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

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Short Title: Cost of breathing during lizard gestation

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Abstract

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/total rib number). Pneumotachography and open flow respirometry were used to measure breathing patterns and metabolic rates. Gestation induced a two fold 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 *Tiliqua rugosa*. This relative hyperventilation 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 3 fold 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.

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 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 (Shaw) is a large, viviparous skink that inhabits vast areas of inland Australia. This species gives birth to 1-4 large young after 4-6 months gestation. The expansion of the body wall to accommodate the developing embryos may be limited in this species due to 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 to 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 1-52%: 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 1-7% (Milsom, 1989; Milsom, 1995). However, the energetic cost of breathing may increase significantly in some circumstances, for example; diseases 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, *Tiliqua rugosa*.

Materials and Methods

Animals

Shingle-back lizards (*Tiliqua rugosa, Gray 1825*) were collected from Burra region South Australia, and a breeding colony established at James Cook University. The animals were housed with a seasonally variable thermal gradient (5-15ºC winter and 20-38 ºC summer), full spectrum lighting (14L:10D), free access to water and were fed a diet of mixed fruit and vegetables, tinned cat food, boiled eggs, and vitamin and mineral supplements (RepCal™ and Herptivite™). Five gravid females and seven non-gravid females were used in this study. Body weight ranged from 688.9 to 1034.6 g (mean ± se, 989.6±76.8g) in the non pregnant females and from 692.1 to 922.2g (mean ± se, 809.9±64.1g) in the gravid females during late gestational period. 6 male lizards were used for radiographic imaging only. Lizards were sexed by eversion of the hemipenes.

Radiography

Radiographs were obtained between 35-44 days and 2-7 days prior to birth and 2-3 days post birth in 4 lizards. Radiographs were also obtained from 7 non-pregnant females and 6 male lizards over the same time period. Lizards were fasted for 3 days and then slowly cooled to approx 20ºC and wrapped loosely in cloth to discourage movement and placed in ventral recumbency. Optimal soft-tissue contrast was achieved using kVp of 55-60, mA of 200, 32m/Sec and mAs of 6.3 (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 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 divided by the total rib number. A lung inflation index of 1 would represent lungs that completely spanned the length of the trunk and 0 would represent completely collapsed lungs. Thus a decrease in the lung inflation index reflects that the caudal lung margin is located at a more cranial rib number due to increased lung compression. The long end inspiratory pauses in this species' breathing pattern, especially when at low body temperatures of 20ºC, ensured that all radiographs were taken after the lizards had inspired. While 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 computerized axial tomography (CT) scans (kVp 120.0, mA 50.0, 500m/Sec, mAs 75 and slice thickness 0.5mm) were taken of one pregnant (18h prior to birth) and one non pregnant female from which three dimensional images were reconstructed. Lizards were slowly cooled to a temperature of 20ºC, 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 noninvasively using pneumotachography (Glass and Johansen, 1979) using techniques similar to those used by the author in previous studies (Munns and Daniels, 2007; Munns et al., 2004; Munns et al., 2005). A small light weight mask constructed from the end of a 20ml syringe barrel. The mask was fitted over the lizard's nostrils and attached (and the mouth sealed) using a dental polyether impression material (Impregum F, Henry Schein Halas, Brisbane, QLD, Australia). A pump (Reciprotor AB, Sweden) pushed fresh room air through the mask at a constant flow set between 0.9-1.2 $L.min^{-1}$, depending on the size of the lizard, and controlled with a mass flowmeter (Sierra Instruments Inc, Monterey, California, USA). Hypercapnic gas mixtures were also delivered to the masks at constant flows (between 0.9-1.2 $L.min^{-1}$) controlled with a mass flowmeter (Sierra Instruments Inc, Monterey, California, USA). 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, AD Instruments, Bella Vista, NSW, Australia) placed upstream of the mask, such that expirations caused an decrease in airflow and inspiration caused a increase in airflow. Pressure gradients induced by alterations in airflow across the pneumotachograph were monitored using a differential pressure transducer (ML141 spirometer, AD Instruments, Bella Vista, NSW, Australia). 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, AD Instruments, Bella Vista, NSW, Australia) and analysed for fractional concentrations of O_2 (F_{O_2}) and CO_2 (F_{CO_2}) (ML206 gas analyser, AD Instruments, Bella Vista, NSW, Australia). 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., 1992). Briefly,

$$\dot{V}_{O_2} = \text{flow}` \times (F`_{IO_2} - F`_{EO_2}) / (1 - F`_{IO_2})$$

where I and E represent incurrent and excurrent gas respectively, and prime ` represents dry $CO_2$-free gas. $CO_2$ was mathematically scrubbed using $F`_{O_2} = F_{O_2} / (1 - F_{CO_2})$.

$$\dot{V}_{CO_2} = \text{flow}` \times (F`_{ECO_2} - F`_{ICO_2}) / (1 - F`_{ICO_2})$$

where prime ` represents dry O_2-free gas. 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 ($\dot{V}_E = V_T \times f$), 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 were 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 4wks and 1 wk prior to birth and in the first 24h after birth. Noninvasively determining the stage of pregnancy in *Tiliqua rugosa* is difficult due 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 two weeks from approx 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 approx 24h 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 this study, voluntarily fed their captive (relatively low protein) diet at 30°C demonstrated that the duration of metabolic rate elevation caused by feeding is approx 48hrs. Thus, the lizards in this study were fasted for a minimum of 48h prior to commencing experiments to avoiding the possible confounding affects of digestion induced alterations in metabolic rate. Experiments were performed at 30ºC and animals were equilibrated at the test temperature for a minimum of 12 h. Breathing masks were fitted and lizards wrapped loosely in cotton cloth to discourage movement. After 60 minutes of breathing air, the incurrent gas mixture was changed to 2.5% CO_2 (in 21% O_2 and balance N_2) for 10 minutes, followed by 5% CO_2 (in 21% O_2 and balance N_2) for 10 minutes. Air was then returned to the incurrent gas line for a minimum of 30 minutes and lizards monitored until normal breathing patterns and $\dot{V}_{O_2}$ had recommenced. Any experiment in which the lizards became active was discarded.

Data collection, analysis and statistics

All signals were collected at 1 kHz using the Powerlab data acquisition system (Model 8/30, AD Instruments, Bella Vista, NSW, Australia) using Chart data acquisition software (AD Instruments, Bella Vista, NSW, Australia). The last 25 consecutive breaths were analysed for each inhaled gas mixture. All data presented are mean±se. Ventilatory volumes are reported at BTPS and $\dot{V}_{O_2}$ at STPD.

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 t-test ($P<0.05$).

Results

The mean relative clutch mass (gestational load) of the five pregnancies (3 singleton births and 2 sets of twins) was 28.3±4.4% of maternal mass, ranging from 19.1% to 37.6%. 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, $P = 0.39$) between one week prior to birth (8.65±0.29 cm) and one week after birth (8.30±0.39 cm). Computerised Tomography (CT scan) of one pregnant female showed that in very late pregnancy (18h 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 (caudal margin of the lung relative to rib number as counted from cranial to caudal direction / total rib number) was measured during gestation (Fig 3, ANOVA, $P<0.00001$). The lung inflation index in non-pregnant females 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 *Tiliqua 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 2.3-2.6 fold in response to 2.5% CO_2 and 2.5-5.2 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 2.1-6.0 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 theses parameters were not significantly elevated in response to 2.5% CO_2. $\dot{V}_{O_2}$ (2.7-5.2 fold) and $\dot{V}_{CO_2}$ (2.3-4.1 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 visualization 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 *Tiliqua nigrolutea* (Gartrell et al., 2002) and in 5 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 (Munns, pers. obs.).

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 prepartum (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. While 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 two fold 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 which produced elevated $\dot{V}_E$ for the least expensive mechanical work, the overall energetic cost of breathing increased 3 fold 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 humans increases in the rate of inspiration during pregnancy reflects 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 to those measured in an earlier study of the same species (Fig 4) in which $\dot{V}_E$ and V_T were reduced 2 weeks prepartum compared to 12-14 weeks prepartum but not significantly reduced relative to non pregnant females and males (Munns and Daniels, 2007). The relative clutch mass of both groups of pregnant lizards were similar (28.3±4.4% in this study compared to 21.6±2.6%) however females from 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 due to a more regular and higher quality diet compared to that available to wild lizards. Females used in this study had significantly greater body mass for the same snout-vent length (mean 989.6±76.8g) compared to those in the previous study (mean 662.2±22.5g). Increased abdominal fat stores may decrease the space available in the body cavity for fetal growth and may result 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 to 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 $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ 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 $\dot{V}_{O_2}$, 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 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 (pers. obs) and the amount of food consumed decreases (Munns and Daniels, 2007), and 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 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 to estimates in Tegu lizards (less than 1%) and American alligators (1-5%) using hypercapnic gases and a similar method to this 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.

There is relatively little comparative data on the cost of breathing in reptiles, and estimates vary considerably from 1-52% 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 and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995). Hypoxia produces higher cost of breathing estimates compared to hypercapnia in reptiles (Jackson et al., 1991; Skovgaard and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995). Hypoxia (2.5-10%) can induced a wide variety of breathing pattern responses in reptiles (reviewed in (Munns, 2000)) and severe hypoxic (6%) 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 to hypoxia (Skovgaard and Wang, 2004) and produces more conservative estimates of the energetic cost of breathing. It has been suggested that a hypercapnic 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 hypercapnic induced depression in non-ventilatory metabolism (while still inducing a steady state alteration in breathing pattern), the exposure time to hypercapnia was limited to 10 minutes in this study, significantly shorter than the 45-60 minutes used in previous studies (Skovgaard and Wang, 2004; Skovgaard and Wang, 2007; Wang and Warburton, 1995).

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*. Gestation increases the energetic cost of breathing 3 fold 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 analysis). This very high energetic cost of breathing exceeds the highest measurement to date in reptiles; 52.3% in hibernating Tegu lizards (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 gestating *T. rugosa*, reducing the energy available for other activities such as exercise. *T. rugosa* has a low maximum metabolic rate (0.722 ml O_2/(g.h) 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* (pers.obs.) 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 Tegu lizards (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, is associated with a relative hyperventilation via increases in *f*. An increase in *f* and a relative hyperventilation was not present during gestation in a previous study using the same species (Munns and Daniels 2007), and may suggest that differences in body condition and abdominal fat stores during pregnancy influence breathing patterns. Gestational alterations in breathing patterns (and presumably chest wall and lung compliance) resulted in 3 fold increases in the energetic cost of breathing which may have significant consequences for the energy budgets of gestating females.

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Literature Cited

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Ballam, G. O. (1985). Breathing response of the Tegu lizard to 1-4% CO_2 in the mouth and nose or inspired into the lungs. *Respir. Physiol.* **62**, 375-386.

Ballam, G. O. and Donaldson, L. A. (1988). Effect of venous (gut) CO_2 loading on intrapulmonary gas fractions and ventilation in the tegu lizard. *J. Comp. Physiol. B* **158B**, 591-600.

Bauwens, D. and Thoen, C. (1981). Escpae tactics and vulnerability to predation associated with reproduction in the *Lacerata vivipara*. *J. Anim. Ecol.* **50**, 733-743.

Beaupre, S. (2005). Ratio representations of specific dynamic action (mass-specific SDA and SDA coefficient) do not standardize for body mass and meal size. *Physiol. Biochem. Zool.* **78**, 126-31.

Bennett, A. F. and Hicks, J. (2001). Postprandial exercise: prioritization or additivity of the metabolic responses? *J. Exp. Biol.* **204**, 2127-2132.

Busa, W. B. and Nuccitelli, R. (1984). Metabolic regulation via intracellular pH. *Am. J. Physiol.* **246**, R409-R438.

Campbell, E., Westlake, E. and Cherniack, R. (1957). Simple methods of estimating oxygen consumption and efficiency of the muscles of breathing. *J. Appl. Physiol.* **11**, 303-8.

Coates, E. L., Furiila, R. A., Ballam, G. O. and Bartlett, D., Jr. (1991). A decrease in nasal CO_2 stimulates breathing in the tegu lizard. *Respir. Physiol.* **86**, 65-75.

Daniels, C. B., McGregor, L. K. and Nicholas, T. E. (1994). The Dragons breath: A model for the dynamics of breathing and faveolar ventilation in agamid lizards. *Herpetol.* **50**, 251-261.

de Andrade, D. V. and Abe, A. S. (1999). Gas exchange and ventilation during dormancy in the tegu lizard *Tupinambis merianae*. *J. Exp. Biol.* **202**, 3677-3685.

Dellweg, D., Haidi, P., Siemon, K., Appelhans, P. and Kohler, D. (2008). Impact of breathing pattern on work of breathing in healthy subjects and patients with COPD. *Resp. Physiol. & Neuro.* **161**, 197-200.

Frappell, P. B., Lanthier, C., Baudinette, R. V. and Mortola, J. P. (1992). Metabolism and ventilation in acute hypoxia: a comparative analysis in small mammalian species. *Am. J. Physiol.* **262**, R1040-R1046.

Garland, R. J. and Milsom, W. K. (1994). End-tidal composition is not correlated with episodic breathing in hibernating ground squirels. *Can. J. Zool.* **72**, 1141-1148.

Gartrell, B. D., Girling, J. E., Edwards, A. and Jones, S. M. (2002). Comparison of noninvasive methods for the evaluation of female reproductive condition in a large vivipারious lizard, *Tiliqua nigrolutea*. *Zoo. Biol.* **21**, 253-268.

Gilman, C. A. and Wolf, B. O. (2007). Use of portable ultrasonography as a nondestructive method for estimating reproductive effort in lizards. *J. Exp. Biol.* **210**, 1859-1867.

Glass, M. L. and Johansen, K. (1979). Periodic breathing in the crocodile *Crocodylus niloticus*: consequences for the gas exchange ratio and control of breathing. *J. Exp. Biol.* **208**, 319-326.

Hartzler, L., Munns, S. L., Bennett, A. F. and Hicks, J. (2006). Metabolic and blood gas dependance on digestive state in the Sannah monitor lizard, *Varanus exanthematicus*: an assessment of the alkaline tide. *J. Exp. Biol.* **209**, 1052-1057.

Hicks, J. W. and Wang, T. (1999). Hypoxic hypometabolism in the turtle, *Trachemys scripta*. *Am. J. Physiol.* **277**, R18-R23.

Hicks, J. W., Wang, T. and Bennett, A. F. (2000). Patterns of cardiovascular and ventilatory response to elevated metabolic states in the lizard *Varanus exanthematicus*. *J. Exp. Biol.* **203**, 2437-2445.

Jackson, D. C., Singer, J. H. and Downey, P. T. (1991). Oxidative cost of breathing in the turtle *Chrysemys picta bellii*. *Am. J. Physiol.* **261**, R1325-R1328.

Jensen, D., Wolfe, L., Slatkovska, L., Webb, K., Davies, G. and O'Donnell, D. (2005). Effects of human pregnancy on the ventilatory chemoreflex response to carbon dioxide. *Am. J. Physiol.* **288**, R1369-R1375.

John-Alder, H. B., Garland, T. and Bennett, A. F. (1986). Locomotory capacities, oxygen consumption and the cost of locomotion of the shingle-back lizard (*Trachydosaurus rugosus*). *Physiol. Zool.* **59**, 523-531.

Jounieaux, V. and Mayeux, I. (1995). Oxygen cost of breathing in patients with emphysema or chronic bronchitis in acute respiratory failure. *Am J Respir Crit Care Med.* **152**, 2181-4.

Kinney, J. L. and White, F. N. (1977). Oxidative cost of ventilation in a turtle *Pseudemys floridana*. *Resp. Physiol.* **31**, 327-332.

Klein, W. and Owerkowicz, T. (2006). Function of intracoelomic septa in lung vntilation of amniotes: lessons from lizards. *Physiol. Biochem. Zool.* **79**, 1019-1032.

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

Kolarzyk, E., Szot, W. and Lyszcarz, J. (2005). Lung function and breathing regulation parameters during pregnancy. *Arch. Gynecol. Obstet.* **272**, 53-58.

Miles, D., Sinervo, B. and Frankino, W. (2000). Reproductive burden, locomotor performance, and the cost of reproduction in free ranging lizards. *Evolution* **54**, 1386-1395.

Milsom, W. K. (1984). The interrelationship between pulmonary mechanics and the spontanteous breathing pattern in the Tokay lizard, *Gekko Gecko. J. Exp. Biol.* **113**, 203-214.

Milsom, W. K. (1988). Control of arrythmic breathing in aerial breathers. *Can. J. Zool.* **66**, 99-108.

Milsom, W. K. (1989). Comparative Aspects of Vertebrate Pulmonary Mechanics. In *Comparative Pulmonary Physiology*, (ed. S. C. Wood), pp. 587-619. New York: Marcel Denker, Inc.

Milsom, W. K. (1995). Regulation of respiration in lower vertebrates: role of CO2/pH chemoreceptors. In *Advances in Comparative and Environmental Physiology.*, vol. 21, pp. 61-104. Berlin: Springer-Verlag.

Milsom, W. K. and Vitalis, T. Z. (1984). Pulmonary mechanics and the work of breathing in the lizard, *Gekko gecko*. *J. Exp. Biol.* **113**, 187-202.

Munns, S. L. (2000). Ventilation in freely-moving reptiles. *Department of Zoology University of Melbourne* **Ph.D.**

Munns, S. L. and Daniels, C. B. (2007). Breathing with big babies: Ventilation and oxygen consumption during pregnancy in the lizard, *Tiliqua rugosa*. *Physiol. Biochem. Zool.* **80**, 35-45.

Munns, S. L., Hartzler, L. K., Bennett, A. F. and Hicks, J. W. (2004). Elevated intra-abdominal pressure limits venous return during exercise in *Varanus exanthematicus*. *J. Exp. Biol.* **207**, 4111-4120.

Munns, S. L., Hartzler, L. K., Bennett, A. F. and Hicks, J. W. (2005). Terrestrial locomotion does not constrain venous return in the American alligator, *Alligator mississippiensis*. *J. Exp. Biol.* **208**, 3331-3339.

Olsson, M., Shine, R. and Bak-Olsson, E. (2000). Locomotor impairment of gravid lizards: is the burden physical or physiological? *J. Evol. Biol.* **13**, 263-268.

Perry, S. F. (1989). Structure and function of the reptilian respiratory system. In *Comparative Pulmonary Physiology - current concepts.*, (ed. S. C. Wood), pp. 193-236. New York: Marcel Dekker Inc.

Perry, S. F. and Duncker, H. R. (1978). Lung architecture volume and static mechanics in five species of lizards. *Respir. Physiol.* **34**, 61-81.

Perry, S. F. and Duncker, H. R. (1980). Interrelationship of static mechanical factors and anatomical structure in lung evolution. *J. Comp. Physiol.* **138**, 321-334.

Powell, F. L., Milsom, W. K. and Mitchell, G. S. (1988). Effects of intrapulmonary CO_2 and airway pressure on pulmonary vagal afferent activity in the alligator. *Resp. Physiol.* **74**, 285-298.

Secor, S., Hicks, J. and Bennett, A. (2000). Ventilatory and cardiovascular responses of a python (*Python molurus*) to exercise and digestion. *J. Exp. Biol.* **203**, 2447-2454.

Secor, S., Stein, E. and Diamond, J. (1994). Rapid upregulation of snake intestine in response to feeding: a new model of intestinal adaptation. *Am. J. Physio.* **266**, G695-G705.

Shine, R. (1980). "Costs" of reproduction in reptiles. *Oecologica* **46**, 92-100.

Sinervo, B., Hedges, R. and Adolph, S. C. (1991). Decreased sprint speed as a cost of reproduction in the lizard *Sceloporous occidentalis*: variation amoung populations. *J. Exp. Biol.* **155**, 323-336.

Skovgaard, N. and Wang, T. (2004). Cost of ventilation and effect of digestive state on the ventilatory response of the tegu lizard. *Resp. Physiol. Neurobiol.* **141**, 85-97.

Skovgaard, N. and Wang, T. (2007). Low cost of ventilation in the vagotomised alligator (*Alligator mississippiensis*). *Resp. Physiol. & Neuro.* **159**, 28-33.

Takayama, T., Shindoh, C., Kurokawa, Y., Hida, W., Kurosawa, H., H, O. and Satomi, S. (2003). Effects of lung volume reduction surgery for emphysema on oxygen cost of breathing. *Chest* **123**, 1847-52.

Toledo, L., Abe, A. and Andrade, D. (2003). Temperature and meal size effects on the postprandial metabolism and energetics in a boid snake. *Physiol. Biochem. Zool.* **76**, 240-6.

van Damme, R., Bauwens, D. and Verheyen, R. F. (1989). Effect of relative clutch mass on sprint speed in the lizard *Lacerta vivipara*. *J. Herpetol.* **23**, 459-461.

Vitalis, T. Z. and Milsom, W. K. (1986). Mechanical analysis of spontaneous breathing the the semi-aquatic turtle, *Pseudemys scripta*. *J. Exp. Biol.* **125**, 157-171.

Wang, Q., Wang, W., Huang, Q., Zhang, Y. and Luo, Y. (2012). Effect of meal size on the specific dynamic action of the juvenile snakehead (*Channa argus*). *Comp Biochem Physiol* **161**, 401-5.

Wang, T. and Warburton, S. J. (1995). Breathing pattern and cost of ventilation in the American Alligator. *Resp. Physiol.* **102**, 29-37.

Table 1: The effect of gestation on breathing patterns and metabolic rate in *T. rugosa* breathing air, 2.5% CO_2 and 5% CO_2. Number in parenthesis indicated standard errors of the mean. † indicates a significant difference relative to air values at the same stage of pregnancy (Dunnetts, $P<0.05$), * indicates a significant difference relative to non pregnant values when breathing the same inhaled gas (Dunnetts, $P<0.05$). n=5 pregnant and n=7 non pregnant.

Fig 1: Radiographs of one pregnant lizard A. 40 days prepartum, B. 3 days prepartum and C. 2 days postpartum. 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 foetal mandibles).

Fig 2: Computerised Tomography scans showing A. Sagittal and B. Coronal sections of a pregnant female (singleton pregnancy) 18h 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: The radiographic imaging was used to determine the lung inflation index (the rib number associated with the caudal margin of the lungs / total number of ribs). 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 postpartum compared to non-pregnant lizards (Dunnett's t-test, $P=0.33$). Data shown are mean±se, n=8 lungs from pregnant lizards, n=26 lungs from non-pregnant lizards.

Fig 4: Inspired tidal volume was not significantly altered during gestation (A). A significant increase in breathing frequency (B) and decrease in the duration of the non ventilatory pause (D) was responsible for the increase in minute ventilation (C). * indicates a significant difference relative to non pregnant values (Dunnetts, $P<0.05$). Data shown are mean±se, n=5 pregnant and n=7 non pregnant. Comparison data from Munns and Daniels (2007) are shown in grey in which minute ventilation and tidal volume were significantly lower in the 2 weeks prepartum to 12-14 weeks prepartum, but not significantly different when compared to non-pregnant females and males.

Fig 5: Despite no significant alteration in inspired tidal volume, the rate of inspiration (gradient of each line) increased significantly (†, Dunnetts, $P<0.05$) relative to non-pregnant (■) values 4 weeks (▲) and 1 week (♦) prior to birth and remained elevated in the first week after birth (●). The duration of inspiration significantly decreased 1 week prior to and 1 week after birth relative to non pregnant values (*, Dunnetts, $P<0.05$). Data shown are mean±se, n=5 pregnant and n=7 non pregnant.

Fig 6: The rates of O_2 consumption (A), CO_2 production (B) and the respiratory exchange ratio (E) were not significantly different during gestation compared to non pregnant values. The air convection requirements for both O_2 (C) and CO_2 (D) were significantly elevated at 4 and 1 week prepartum and in the first week postpartum. * indicates a significant difference relative to non pregnant values (Dunnetts, $P<0.05$). Data shown are mean±se, n=5 pregnant and n=7 non pregnant.

Fig 7: The energetic cost of breathing for one representative pregnant female at 4 weeks and 1 week prepartum and in the first week postpartum. 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 prior to birth and increased to 75.4% one week prepartum (for comparison non pregnant cost of breathing is 19.96±3.85%). In the first week postpartum 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: The energetic cost of breathing significantly increases at 4 weeks and 1 week prepartum and in the first week postpartum. * indicates a significant difference relative to non pregnant values (Dunnetts, $P<0.05$). Data shown are mean±se, n=5 pregnant and n=7 non pregnant.