RESEARCH ARTICLE

Gestation increases the energetic cost of breathing in the lizard *Tiliqua rugosa*

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SUMMARY

High gestational loads result in fetuses that occupy a large proportion of the body cavity and may compress maternal organs. Compression of the lungs results in alterations in breathing patterns during gestation, which may affect the energetic cost of breathing. In this study, the energetic cost of breathing during gestation was determined in the viviparous skink *Tiliqua rugosa*. Radiographic imaging showed progressive lung compression during gestation and a 30% reduction in the lung inflation index (rib number at which the caudal margin of the lung was imaged divided by total rib number). Pneumotachography and open flow respirometry were used to measure breathing patterns and metabolic rates. Gestation induced a twofold increase in minute ventilation *via* increases in breathing frequency, but no change in inspired tidal volume. The rates of O_2 consumption and CO_2 production did not change significantly during gestation. Together, these results suggest that a relative hyperventilation occurs during gestation in *T. rugosa*, which in turn suggests that diffusion and/or perfusion limitations may exist at the lung during gestation. The energetic cost of breathing was estimated as a percentage of resting metabolic rate using hypercapnia to stimulate ventilation at different stages of pregnancy. The energetic cost of breathing in non-pregnant lizards was 19.96±3.85% of resting metabolic rate and increased threefold to 62.80±10.11% during late gestation. This significant increase in the energetic cost of breathing may have significant consequences for energy budgets during gestation.

Key words: ventilation, breathing pattern, oxygen consumption, hypercapnia, reptile, lung.

INTRODUCTION

Lungs are highly plastic organs: lung volumes change during the normal breathing cycle and are subject to compressive forces from nearby organs, such as the liver and the gastrointestinal tract. Compression of the lung is likely to alter the pressure required to achieve normal tidal volume and the energetic cost of lung ventilation. The shingleback lizard, *Tiliqua rugosa*, experiences significant lung compression during gestation and thus is used in this study as a model to investigate the effects of lung compression on ventilation and the energetic cost of breathing.

Tiliqua rugosa is a large, viviparous skink that inhabits vast areas of inland Australia. This species gives birth to one to four large young after 4–6 months gestation. The expansion of the body wall to accommodate the developing embryos may be limited in this species because of the presence of thick, ossified scales. The developing embryos occupy a large proportion of the body cavity and can compress and regionally collapse the lungs during gestation (Munns and Daniels, 2007).

The unicameral lungs of this and other scincid and agamid species are large and baglike, lacking the higher degree of internal compartmentalization characteristic of paucicameral and multicameral lungs (Perry, 1989). The lack of a muscular diaphragm and both post-pulmonary and post-hepatic septa in skinks (Klein and Owerkowicz, 2006) means that the lungs can expand to occupy a large portion of the body cavity. Being highly distensible, the lungs are subject to distortion and compression from surrounding internal organs such as the liver and gastrointestinal tract (Daniels et al., 1994), and from the developing embryos during gestation (Munns and Daniels, 2007). Thus, the spatial requirements of the developing embryos and adequate maternal lung expansion may conflict, especially during late gestation. The resulting lung compression during gestation can alter breathing patterns, decreasing tidal volume and minute ventilation in the two weeks preceding parturition (Munns and Daniels, 2007).

Reptilian breathing patterns normally consist of ventilatory periods, made up of single or multiple breaths, interspersed with breath holds (non-ventilatory periods) of variable duration (Milsom, 1988). Breathing patterns are highly plastic, with alterations in minute ventilation being achieved by alterations in tidal volume, breathing frequency and the duration of the non-ventilatory pause, either independently or in combination. The mechanical act of ventilation is a muscular activity and as such incurs an energetic cost. For any given minute ventilation, there is an optimum combination of tidal volume and breathing frequency at which the energetic cost of breathing is minimized (Milsom, 1989; Perry, 1989; Perry and Duncker, 1980). The mechanical work of ventilation increases in direct proportion with breathing frequency, but increases with the square of tidal volume (Milsom and Vitalis, 1984; Perry, 1989). As a result, increases tidal volume are a more energetically costly option for increasing minute ventilation compared with the same change in minute ventilation achieved *via* increases in breathing frequency.

Gestation-induced alterations in breathing patterns, and the likely decrease in lung compliance associated with gestational lung compression, may significantly alter the energetic cost of breathing during pregnancy. An increase in the energetic cost of breathing may have importance ramifications for the energy budgets of pregnant females.

The energetic cost of breathing cannot be measured directly; instead, estimates of the energetic cost of breathing (as a percentage of resting metabolic rate) have been made in a small number of reptiles. Estimates of the cost of breathing in reptiles range from 1 to 52%: 1–15% in hatchling alligators (Wang and Warburton, 1995), up to 17% for fasted and digesting tegu lizards (Skovgaard and Wang, 2004), 52% in dormant tegus (de Andrade and Abe, 1999) and 1–30% in chelonians (Jackson et al., 1991; Kinney and White, 1977). In contrast, most mammals have relatively low energetic costs of breathing, averaging between 1 and 7% (Milsom, 1989; Milsom, 1995). However, the energetic cost of breathing may increase significantly in some circumstances, for example, disease states such as chronic obstructive pulmonary disease (emphysema, chronic bronchitis or a mixture of both) in humans (Dellweg et al., 2008; Jounieaux and Mayeux, 1995). The energetic cost of breathing in human emphysema patients has been estimated at 23.1% at rest and 55.5% during exercise (Takayama et al., 2003), and the oxygen consumption of the respiratory muscles has been shown to increase 28-fold in emphysema patients during maximal ventilation (Campbell et al., 1957). Extremely high costs of breathing have been found in hibernation squirrels with estimates of 90% of resting metabolic rate (Garland and Milsom, 1994). The aim of this study was to determine the energetic cost of breathing during pregnancies with high gestational loads in the viviparous skink *T. rugosa*.

MATERIALS AND METHODS
Animals
Shingleback lizards [*Tiliqua rugosa* (Gray 1825)] were collected from the Burra region of South Australia, and a breeding colony was established at James Cook University. The animals were housed with a seasonally variable thermal gradient (5–15°C winter and 20–38°C summer), full-spectrum lighting (14 h:10 h light:dark) and free access to water, and were fed a diet of mixed fruit and vegetables, tinned cat food, boiled eggs, and vitamin and mineral supplements (ReptiCal and Herptivite, Beaphar Australia, Eagle Farm, QLD, Australia). Five gravid and seven non-gravid females were used in this study. Body mass ranged from 688.9 to 1034.6 g (mean ± s.e.m., 989.6±76.8 g) in the non-pregnant females and from 692.1 to 922.2 g (mean ± s.e.m., 809.9±64.1 g) in the gravid females during the late gestational period. Six male lizards were used for radiographic imaging only. Lizards were sexed by eversion of the hemipenes.

Radiography
Radiographs were obtained between 35 and 44 days and 2–7 days prior to birth and 2–3 days post-birth in four lizards. Radiographs were also obtained from seven non-pregnant females and six males over the same time period. Lizards were fasted for 3 days and then slowly cooled to ~20°C, wrapped loosely in cloth to discourage movement and placed in ventral recumbency. Optimal soft-tissue contrast was achieved using a peak kilovoltage (kVp) of 55–60, a tube current giving an amperage of 200 mA, and a time selection of 32 ms^{-1}, resulting in a radiographic exposure of of 6.3 mAs (Shimadzu general unit, Kyoto, Japan, and digital detector plate, Canon CXDi-50G, Kyoto, Japan). Radiographs were used to determine the maximum body width in the week prior to birth and in the week after birth, and differences were analysed with a paired *t*-test (*P*<0.05). The lung margin was determined from the difference in radio-opacity, with the lungs being less opaque than the surrounding abdominal contents. The lung inflation index was calculated by determining the rib number (counted from the most cranial rib in a caudal direction) at which the most caudal margin

of each lung was imaged by the total rib number. A lung inflation index of 1 represents lungs that completely spanned the length of the trunk and an index of 0 represents completely collapsed lungs. Thus a decrease in the lung inflation index indicates that the caudal lung margin is located at a more cranial rib number as a result of increased lung compression. The long end-inspiratory pauses in this species' breathing pattern, especially when at low body temperatures of 20°C, ensured that all radiographs were taken after the lizards had inspired. Although this method cannot assess the degree of dorso-ventral lung compression caused by the developing fetuses or by the displacement of other internal organs such as the intestines, it may be a useful tool in the early determination of pregnancy in this species.

Computerised axial tomography scans
Serial computerised axial tomography (CT) scans (kVp 120.0, 50.0 mA, 500 m s^{-1}, 75 mAs and slice thickness 0.5 mm) were taken of one pregnant (18 h prior to birth) and one non-pregnant female from which three-dimensional images were reconstructed. Lizards were slowly cooled to a temperature of 20°C, loosely wrapped in a cloth and placed directly on the scanner bed. Lizards were observed *via* monitors and remained still during the scanning procedure.

Measurement of lung ventilation and gas exchange
Breathing patterns were measured non-invasively using pneumotachography (Glass and Johansen, 1979) using techniques similar to those used in previous studies (Munns and Daniels, 2007; Munns et al., 2004; Munns et al., 2005). A small lightweight mask constructed from the end of a 20 ml syringe barrel was fitted over the lizard's nostrils and attached (and the mouth sealed) using dental polyether impression material (Impregum F, Henry Schein Halas, Brisbane, QLD, Australia). A pump (Reciprotor AB, Scara, Sweden) pushed fresh room air through the mask at a constant flow set between 0.9 and 1.2 l min^{-1}, depending on the size of the lizard, and controlled with a mass flowmeter (Sierra Instruments, Monterey, CA, USA). Hypercapnic gas mixtures were also delivered to the mask at constant flow (between 0.9 and 1.2 l min^{-1}) controlled with a mass flowmeter (Sierra Instruments). Care was taken to ensure that the flow rate though the mask exceeded the rate of expiration, thus minimising the possibility of rebreathing. An opening was made in the syringe barrel for excurrent airflow. Alterations in airflow due to ventilation were measured using a pneumotachograph (MLT1L Respiratory Flow Head, ADInstruments, Bella Vista, NSW, Australia) placed upstream of the mask, such that expirations caused a decrease in airflow and inspiration caused an increase in airflow. Pressure gradients induced by alterations in airflow across the pneumotachograph were monitored using a differential pressure transducer (ML141 spirometer, ADInstruments). The signal was calibrated by injecting and withdrawing known volumes of gas from the sealed mask, and was integrated to obtain tidal volumes. Gas exiting the mask was sub-sampled, passed through an indicating molecular sieve desiccant (MLA6024, ADInstruments) and analysed for fractional concentrations of O_2 (F_{O_2}) and CO_2 (F_{CO_2}) (ML206 gas analyser, ADInstruments). The rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were determined as previously described by Frappell et al. (Frappell et al., 1992). Briefly:

$$\dot{V}_{O_2} = v' \times (F'_{I_{O_2}} - F'_{E_{O_2}}) / (1 - F'_{I_{O_2}}), \qquad (1)$$

$$\dot{V}_{CO_2} = v' \times (F'_{E_{CO_2}} - F'_{I_{CO_2}}) / (1 - F'_{I_{CO_2}}), \qquad (2)$$

where v represents flow, I and E represent incurrent and excurrent gas, respectively, and the primes represent dry CO_2-free and O_2-

free gas in Eqns 1 and 2, respectively. CO_2 was mathematically scrubbed using $F'_{O_2}=F_{O_2}/(1-F_{CO_2})$. Metabolic gas values are reported at STPD (standard temperature and pressure, dry).

Breathing patterns were analysed in terms of inspired tidal volume (V_T), breathing frequency (f), minute ventilation ($\dot{V}_E=V_T\cdot f$), inspiratory duration (T_I), the duration of the non-ventilatory period (T_{NVP}) and inspiratory airflow rate (V_{TI}/T_I). An average of 25 consecutive breaths was analysed and ventilatory volumes are reported at BTPS (body temperature and barometric pressure, saturated). The air convection requirements for O_2 (ACR O_2) and CO_2 (ACR CO_2) and respiratory exchange ratio (RER) were also calculated.

Experimental protocol
Metabolic rate and breathing patterns were obtained from gravid lizards 4 weeks and 1 week prior to birth and in the first 24 h after birth. Non-invasively determining the stage of pregnancy in *T. rugosa* is difficult because of the presence of heavily ossified scales, which disrupt signal transmission of both ultrasound and traditional X-ray imaging modalities. As a result, data are expressed as weeks prior to birth rather than time post-conception, thus enabling comparisons to be made between animals without the complication of potentially variable developmental times and unknown conception dates. Breathing patterns were measured every 2 weeks from approximate mid-gestation and time-matched data collated post-birth. Measurements from non-gravid females were also made in the same time period.

Digestion in lizards induces peak increases in $\dot{V}_E$ and $\dot{V}_{O_2}$ that occur ~24 h post-feeding (Hicks et al., 2000). However, body temperature as well as the size, composition and frequency of meals can alter the metabolic response to feeding in reptiles (Beaupre, 2005; Bennett and Hicks, 2001; Hartzler et al., 2006; Hicks et al., 2000; Klein et al., 2006; Secor et al., 2000; Toledo et al., 2003; Wang et al., 2012). Pilot data from lizards in the present study, voluntarily fed their captive (relatively low protein) diet at 30°C, demonstrated that the duration of metabolic rate elevation caused by feeding is ~48 h. Thus, the lizards in this study were fasted for a minimum of 48 h prior to commencing experiments to avoid the possible confounding affects of digestion-induced alterations in metabolic rate. Experiments were performed at 30°C and animals were equilibrated at the test temperature for a minimum of 12 h. Breathing masks were fitted and lizards were wrapped loosely in cotton cloth to discourage movement. After 60 min of breathing air, the incurrent gas mixture was changed to 2.5% CO_2 (in 21% O_2 and balance N_2) for 10 min, followed by 5% CO_2 (in 21% O_2 and balance N_2) for 10 min. Air was then returned to the incurrent gas line for a minimum of 30 min and lizards were monitored until normal breathing patterns and $\dot{V}_{O_2}$ had recommenced. Any experiment in which the lizards became active was discarded from further analysis.

Data collection, analysis and statistics
All signals were collected at 1 kHz using the Powerlab data acquisition system (Model 8/30, ADInstruments) using Chart data acquisition software (ADInstruments). The last 25 consecutive breaths were analysed for each inhaled gas mixture. All data presented are means ± s.e.m.

Estimates of the energetic cost of breathing were calculated in individual lizards as a percentage of resting metabolic rate using a method previously described (Jackson et al., 1991; Skovgaard and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995). A regression line was plotted between $\dot{V}_E$ and $\dot{V}_{O_2}$ in response

to breathing air, 2.5% CO_2 and 5% CO_2. From this relationship, the cost of all metabolic activities other than ventilation (non-ventilatory metabolic cost) could be derived from the y-intercept (i.e. $\dot{V}_{O_2}$ where $\dot{V}_E=0$). Assuming that the relationship between $\dot{V}_E$ and $\dot{V}_{O_2}$ is linear and that there is no change in non-ventilatory metabolic rate during hypercapnic exposure, the percentage energetic cost of breathing can be calculated from the non-ventilatory and resting metabolic rates.

Statistical analysis of breathing patterns, metabolic rates and position of caudal lung margin during gestation were analysed using two-way ANOVA ($P<0.05$), followed by Dunnett's t-test ($P<0.05$).

RESULTS
The mean relative clutch mass (gestational load) of the five pregnancies (three singleton births and two sets of twins) was 28.3±4.4% of maternal mass, ranging from 19.1 to 37.6%. The estimated duration of pregnancies was 4.5 months.

Imaging
Radiographs were unable to image the developing fetuses at 5–6 weeks prior to birth. Despite this, the lung compression caused by the developing fetuses was evident in radiographs by 5–6 weeks prior to birth (Fig. 1). By the week prior to birth, radiographic imaging clearly revealed the mandible skeletal elements of the fetuses, although no clear image of the spine or other skeletal elements was visible (Fig. 1). The maximum body width was not significantly different (paired t-test, $P=0.39$) 1 week prior to birth (8.65±0.29 cm) compared with 1 week after birth (8.30±0.39 cm). A CT scan of one pregnant female showed that in very late pregnancy (18 h prior to birth) the fetus occupied a significant proportion of the body cavity in both the dorso-ventral and anterior–posterior dimensions (Fig. 2). A significant difference in the lung inflation index was measured during gestation (ANOVA, $P<0.00001$; Fig. 3). The lung inflation index in non-pregnant females
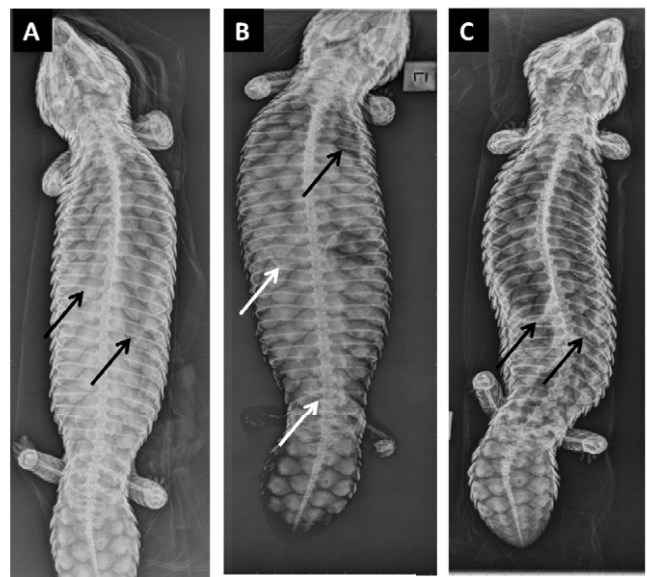

Fig. 1. Radiographs of one pregnant *Tiliqua rugosa* lizard (A) 40 days pre-partum, (B) 3 days pre-partum and (C) 2 days post-partum. An anterior displacement of the caudal margin of the lung (black arrows) was evident in all pregnant lizards in the last 5–6 weeks of gestation, and was most extreme in this individual, who carried twins (white arrows mark fetal mandibles).
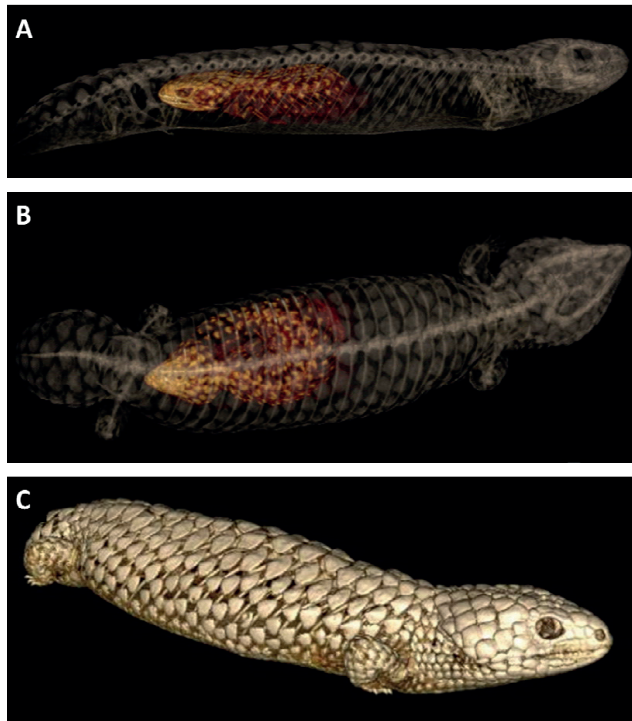
Fig. 2. Computerised tomography scans showing (A) sagittal and (B) coronal sections of a pregnant female *Tiliqua rugosa* (singleton pregnancy) 18 h prior to parturition. (C) A reconstruction of the surface bony elements shows the heavily ossified scales, which prevent significant expansion of the body wall during gestation.
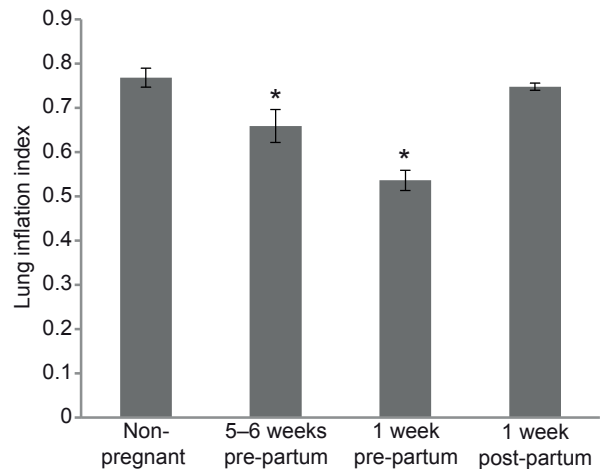

Fig. 3. Radiographic imaging was used to determine the lung inflation index (the rib number associated with the caudal margin of the lungs divided by the total number of ribs) of *Tiliqua rugosa*. A significant decrease in the lung inflation index and thus an anterior displacement of the lungs during gestation (ANOVA, $P<0.00001$) was measured during the periods 35–44 days (Dunnett's t-test, $P<0.00001$) and 2–7 days (Dunnett's t-test, $P<0.00001$) prior to birth. There was no significant difference in the lung inflation index 2–3 days post-partum compared with non-pregnant lizards (Dunnett's t-test, $P=0.33$). Data shown are means ± s.e.m., $N=8$ lungs from pregnant lizards and $N=26$ lungs from non-pregnant lizards.

and males did not differ and averaged 0.77±0.01. In the period 35–44 days prior to birth, the average lung inflation index was significantly reduced to 0.66±0.02 ($P<0.0001$), which was further reduced to 0.54±0.04 in the 2–7 days prior to birth ($P<0.0001$). In the first 2–3 days after birth the lung compression index (0.75±0.02) was not significantly different from that in non-pregnant lizards ($P=0.33$).

Breathing patterns during gestation

Both pregnant and non-pregnant *T. rugosa* had a breathing pattern that consisted of single breaths, in which expiration always preceded inspiration, interspersed with non-ventilatory pauses.

$\dot{V}_E$ was elevated in the week prior to and after birth relative to non-pregnant lizards (Fig. 4). These increases in $\dot{V}_E$ were induced by increases in f and decreases in T_{NVP}, without any significant alteration in V_T (Table 1). Despite no significant alteration in V_T, T_I decreased in the week prior to and after birth, and V_{TI}/T_I increased at 4 weeks and 1 week prior to birth and 1 week after birth (Fig. 5). $\dot{V}_{O_2}$, $\dot{V}_{CO_2}$ and RER were not significantly altered during gestation (Fig. 6). As a result of the significant increases in $\dot{V}_E$ without increases in $\dot{V}_{O_2}$ or $\dot{V}_{CO_2}$, both ACR O_2 and ACR CO_2 increased relative to non-pregnant values at 4 weeks and 1 week prior to birth and in the first week after birth (Fig. 6).

Breathing patterns in response to hypercapnia

$\dot{V}_E$ increased 2.3- to 2.6-fold in response to 2.5% CO_2 and 2.5- to 5.2-fold in response to 5% CO_2 (Table 1). f and T_{NVP} were not significantly altered by hypercapnia, thus the increases in $\dot{V}_E$ were induced solely by 2.1- to 6.0-fold increases in V_T. V_T changes in response to 5% CO_2 were accomplished *via* increases in both V_{TI}/T_I

and T_I, although these parameters were not significantly elevated in response to 2.5% CO_2. $\dot{V}_{O_2}$ (2.7- to 5.2-fold) and $\dot{V}_{CO_2}$ (2.3- to 4.1-fold) increased in response to 5% CO_2, although no significant changes in ACR O_2, ACR CO_2 or RER were measured (Table 1). There were no significant interaction effects between stage of pregnancy and inhaled gas composition in any metabolic or ventilatory parameter (two-way ANOVA, $P>0.05$).

Energetic cost of breathing

The energetic cost of breathing was estimated as a percentage of resting metabolic rate from the linear relationship between $\dot{V}_E$ and $\dot{V}_{O_2}$ when breathing air, 2.5% CO_2 and 5% CO_2 (Fig. 7). The energetic cost of breathing in non-pregnant lizards was 19.96±3.85% of resting metabolic rate. Gestation significantly increased the energetic cost of breathing to 34.67±0.50% at 4 weeks prior to birth, 62.80±10.11% 1 week prior to birth and 49.25±14.02% in the first week after birth (Fig. 8).

DISCUSSION
Imaging

In this study, fetal tissues could not be detected using radiographic images. As a result, the number of fetuses present could not be determined until the week prior to birth, when fetal ossification permitted the visualisation of fetal mandibles (Fig. 1). A similar result was found in the closely related viviparous blotched blue-tongue lizard, *Tiliqua nigrolutea*, in which gestation could not be confirmed radiographically until the presence of fetal skulls and mandibles in late gestation (Gartrell et al., 2002). Ultrasonography was found to have moderate to high accuracy in determining gestation throughout the reproductive cycle in *T. nigrolutea* (Gartrell et al., 2002) and in five species of oviparous lizards (Gilman and Wolf, 2007); however, the heavily ossified scales in *T. rugosa* result in poor signal penetrance and thus ultrasonography is not useful in determining gestation in this species (S.L.M., personal observation).
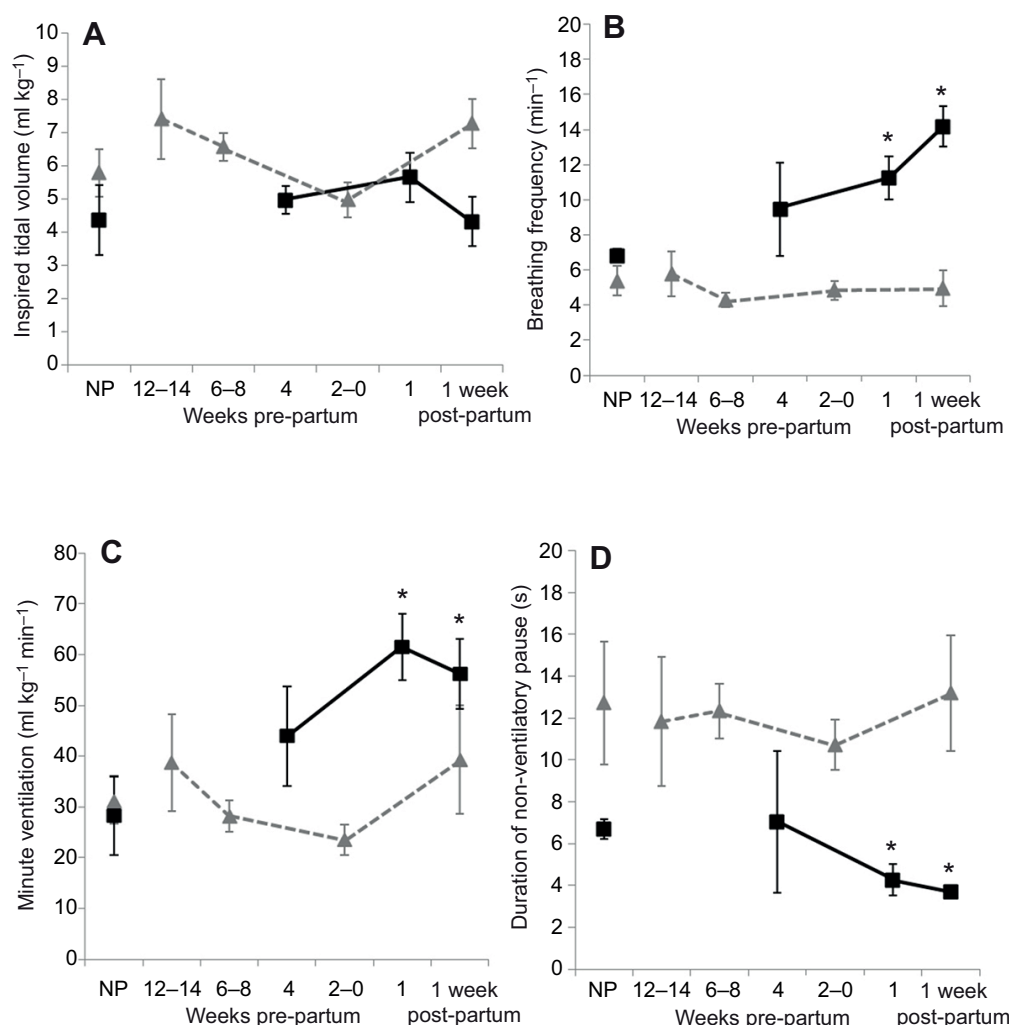
Fig. 4. Inspired tidal volume was not significantly altered during gestation in *Tiliqua rugosa* (A). A significant increase in breathing frequency (B) and a decrease in the duration of the non-ventilatory pause (D) were responsible for the increase in minute ventilation (C). Asterisks indicate a significant difference relative to non-pregnant values (Dunnett's *t*-test, *P*<0.05). Data shown are means ± s.e.m., *N*=5 pregnant lizards and *N*=7 non-pregnant lizards. Comparison data from Munns and Daniels (Munns and Daniels, 2007) are shown in grey, in which minute ventilation and tidal volume were significantly lower in the 2 weeks pre-partum to 12–14 weeks pre-partum, but not significantly different when compared with non-pregnant females and males.

During gestation, the increasing size of the fetuses resulted in no significant change in body width (*P*=0.39) but significant lung compression, and it may be possible to diagnose gestation based on the degree of lung compression. In fasted lizards, the mean lung inflation index decreased by 30% one week pre-partum (Fig. 3). In one individual that carried twins, the lung compression index was 0.40 three days prior to birth, representing a 48% reduction during gestation. Although this method of indexing lung inflation does not yield any data on lung volumetric changes occurring during gestation, it may be useful as a method of radiographically diagnosing gestation prior to fetal ossification, especially in species possessing dermal ossification, and will provide qualitative information regarding the degree of lung compression during gestation.

Breathing patterns during gestation

Gestation in *T. rugosa* induced a twofold increase in $\dot{V}_E$ *via* an increase in f with no concurrent increase in V_T (Fig. 4). The combination of f and V_T used to produce a particular $\dot{V}_E$ has a direct impact on the energetic cost of breathing. The mechanical work of breathing increases in direct proportion with f, but increases with the square of V_T (Milsom and Vitalis, 1984; Perry, 1989). As a result, it is more economical to increase $\dot{V}_E$ *via* increases in f rather than *via* increases in V_T (Milsom, 1984; Milsom and Vitalis, 1984; Vitalis and Milsom, 1986). During gestation, *T. rugosa* increases $\dot{V}_E$ using solely increases in f, a breathing pattern that results in a lower

mechanical work compared with that resulting from increases in V_T (or combinations of both V_T and f). Despite the adoption of a breathing pattern that produced elevated $\dot{V}_E$ for the least expensive mechanical work, the overall energetic cost of breathing increased threefold during gestation (Fig. 8).

Gestation did not induce an increase in V_T; however, T_I was reduced at the same time as V_{TI}/T_I was increased (Fig. 5). The resulting shorter inspirations with higher rates of airflow produced a gasp-like inspiration, particularly during late gestation. It is likely that an increase in the rate of inspiratory airflow would require increased respiratory muscle recruitment during inspiration and thus is likely to contribute to the increased energetic cost of breathing during gestation (Fig. 8). In this study and in studies in humans, increases in the rate of inspiration during pregnancy reflect an increased respiratory drive (Kolarzyk et al., 2005). In humans, at least, this increase in the rate of inspiration during pregnancy may be associated with overcoming increased respiratory system resistance (Kolarzyk et al., 2005) and/or may be linked with progesterone-associated changes in central chemosensitivity (Jensen et al., 2005). It is likely that lung compression during pregnancy in *T. rugosa* decreases lung compliance and an increase in the rate of inspiration may be an advantageous compensatory response.

The breathing pattern alterations measured during gestation in this study followed a different pattern compared with those measured in an earlier study of the same species (Fig. 4) in which $\dot{V}_E$ and V_T were reduced 2 weeks pre-partum compared with

Table 1. The effect of gestation on breathing patterns and metabolic rate in *Tiliqua rugosa* breathing air, 2.5% CO_2 and 5% CO_2

	Non-pregnant			4 weeks pre-partum			1 week pre-partum			1 week post-partum		
	Air	2.5% CO_2	5% CO_2	Air	2.5% CO_2	5% CO_2	Air	2.5% CO_2	5% CO_2	Air	2.5% CO_2	5% CO_2
$\dot{V}_E$ (ml kg⁻¹ min⁻¹)	28.32±7.80	87.70±19.93†	115.27±8.99†	43.97±9.81	100.61±25.25	229.73±88.98†	61.51±6.50*	155.78±36.98†*	200.58±55.33†	56.21±6.88*	143.47±50.59	139.78±47.00
V_T (ml kg⁻¹)	4.37±1.06	11.26±1.02†	14.54±2.52†	4.97±0.41	10.69±2.10	21.13±5.39†	5.65±0.75	16.29±6.34	34.11±10.17†*	4.33±0.75	13.07±2.21†	19.09±2.84†
f (min⁻¹)	6.81±0.38	7.41±1.43	9.56±1.99	9.47±2.65	10.98±3.77	10.46±2.31	11.23±1.22*	12.70±5.22	6.20±1.32	14.18±1.16*	10.98±1.65	7.69±3.72
T_{NVP} (s)	6.69±0.48	6.27±1.66	4.03±1.04	7.04±3.40	6.11±3.99	2.78±1.98	4.27±0.75*	3.83±1.24	8.00±3.91	3.70±0.01*	3.67±1.24	5.36±3.10
T_I (s)	1.38±0.18	1.91±0.26	2.25±0.34†	1.07±0.30	1.46±0.35	2.04±0.17†	0.79±0.09*	1.58±0.39	2.45±0.42†	0.68±0.10*	1.36±0.26	2.04±0.83†
V_{TI}/T_I (ml kg⁻¹ s⁻¹)	3.39±0.46	5.52±0.86	6.74±1.03†	5.14±2.28*	7.51±1.02	10.34±2.28†	7.42±3.05*	9.39±1.83*	13.63±3.05†*	6.81±1.65*	9.71±0.71*	9.89±2.64
$\dot{V}_{O2}$ (ml kg⁻¹ min⁻¹)	4.88±0.51	7.47±1.08	13.05±3.65†	3.04±0.75	7.78±2.76	12.66±5.71	5.47±1.37	7.25±1.04	19.21±1.44†	4.51±0.80	7.66±1.18	9.94±2.00
$\dot{V}_{CO2}$ (ml kg⁻¹ min⁻¹)	3.23±0.55	5.58±1.58	8.97±2.75	2.75±0.33	5.98±2.24	9.29±5.27	3.56±0.97	5.89±0.88	14.47±1.71†	3.45±1.37	5.62±2.14	7.88±2.18
RER	0.68±0.09	0.75±0.16	0.67±0.03	0.99±0.14	0.77±0.20	0.73±0.29	0.65±0.03	0.81±0.08	0.75±0.14	0.79±0.09	0.81±0.11	0.79±0.11
ACR O_2	6.05±1.15	12.32±3.30	11.98±2.64	15.26±3.91*	17.64±4.80	18.90±2.04	13.29±3.98*	22.66±6.71	10.30±2.40	12.91±1.67*	18.13±3.85	10.13±7.00
ACR CO_2	8.77±1.80	14.82±4.78	12.86±4.99	16.02±3.99*	16.80±3.58	24.72±4.84	17.30±5.76*	26.42±8.28	13.86±5.04	16.27±1.11*	25.52±4.31	17.72±7.39

$\dot{V}_E = V_T \cdot f$, minute ventilation; V_T, inspired tidal volume; f, breathing frequency; T_{NVP}, duration of the non-ventilatory period; T_I, inspiratory duration; V_{TI}/T_I, inspiratory airflow rate; $\dot{V}_{O2}$, rate of oxygen consumption; $\dot{V}_{CO2}$, rate of carbon dioxide production; RER, respiratory exchange ratio; ACR O_2 and ACR CO_2, air convection requirements for O_2 and CO_2, respectively.
Data are means ± s.e.m. †Significant difference relative to air values at the same stage of pregnancy (Dunnett's *t*-test, $P<0.05$); *significant difference relative to non-pregnant values when breathing the same inhaled gas (Dunnett's *t*-test, $P<0.05$) (N=5 pregnant and N=7 non-pregnant).
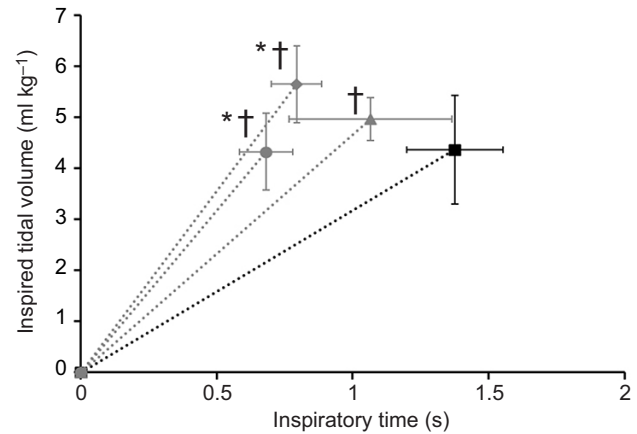

Fig. 5. Despite no significant alteration in inspired tidal volume, the rate of inspiration (gradient of each line) increased significantly (Dunnett's *t*-test, †$P<0.05$) relative to non-pregnant (black square) values 4 weeks (gray triangle) and 1 week (gray diamond) prior to birth, and remained elevated in the first week after birth (gray circle) in *Tiliqua rugosa*. The duration of inspiration significantly decreased 1 week prior to and 1 week after birth relative to non-pregnant values (Dunnett's *t*-test, *$P<0.05$). Data shown are means ± s.e.m., N=5 pregnant lizards and N=7 non-pregnant lizards.

12–14 weeks pre-partum, but not significantly reduced relative to non-pregnant females and males (Munns and Daniels, 2007). The relative clutch masses of both groups of pregnant lizards were similar (28.3±4.4% compared with 21.6±2.6% in this study); however, females from a previous study (Munns and Daniels, 2007) were caught from the field during early gestation, whereas captive breeding was employed in this study. Captive *T. rugosa* are likely to have increased abdominal fat stores as a result of a more regular and higher quality diet compared with that available to wild lizards. Females used in this study had significantly greater body mass for the same snout–vent length (mean 989.6±76.8 g) compared with those in the previous study (mean 662.2±22.5 g). Increased abdominal fat stores may decrease the space available in the body cavity for fetal growth and may result in a greater degree of lung compression and thus alter gestational breathing patterns. This hypothesis could be tested using a detailed analysis of the breathing patterns induced by singleton compared with twin pregnancies (with twin pregnancies likely to induce greater lung compression); however, insufficient data from twin pregnancies are presently available to make this comparison.

The maintenance of $\dot{V}_{O2}$ and $\dot{V}_{CO2}$ during gestation combined with an elevated $\dot{V}_E$ resulted in increases in both ACR O_2 and ACR CO_2 (Fig. 6). An increase in ACR O_2 is produced when an elevated $\dot{V}_E$ is used to achieve the same $\dot{V}_{O2}$, and thus reflects a relative hyperventilation and a decrease in pulmonary O_2 extraction efficiency. The relative hyperventilation that was induced during gestation in *T. rugosa* may be the result of either a diffusion and/or perfusion limitation to the rate of gas exchange in the maternal lung. The decrease in the lung inflation index during gestation (Fig. 3) indicates that there was progressive lung compression during gestation in this study, which may reduce the surface area available for gas exchange and produce a diffusion limitation to gas exchange. It is possible that the progressive lung compression may also increase pulmonary vascular resistance and may produce a perfusion limitation to pulmonary gas exchange by increasing ventricular afterload. Lung diffusing capacity and pulmonary vascular resistance were not measured in this study
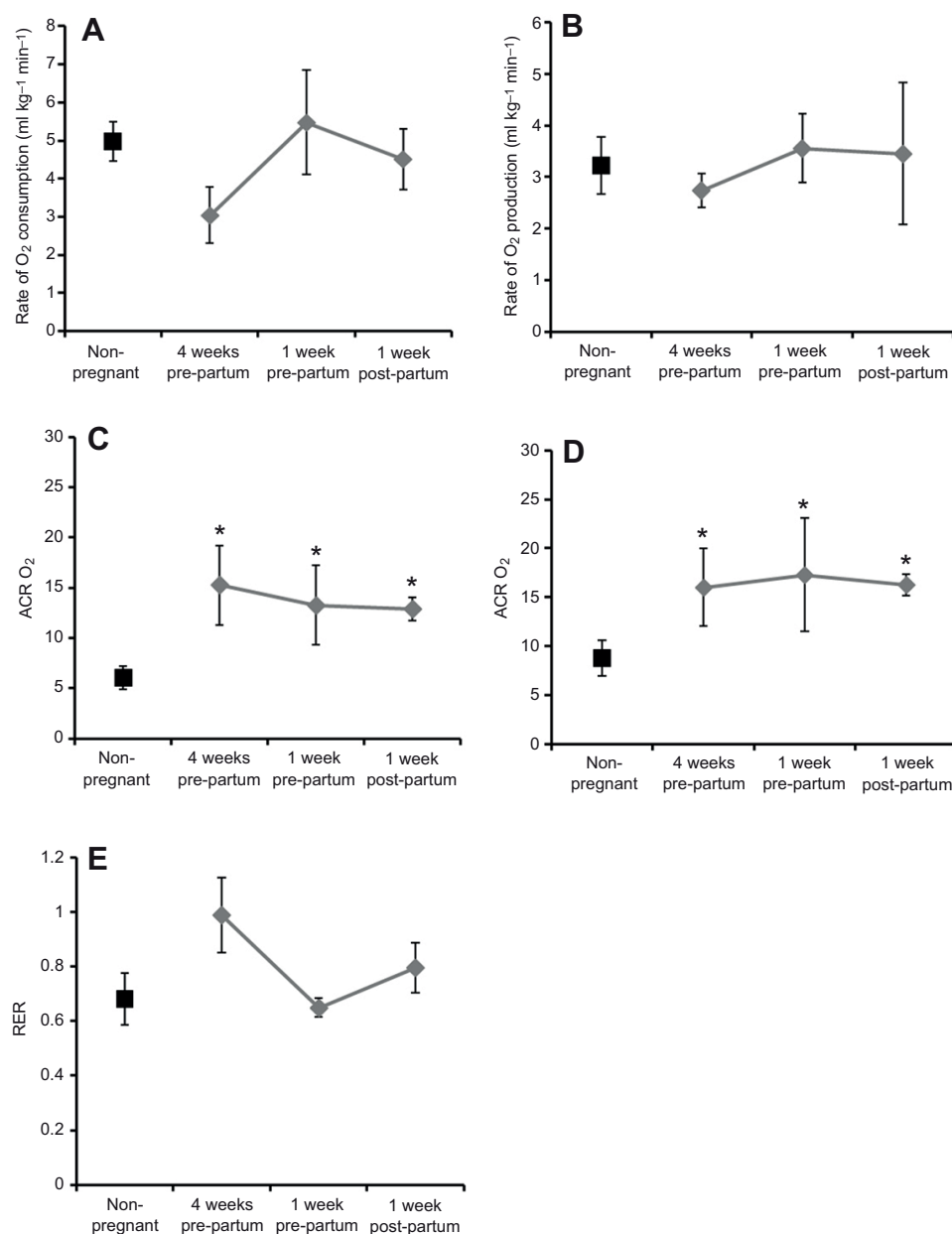
Fig. 6. Rates of O_2 consumption (A), CO_2 production (B) and the respiratory exchange ratio (RER; E) were not significantly different during gestation compared with non-pregnant values in *Tiliqua rugosa*. The air convection requirements (ACR) for both O_2 (C) and CO_2 (D) were significantly elevated at 4 weeks and 1 week pre-partum and in the first week post-partum. Asterisks indicate a significant difference relative to non-pregnant values (Dunnett's *t*-test, $P<0.05$). Data shown are means ± s.e.m., *N*=5 pregnant lizards and *N*=7 non-pregnant lizards.

and the changes in these parameters during gestation should be the subject of future studies.

In this study, gestational $\dot{V}_{O_2}$ represents the sum of both maternal and fetal tissues, thus it is possible that maternal $\dot{V}_{O_2}$ decreases during gestation while fetal $\dot{V}_{O_2}$ increases, thus resulting in no net change in total $\dot{V}_{O_2}$. During gestation, activity levels (S.L.M., personal observation) and the amount of food consumed decrease (Munns and Daniels, 2007), which may be associated with a decrease metabolic cost of gastrointestinal tract maintenance (Secor et al., 1994) and, as a result, in maternal metabolic rate. A decrease in metabolic rate would act to lower maternal oxygen demand, and thus may be an advantage if lung compression decreases the efficiency of gas exchange at the respiratory membrane. If an overestimate of maternal $\dot{V}_{O_2}$ occurred in this study, it would result in an underestimate of ACR O_2 during gestation and an underestimate in the degree of relative hyperventilation. Thus the impact of gestational lung compression

and breathing pattern alterations on pulmonary gas exchange described here may be an underestimate.

Energetic cost of breathing

The energetic cost of breathing in non-pregnant *T. rugosa* was 19.96±3.85% of resting metabolic rate (Fig. 8). This represents a relatively high cost of breathing compared with estimates in tegus (less than 1%) and American alligators (1–5%) using hypercapnic gases and a method similar to that used in the present study (Skovgaard and Wang, 2004; Wang and Warburton, 1995). Lizards have relatively simple (unicameral) and highly compliant lungs (Perry and Duncker, 1978). As a result, most of the work of breathing is used to overcome elastic forces in the chest wall (Skovgaard and Wang, 2004). Given the presence of ossified scales in *T. rugosa* and the likely decrease in chest wall compliance, a higher resting energetic cost of breathing is not surprising in this species.
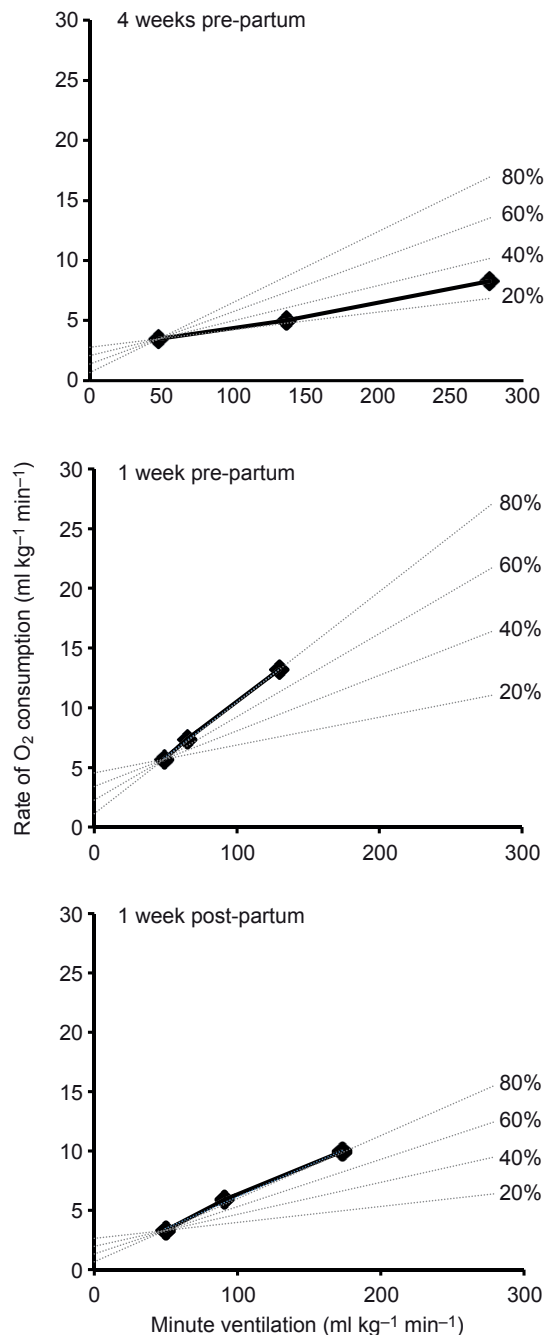
Fig. 7. Energetic cost of breathing for one representative pregnant female *Tiliqua rugosa* at 4 weeks and 1 week pre-partum and in the first week post-partum. The response to hypercapnia was linear in this and all other lizards throughout pregnancy. The energetic cost of breathing was 34.9% of resting metabolic rate at 4 weeks pre-partum and increased to 75.4% 1 week pre-partum (for comparison non pregnant cost of breathing is 19.96±3.85%). In the first week post-partum the energetic cost of breathing remained high at 74.9% of resting metabolic rate. Theoretical energetic cost of breathing isopleths (dotted lines) are shown for 20, 40, 60 and 80% of resting metabolic rate.
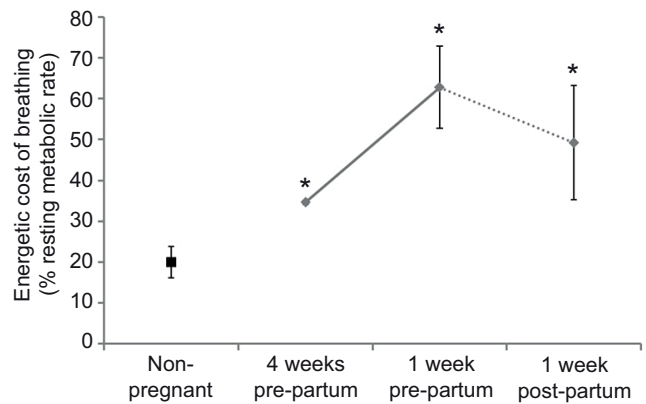

Fig. 8. Energetic cost of breathing in *Tiliqua rugosa* significantly increases at 4 weeks and 1 week pre-partum and in the first week post-partum. Asterisks indicate a significant difference relative to non-pregnant values (Dunnett's *t*-test, $P<0.05$). Data shown are means ± s.e.m., N=5 pregnant lizards and N=7 non-pregnant lizards.

and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995). Hypoxia produces higher cost of breathing estimates compared with hypercapnia in reptiles (Jackson et al., 1991; Skovgaard and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995). Hypoxia (2.5–10% O_2) can induced a wide variety of breathing pattern responses in reptiles (reviewed in Munns, 2000), and severe hypoxia (6% O_2) has been shown to induce agitation and increase movement (Skovgaard and Wang, 2004). Cost of breathing calculations make the assumption that non-ventilatory metabolism remains constant. Movement induced by severe hypoxia would increase the non-ventilatory metabolic rate and thus void one of the main assumptions made during cost of breathing calculations. In this study, hypercapnia was used to trigger breathing pattern alterations in resting lizards because it generally produces larger and more linear changes in $\dot{V}_E$ compared with hypoxia (Skovgaard and Wang, 2004), and produces more conservative estimates of the energetic cost of breathing. It has been suggested that a hypercapnia-induced acidosis may lower non-ventilatory metabolic rate (Busa and Nuccitelli, 1984), which would result in an underestimation of the cost of breathing. However, metabolic depression was not induced by hypercapnia in artificially ventilated turtles (Hicks and Wang, 1999), so the effect of hypercapnia on non-ventilatory metabolism in reptiles remains unclear. To reduce the possibility of a hypercapnia-induced depression in non-ventilatory metabolism (while still inducing a steady-state alteration in breathing pattern), the exposure time to hypercapnia was limited to 10min in this study, significantly shorter than the 45–60min used in previous studies (Skovgaard and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995).

Skovgaard and Wang (Skovgaard and Wang, 2004) have shown that ventilation can be elevated for a low energetic cost in lizards; however, this was not the case during gestation in *T. rugosa* in the present study. Gestation increases the energetic cost of breathing threefold to 62.8±10.1% of resting metabolic rate (Fig. 8). This increase in the energetic cost of breathing is the first measured for a gestating reptile, and may be due a combination of factors: the energetic cost of increased respiratory muscle recruitment required to increase $\dot{V}_E$ and the rate of inspiration, the energetic cost associated with overcoming any decrease in lung and/or chest wall compliance, and any increase in flow-resistive forces associated with increasing the rate of inspiration (which cannot be directly accounted for in this

There are relatively few comparative data on the cost of breathing in reptiles, and estimates vary considerably from 1 to 52% of resting metabolic rate and depend on the methods employed and the type of gases used to induce ventilatory changes (de Andrade and Abe, 1999; Jackson et al., 1991; Kinney and White, 1977; Skovgaard

analysis). This very high energetic cost of breathing exceeds the highest measurement to date in reptiles: 52.3% in hibernating tegus (de Andrade and Abe, 1999), although as these hibernating lizards were at a body temperature of 17°C, their energetic cost of breathing is high in relative terms due to metabolic depression but may low in absolute terms. However, the energetic cost of breathing during gestation in *T. rugosa* does not exceed the extremely high energetic cost of breathing estimates (90%) from hibernating squirrels (Garland and Milsom, 1994). Elevated energetic costs of breathing may have a considerable impact on the energy budgets of gesting *T. rugosa*, reducing the energy available for other activities such as exercise. *Tiliqua rugosa* has a low maximum metabolic rate $[0.722\,\text{ml}\,O_2\,g^{-1}\,h^{-1}$ at 35°C (John-Alder et al., 1986)], being in the lower 50% for all values for lizards at 35°C (John-Alder et al., 1986). The species is described as being unusually slow, with limited stamina, low sprint speeds and low maximum aerobic speeds (John-Alder et al., 1986). Activity levels decline and levels of aggression increase as gestation progresses in *T. rugosa* (S.L.M., personal observation), which may be due in part to the elevated costs associated with ventilation. Exercise capacity during gestation, including the ability to forage for food and escape predators, may be crucial for survival. A decrease in sprint speed and/or endurance in gestating lizards is common (Bauwens and Thoen, 1981; Miles et al., 2000; Olsson et al., 2000; Shine, 1980; Sinervo et al., 1991; van Damme et al., 1989), and may be partially responsible for the decline in survival rates during gestation in some squamate reptiles (Miles et al., 2000), although the physiology underpinning this finding is poorly understood. The effect of progressive lung compression and increased energetic cost of breathing on locomotion in pregnant *T. rugosa* is the subject of current experiments.

Breathing patterns in response to hypercapnia

An increase in V_T was induced by hypercapnia in both pregnant and non-pregnant lizards; however, during hypercapnia, the gestation-induced increase in f (and thus $\dot{V}_E$) was abolished (Table 1). The blunting of the breathing pattern response to gestation during hypercapnia may indicate a decreased sensitivity of CO_2 chemoreceptors during pregnancy. The sensitivity of pulmonary stretch receptors (which are mildly CO_2 sensitive) is depressed by hypercapnia, which reduces the negative feedback during lung inflation, and results in elevated V_T (Milsom, 1995; Powell et al., 1988). In addition, hypercapnic stimulation of pulmonary and upper airway chemoreceptors has been shown to reduce f (and hence $\dot{V}_E$) in tegus (Ballam, 1985; Ballam and Donaldson, 1988; Coates et al., 1991). Whether gestation induces any alterations in the sensitivity of CO_2 chemoreceptors in lizards is unclear.

In conclusion, gestation resulted in significant lung compression in *T. rugosa* and, in this study, was associated with a relative hyperventilation *via* increases in f. This increase in f and a relative hyperventilation were not present during gestation in a previous study using the same species (Munns and Daniels, 2007), which suggests that differences in body condition and abdominal fat stores during pregnancy may influence breathing patterns. Gestational alterations in breathing patterns (and presumably chest wall and lung compliance) resulted in threefold increases in the energetic cost of breathing, which may have significant consequences for the energy budgets of gestating females.

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