development was underway.

2.2. Incubation conditions

A temperature probe (Grant Instruments, Cambridge, UK) was inserted into one of the boxes in each incubator via the lid to allow regular monitoring (Squirrel temperature meter model SQ8-4U, Grant Instruments, Cambridge, UK). Tank temperatures were also logged at hourly intervals in the centre of each tank (outside the egg boxes) using a pre-calibrated Tiny-Tag datalogger (Gemini Dataloggers, Cambridge, UK). Calibration was performed by positioning the dataloggers inside a Styrofoam box within an incubator. Accuracies of the dataloggers were checked against a UKAS certified thermometer over a range of temperatures between 20.0 and 35.0 °C. We aimed to incubate at 28.0, 30.0 and 32.0 °C. Actual mean temperatures (±SD) recorded inside each tank were 27.6 °C (±0.4), 30.0 °C (±0.5) and 31.8 °C (±0.4). Due to space constraints, it was not possible to position dataloggers inside the egg boxes during incubation.

Heaters and ventilation holes were located at opposite ends of the water bath. Weekly rotations of box positions were carried out to minimise potential position effects within each treatment. Distilled water was added in 5 ml aliquots periodically to each egg box to counteract evaporative water loss.

2.3. Incubation duration

The start of incubation was defined as 11 am in the morning after the eggs were laid, the approximate time they were set in the incubators. When the anticipated hatching date approached, incubators were checked at least twice daily, and the numbers of hatchlings hatched or in the process of hatching were recorded.

2.4. Hatching success and observation of hatchlings

Following complete emergence of the hatchling from the egg, empty shells were removed to provide more space. External yolk sac contents typically required 12–24 h for complete absorption. The following morning hatchlings were transferred to nesting beach sand (dampened with distilled water) and maintained at 30.0 °C, undisturbed and under darkened conditions, until evening. Hatchling straight carapace length and widths were recorded (Vernier callipers, AR/CAL 6901-SE, Camlab, Cambridge, UK), together with body mass to the nearest 0.01 g by electronic balance. Prior to the release vertebral carapace scutes were counted to assess the frequency of developmental abnormalities at each temperature. The normal arrangement is 5 vertebral scutes (Pritchard and Mortimer, 1999). All hatchlings were then released on to the nesting beach.

2.5. Respirometry

VO₂ and VCO₂ were measured with an open-flow respirometry system (Arch et al., 2006). Air was drawn from outside the building and dried over silica gel, passed into a pump, re-dried and passed through a Wright's flow meter (Zeal Group, London, UK), where air again became saturated with water. The air then passed into the respirometry chamber containing the eggs. During respirometry the eggs remained in boxes but the lids were removed. The respirometry chamber (a polycarbonate IP67 standard enclosure, 250×175×150 mm) was submerged in a water bath maintained at the same temperature that the eggs were incubated. On leaving the enclosure, the air was re-dried and passed through a Servomex Xentra 4100 gas purity analyser, where O₂ and CO₂ concentrations were logged at 60-second intervals over a period of 330 min. Flow rate was maintained at around 200 ml/min. O₂ and CO₂ values were corrected for flow rate and flow temperature (recorded at the beginning and end of each measurement trial), and air pressure (recorded at hourly intervals at Pyrgos (37.40 °N, 27.27 °E) weather station by the Hellenic National Meteorological Service).

Mean respirometry chamber temperature (calculated over 15 measurement trials) for the eggs incubated at 27.6 °C was 27.1 °C, for the eggs incubated at 30.0 °C was 29.6 °C ($n = 25$ trials) and for the eggs at 31.8 °C, the chamber was a mean 30.9 °C ($n = 27$ trials). The relative humidity of the respirometry chamber ranged from 40–70% on the substrate surface ($n = 67$ trials) and was greatest at lower temperatures.

Prior to the experiment we determined through measurements of the control boxes (containing only the incubation medium) that no change in VO₂ or VCO₂ occurred. The system O₂ sensor was calibrated at 3-week intervals, and CO₂ was spanned prior to each respirometry measurement. Before adding the eggs and following their removal, baseline readings of O₂ and CO₂ were recorded for 15 min after the system had equilibrated. Due to the low flow rate, we calculated that the chamber required 30 min to equilibrate, and have excluded 60 min following the addition of the eggs from the analyses.

2.6. Calculation of energy expenditure

From day 11 of incubation, we aimed to carry out three respirometry measurements each day, using one box of eggs (e.g. Box 1), from each temperature treatment. In total 33 respirometry measurements were performed on eggs incubating at 27.6 °C, and 29 measurements were made from both the 30.0 and 31.8 °C treatments. To calculate total energy expenditure we needed to know the metabolism on each day throughout incubation, but we only measured metabolism on average every third day. On days respirometry was not carried out, VO₂ and VCO₂ were therefore calculated by linear interpolation of the values on either side. If more than one box from a given temperature was measured on a single day, the average of these values was used.

Data were processed using customised software written in-house, and VO₂ and VCO₂ calculated as the lowest 10 minute period recorded

in ml/min during a given respirometry measurement (see Arch et al., 2006 for calculations). These values were converted to ml/egg/day. Daily energy expenditure (DEE) of the developing embryos was estimated by multiplying the VO₂ by an energy equivalent conversion factor (see below) and converted to Joules. At each temperature, total energy expenditure was calculated by summing the DEE across the entire incubation. Three different energy equivalents were used in the calculation of DEE, described as follows:

Method 1

When protein metabolism occurs, the use of a fixed RQ of 0.8 (producing an energy equivalent of 20.1 J/ml⁻¹ O₂) has been suggested (Brody, 1945; McDonald, 1976; Gessaman and Nagy, 1988). Energy expenditure was therefore calculated as:

$$\Sigma VO_2(\text{ml / egg / day}) * 20.1$$

Method 2

Following the methodology adopted in previous studies (e.g. Booth and Astill, 2001) an energy equivalent of 19.7 J/ml⁻¹ O₂ was used. Total energy expenditure at each incubation temperature was calculated using the following Equation:

$$\Sigma VO_2(\text{ml / egg / day}) * 19.7$$

Method 3

Through the measurement of VO₂ and VCO₂, RQs were determined following each respirometry measurement using the equation: RQ = VCO₂/VO₂ (Kleiber, 1987). Actual energy equivalents in kcal (K) were calculated from these RQ values using the Weir Equation (Weir, 1949): $K = 3.941 + (1.1 \text{ (RQ)})$ and converted to Joules (4.1868 kJ/kcal; Bartholemew, 1982). Energy expenditure was then calculated as:

$$\Sigma VO_2(\text{ml / egg / day}) * K$$

3. Results

3.1. Incubation duration

High, low and intermediate temperatures generated markedly different incubation durations, higher temperatures resulting in shorter incubation durations (Table 1). Hatching was asynchronous. Mean incubation durations for each temperature were calculated from incubation durations for individual eggs in all three boxes, except at 31.8 °C where results were taken from Box 3 only. At this temperature, Boxes 1 and 2 hatched between 21/09/02 18:35 and 23/09/02 06:10, therefore incubation durations could not be accurately calculated.

3.2. Hatching success and hatchling body size

At each incubation temperature 1 of the 30 eggs failed to hatch, giving a hatching success rate of 96.7%. Unhatched eggs were opened at 68–70 days incubation, and showed no visible signs of embryonic development. As these eggs probably made a negligible contribution to total respiration, calculations of VO₂ and VCO₂ for these boxes were based on 9 rather than 10 eggs. Summary statistics for egg and hatchling measurements are detailed in Table 1. A significant clutch effect on the initial egg mass was apparent, with eggs from Clutch 2 (Box 3, mean 31.86 g) having significantly heavier eggs than Clutch 1 (Boxes 1 and 2, mean 29.57 g; one-way ANOVA, $F = 61.99$, $p < 0.001$, $n = 87$ eggs). Between temperature treatments there were no significant differences in egg mass ($F = 0.20$, $p > 0.05$, $n = 87$).

Analyses of temperature effects on hatchling body size were carried out using General Linear Modelling, with temperature treatment as a factor and clutch as a nested factor. Neither factor had a significant effect on hatchling body mass ($n = 87$ hatchlings). Clutch had no effect on carapace length, but hatchlings from lower temperatures had slightly

Table 1

Mean (\pm SD) values for initial egg mass and body mass, carapace length and carapace width for hatchlings, and incubation duration for each temperature treatment.

	Incubation temperature		
	27.6 °C	30.0 °C	31.8 °C
<i>Egg and hatchling size parameters</i>			
Mean egg mass (g)	30.48 (\pm 1.62)	30.21 (\pm 1.65)	30.31 (\pm 1.79)
Mean hatchling mass (g)	16.74 (\pm 0.82)	16.72 (\pm 1.02)	16.59 (\pm 0.90)
Mean hatchling carapace length (cm)	4.29 (\pm 0.09)	4.24 (\pm 0.10)	4.22 (\pm 0.10)
Mean hatchling carapace width (cm)	3.26 (\pm 0.08)	3.20 (\pm 0.10)	3.19 (\pm 0.11)
<i>Incubation duration</i>			
Mean incubation duration (days)	62.53 (\pm 0.51)	49.08 (\pm 0.53)	44.87 (\pm 0.66)
<i>Peak VO₂ and VCO₂</i>			
Peak VO ₂ (ml/egg/day)	74.8	91.9	97.9
Peak VCO ₂ (ml/egg/day)	51.6	73.8	73.3
Time of peak (% incubation)	77.8	79.6	86.7
<i>Total VO₂ and VCO₂</i>			
VO ₂ (ml/egg)	1777 ml	1680 ml	1613 ml
VCO ₂ (ml/egg)	1226 ml	1235 ml	1191 ml
<i>Total energy expenditure</i>			
Method 1 (J/egg)	35714 J	33760 J	32431 J
Method 2 (J/egg)	35003 J	33088 J	31786 J
Method 3 (J/egg)	34963 J	33403 J	32107 J

Summary statistics for respirometry results are also shown, including peak and total oxygen consumption and carbon dioxide production at each temperature, when the peak occurred (as a % of the total incubation), and total energy expenditure as calculated by Methods 1, 2 and 3.

longer carapaces ($F=4.51$, $p=0.014$, $n=87$). Hatchlings from lower temperatures also had greater carapace widths ($F=6.52$, $p=0.002$, $n=87$), and hatchlings from Clutch 1 had wider carapaces than those from Clutch 2 ($F=6.35$, $p=0.014$, $n=87$). Supernumerary accounted for all abnormalities in the vertebral carapace scutes, and the frequency increased with the incubation temperature. At the highest temperature treatment 14.3% hatchlings had more than 5 scutes ($n=4/28$ hatchlings), at the intermediate temperature 6.9% ($n=2/29$) and at the lowest temperature 3.4% ($n=1/29$). Abnormalities occurred in both clutches.

3.3. Oxygen consumption and carbon dioxide production

Patterns of VO₂ and VCO₂ are detailed for each incubation temperature in Fig. 1A and B respectively. Fitting a polynomial regression line to the data indicated a rapid increase in VO₂ and VCO₂ approximately half way through incubation towards a maximum, beyond which a decline occurred producing a peaked pattern. Maximum levels of VO₂ and VCO₂ increased with incubation temperature, and occurred progressively later in the incubation at higher temperatures (Table 1). This meant that the peak occurred about 6 days before hatching at 31.8 °C, 10 days before at 30.0 °C and 14 days before hatching at 27.6 °C. Over the whole incubation duration, total VO₂ was greatest at 27.6 °C and least at 31.8 °C, with an intermediate volume at 30.0 °C (Table 1).

3.4. Energy expenditure

Total energy expenditures over the course of development for each temperature, as determined by Methods 1–3, are detailed in Table 1. Regardless of the method used, embryos developing at the lowest incubation temperature experienced greatest energy expenditure, and those developing at the highest temperature, the lowest. Energy expenditure was on average 10.0% higher for embryos developing at 27.6 °C than 31.8 °C, and 5.8% higher than at 30.0 °C. When energy equivalents were calculated from actual RQ values, the difference between the high and low temperature treatments was slightly less (8.9%).

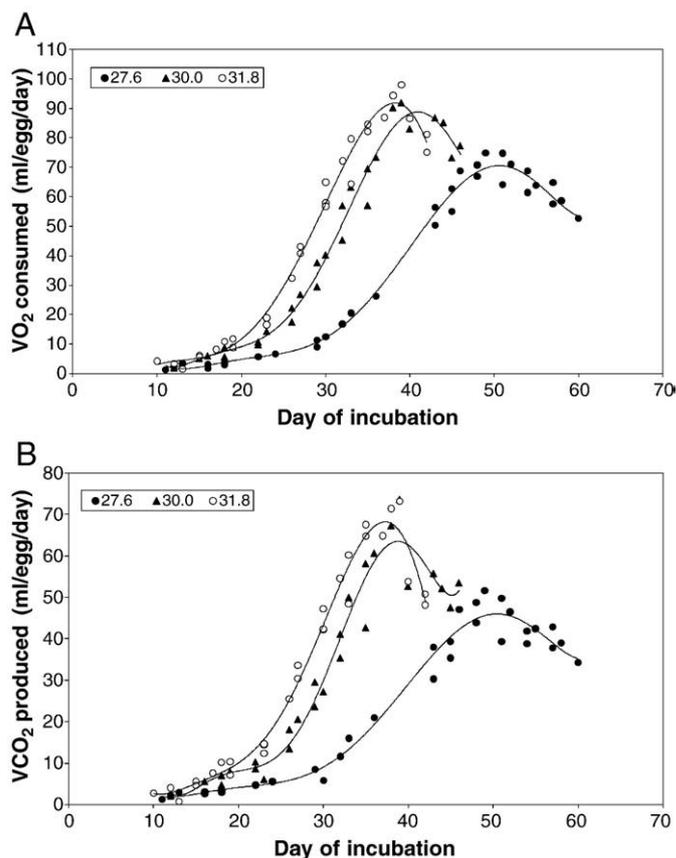


Fig. 1. (A) Oxygen consumption (ml/egg/day) and (B) carbon dioxide production by developing embryos incubated at 27.6, 30.0, and 31.8 °C followed a peaked pattern.

3.5. RQ values and patterns of substrate oxidation

Around 30% of the RQ values were found to be outside the normal range of 0.7–1.0, with abnormal values occurring primarily at the start ($RQ>1$) and end ($RQ<0.7$) of the measured period of incubation. Fig. 2 illustrates actual RQ values ($n=86$) calculated for each respirometry measurement, and shows an apparent decline in RQ over time. To provide more insight into the nature of this decline we used General Linear Modelling, with \log_e RQ as the response variable, day as a covariate, temperature as a factor and the interaction effect between these two variables. RQ was not related to temperature ($F=1.20$, $p>0.05$) and there was no interaction effect between the temperature and the day of incubation on RQ ($F=2.50$, $p>0.05$). A significant effect of day was found, showing a decline in RQ as the incubation progressed ($F=207.43$, $T=-14.40$, $p<0.001$, $n=86$). As no temperature effect was detectable, RQ values from each temperature treatment were pooled for all subsequent calculations.

Quadratic regression confirmed that day was a significant predictor of RQ ($r^2=0.76$, $F=133.304$, $p<0.001$, $n=86$), suggesting a temporal shift in the nature of the substrate oxidation that may have occurred. From the existing dataset, using daily averages where appropriate, fitted RQ values were generated for days 1–63 of incubation. Between days 1–11 RQ values exceeded 1, declining to 0.81 by day 26, and 0.71 by day 38. Fitted RQ values were below 0.70 for the remainder of the incubation.

4. Discussion

When laid, reptile eggs contain energy deposited in the yolk. Most of this energy will be converted to the hatchling, and a proportion will be used to support the energetic costs of development (Vleck and

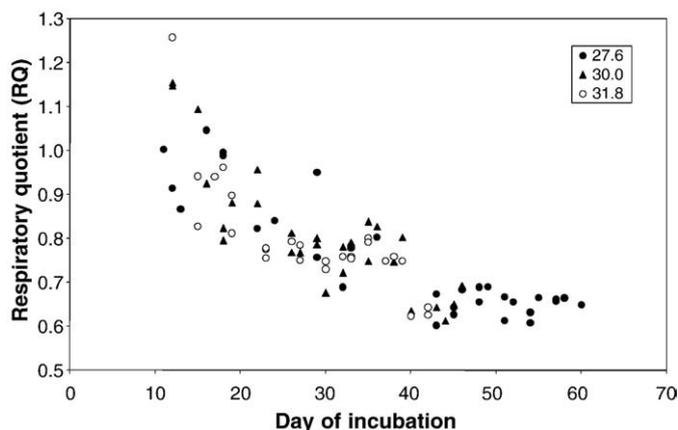


Fig. 2. Calculated RQ for days on which VO_2 and VCO_2 were measured in eggs incubated at 27.6, 30.0 and 31.8 °C.

Hoyt, 1991). Any yolk remaining at the time of hatching provides a supply of energy to the hatchling until it can feed independently and may also, in some reptile species, contribute towards post-hatching growth (Troyer, 1983). In sea turtles, the choice of nest site may affect a number of phenotypic traits. During this study we have determined the effects of incubation temperature on energy expenditure during loggerhead sea turtle development, thus allowing consideration of this variable within the context of other traits potentially affecting reproductive fitness.

4.1. Hatchling body size and developmental abnormalities

Hatchlings incubated at lower temperatures had slightly longer and wider carapaces, but we found no differences in body mass. In birds and oviparous reptiles, the frequency of developmental abnormalities tends to be more common at incubation temperatures towards the extremities of their range (Deeming and Ferguson, 1991). Here we have demonstrated that the incidence of abnormalities in hatchling carapace scute patterns increased at higher incubation temperatures. Miller (1985) noted that supernumerary was one of the most common morphological abnormalities in sea turtles, but typical frequencies were lower than observed here.

4.2. Patterns of VO_2 and VCO_2

Embryos had low rates of VO_2 and VCO_2 during the first half of incubation, the period coinciding with the formation of the basic body plan (Miller, 1985). During the second half of incubation, a rapid increase in metabolism towards a peak occurred, presumably reflecting higher embryonic growth rates which incur greater biosynthesis and maintenance costs (Ackerman, 1981b; Booth and Astill, 2001). This pattern is typical of sea turtle embryos (Ackerman, 1981a; Thompson, 1993; Booth and Astill, 2001). Beyond the peak, patterns of VO_2 and VCO_2 at all temperatures declined towards hatching. In this respect, embryos behaved similarly to both green turtle embryos from Heron Island (Booth and Astill, 2001) and leatherback turtle embryos (Thompson, 1993). The peaked pattern has not previously been described for the loggerhead turtle – earlier studies found that VO_2 in both loggerhead and green turtles (incubated and measured at 30.0 °C) did not demonstrate a marked decline towards hatching, despite a moderate decline in the embryonic growth rate (Ackerman, 1981a).

The peaked pattern was shown at all temperatures, but the details differed slightly according to the incubation temperature. We recorded a more marked decline in VO_2 and VCO_2 to hatching at higher incubation temperatures, as has been previously observed in the VO_2 of two lizard species (Booth et al., 2000) and the Brisbane river turtle *Emydura signata* (Booth, 1998b).

Asynchronous rates of sea turtle development may occur due to the thermal gradients within the nest (Houghton and Hays, 2001). Thompson (1993) proposed that the peaked pattern of oxygen consumption might arise because, although embryos had effectively stopped growing, delayed hatching might be beneficial in encouraging hatching synchrony. Our study does not lend strong support to this theory as, despite the peaked pattern we have observed, studies on the emergence patterns of the loggerhead turtles in Kyparissia Bay (Rees, 2005) and the nearby island of Cephalonia (Houghton and Hays, 2001) have described them as asynchronous.

Dietz et al. (1998) proposed that, in the bird embryos, a plateau in metabolic rate is achieved close to the end of incubation in response to the increased synthesis efficiency, rather than because of a markedly reduced embryonic growth rate. Dietz et al. (1998) suggested that the plateau in energy expenditure corresponded with a period of fat deposition in the embryo. If the timing of fat deposition was similar in the loggerhead embryos, increased synthesis efficiency may have been achieved through the oxidation of fat, as suggested by the RQ values.

4.3. Costs of development

Regardless of the method used to calculate energy expenditure, the same general conclusion for this study can be drawn. Despite higher rates of VO_2 at higher incubation temperatures, a greater amount of total energy is required for the incubation at lower temperatures. This conclusion parallels findings in the bird embryos, and probably occurs because slower growth and longer incubation durations lead to increased tissue maintenance costs (Vleck and Vleck, 1987).

The difference in oxygen consumption between 31.8 and 27.6 °C was around 10%, or equivalent to about 3217 J. Assuming a post-hatching oxygen consumption rate of 0.16 $mL O_2/g/min$ (Wyneken, 1997) is similar across all incubation temperatures, this additional energy could sustain hatchlings from higher temperatures for approximately 10 additional hours. Booth and Astill (2001) found the total oxygen consumption of green turtle embryos at 26.0 °C was approximately 5% greater than at 30.0 °C. In *Emydura signata*, Booth (1998a) found an 11% increase in oxygen consumed at 24 °C, but no significant difference between 26, 28, and 31 °C. It was concluded that higher costs at 24 °C occurred as this was close to the lower critical temperature at which development could proceed. It is possible that a constant temperature of 27.6 °C is close to the lower limits of incubation temperature for the loggerheads in Kyparissia Bay. Certainly the corresponding incubation duration (62.5 days) approaches the maximum reported for this area (67 days, Margaritoulis et al., 2003). Assuming egg composition does not vary seasonally, the implications from our findings are that hatchlings emerging from the lower incubation temperatures would be expected to have less energy available in the form of residual yolk. Although in this study the effect of the incubation temperature on hatchling carapace dimensions was small, and no significant effect on the hatchling body mass was found, the incubation temperature has been widely reported to influence the hatchling phenotype. For example, green turtle hatchlings developing on cooler nesting beaches on Ascension Island are longer, heavier, and have greater hind and fore-flipper areas than those developing on warmer beaches (Glen et al., 2003), the characteristics which may influence swimming ability. Consequently available energy resources for offshore swimming may interact with hatchling phenotype to impact fitness.

The method used to calculate energy expenditure influenced the result to a small extent. Recent studies have favoured the use of a fixed RQ value to calculate energy expenditure (e.g. Booth and Astill, 2001), which given the apparently complex nature of substrate metabolism would appear to currently provide the most reliable estimate. Gessaman and Nagy (1988) assessed the amount of error incurred by failing to correct for the metabolism of protein. When measured or assumed RQ values were used to calculate energy equivalents and

energy expenditure for ureotelic animals oxidising mixed substrates, they estimated that the maximum error incurred was less than 3%, providing the carbohydrate contribution was low.

Despite similar incubation durations, it is notable that the (mass-specific) peak rate of oxygen consumption was higher, and total costs of development at 30.0 °C more expensive, in the current study than that previously reported for the loggerhead turtles (Ackerman, 1981a). When energy expenditure was calculated (assuming an energy equivalent of 19.7 J/mlO₂) and scaled to the initial egg mass, the total was 22% higher in this study. It is unclear whether this infers that the peaked pattern is more expensive, or reflects inter-population differences in the rate of the oxygen consumption, as has been previously suggested for the green turtle (Booth and Astill, 2001). If production costs are scaled to the mass of the hatchling produced, it is apparent that eggs in our study produced relatively large hatchlings, reducing the difference to 14%.

The mean energy content of a green turtle egg from Costa Rica is 259.7 kJ (Bjorndal, 1995). Assuming an energy equivalent of 19.7 J/ml O₂, an embryo from this egg would therefore use about 61.9 kJ, or 24%, of the total energy during development (Ackerman, 1981a). Hays and Speakman (1991) estimated the energy content of an average sized Greek loggerhead egg to be 165 kJ, presuming that the volume-specific energy content of an egg has little inter-specific or inter-population variation. Assuming an energy equivalent of 19.7 J/ml O₂, our results suggest that Kyparissia Bay embryos expend between 19 and 22% of this energy on developmental costs.

4.4. RQ values and patterns of substrate oxidation

The significant decline in RQ during the course of incubation would suggest that the energy sources within the egg were not oxidised proportionately throughout incubation. Although determining the RQ does not allow us to ascertain what the relative proportions of each substrate might be, one possible interpretation is that carbohydrate was oxidised initially, followed by protein and ultimately fat. The use of protein as a metabolic substrate in loggerhead embryos has been previously indicated through the measurement of urea excretion during incubation (Nakamura, 1929; cited from Wilhoft, 1986).

An alternative scenario is that the declining RQ indicates a shift from carbohydrate to fat oxidation. If either of these scenarios were correct, we could speculate that the temporal effect on the nature of the oxidation substrate would result in embryos with longer incubation durations deriving a greater amount of energy from the oxidation of fat.

High RQ values at the start of incubation could be explained by low O₂ conductance due to the higher water content of the eggshell during early incubation (Deeming and Thompson, 1991). Thompson and Stewart (1997) also recorded high RQ values (greater than 1) at the start of the incubation in the lizard *Eumeces fasciatus*, and suggested this may be due to the secretion of the CO₂ stored during formation of the eggshell. The authors determined that during the 25-day incubation period, lizard embryos demonstrated an initially high RQ, which declined until day 15 then levelled to a mean 0.75, suggesting a combination of protein and fat oxidation.

Towards the end of the incubation, we recorded RQ values below that expected from the oxidation of fat. Possible explanations include CO₂ retention or the synthesis of carbohydrate from fat (Kleiber, 1987). Glycogen formation has been a suggested explanation for the low RQ values obtained from the bird embryos during late incubation (Freeman, 1969). Although glycogen comprises a more bulky form of energy storage, it is a means by which energy can become rapidly available, and can be used to provide energy under anaerobic conditions (Schmidt-Nielsen, 1977). During hatching and emergence from the nest the availability of such an energy source would clearly be beneficial.

During this study eggs were incubated under artificial conditions using constant temperatures. Sea turtle eggs incubating under natural

conditions may be subjected to fluctuating temperatures, for example as a result of sea water over-wash, rainfall or diel patterns (Kaska et al., 1998, Broderick et al., 2001, Houghton et al., 2007). The implications of these temperature excursions for the metabolic rate of naturally incubating sea turtle eggs are unknown, but the results presented here probably still have wide applicability. We also acknowledge that during this experiment measurements were made using open-flow respirometry, and eggs were therefore not limited by available oxygen. These conditions may differ to the natural nest environment, where eggs incubate in a sand medium where gas diffusion rates may be reduced to 6–12% that of air (Ackerman, 1977). Our results therefore cannot predict the outcome of the combined effects of the incubation temperature and the limited oxygen diffusion rates on the embryonic metabolism in natural nests.

As lower incubation temperatures generate male embryos, we cannot distinguish between the sex and the temperature effects, or exclude the possibility that development at lower temperatures was more costly due to the production of the male hatchlings. The pivotal temperature for the loggerhead population in Kyparissia Bay is 29.3 °C (Mrosofsky et al., 2002), indicating the lowest incubation temperature used here produced primarily males, the highest temperature mainly females, and the intermediate temperature probably a mixture of both sexes. Results from the snapping turtle *Chelydra serpentina* suggest that it is temperature, rather than sex, which affects the amount of the residual yolk in emerging hatchlings (Rhen and Lang, 1999). In our study, regardless of the causative factor, it is apparent that developmental costs for a male embryo are more expensive. The biological significance of the subtle differences in energy utilisation and body size suggested by our study will depend on whether these translate into viability differences, during early or later life-history stages.

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