RESEARCH ARTICLE

Pregnancy limits lung function during exercise and depresses metabolic rate in the skink *Tiliqua nigrolutea*

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ABSTRACT

High gestational loads have been associated with a range of ecological costs, such as decreased locomotor ability; however, the physiological mechanisms that underpin these changes are poorly understood. In this study, breathing patterns, metabolic rates, lung volume and lung diffusing capacity were measured at rest and during exercise in the pregnant skink *Tiliqua nigrolutea*. Breathing patterns were largely unaffected by gestation; however, decreases in metabolic rate (rate of oxygen consumption) in the late stages of pregnancy induced a relative hyperventilation. The reductions in metabolic rate during late pregnancy prevent the calculation of the maintenance cost of pregnancy based on post-partum and neonatal metabolic rates. Despite the high relative litter mass of 38.9±5.3%, lung diffusing capacity was maintained during all stages of pregnancy, suggesting that alterations in diffusion at the alveolar capillary membrane were not responsible for the relative hyperventilation. Lung volume was increased during pregnancy compared with non-pregnant females, but lung volume was significantly lower during pregnancy compared with post-partum lung volume. Pregnant females were unable to produce the same metabolic and ventilatory changes induced by exercise in non-pregnant females. This lack of ability to respond to increased respiratory drive during exercise may underpin the locomotor impairment measured during gestation in previous studies.

KEY WORDS: Ventilation, Breathing pattern, Oxygen consumption, Gestation, Lizard, Reptile, Pulmonary gas exchange, Lung diffusion capacity, Lung volume, Cost of pregnancy

INTRODUCTION

Gestation in reptiles has been associated with a number of physiological and ecological costs, ranging from changes in maternal metabolic rate (DeMarco, 1993; DeMarco and Guillette, 1992; Ladyman et al., 2003) and shifts in thermal preference (Lin et al., 2008), to impaired locomotor ability (Bauwens and Thoen, 1981; Miles et al., 2000; Olsson et al., 2000; Shine, 1980; Sinervo et al., 1991; van Damme et al., 1989) and declining survival rates (Miles et al., 2000). However, the physiological mechanisms that underpin these gestational costs are poorly understood. Where climatic conditions are suitable, oviparity is the preferred means of reproduction in squamates. Viviparity allows reptiles to extend their range into regions where low temperatures would otherwise reduce reproductive success (Blackburn, 2006; Watson et al., 2014). A recent study suggests that parity mode is a labile trait that shifts

frequently in response to ecological conditions (Pyron and Burbrink, 2014). Such shifts in populations or species must be driven by the balance between the relative costs and benefits of the two modes of parity. A number of viviparous lizards have relatively high gestational loads, such as 19–38% in *Tiliqua rugosa* (Munns, 2013) and 43% in *Tiliqua nigrolutea* (Edwards et al., 2002). In species with high gestational loads, the maintenance costs of gestation are likely to be high and thus these species are ideal for investigating physiological mechanisms that result in the physiological and ecological costs of gestation.

Previous studies have shown that gestation in *T. rugosa* is associated with a relative hyperventilation, a threefold increase in the energetic cost of breathing (from 20% in non-pregnant lizards to 63% in late pregnancy), and a decreased ability to respond to increased respiratory drive (Munns, 2013; Munns and Daniels, 2007). A relative hyperventilation can occur when changes in minute ventilation exceed those in metabolic rate. The hyperventilation measured during gestation in previous studies (Munns, 2013; Munns and Daniels, 2007) may be driven by a decrease in lung volume or a decrease in the rate of pulmonary oxygen diffusion. Elevated ventilatory rates may compensate for a decrease in lung volume or the rate of pulmonary gas diffusion by ensuring faster replenishment of fresh air in the lungs. Radiography and computerised tomography (CT) of pregnant *T. rugosa* (Munns, 2013; Munns and Daniels, 2007) suggest that lung volume decreases during pregnancy as a result of compression from the large developing fetuses, but no direct measurements of lung volume or lung diffusing capacity have been made in pregnant or gravid reptiles to date.

The rate of gas diffusion across the pulmonary membranes is described by Fick's law, where the rate of gas diffusion increases with increasing surface area of the membrane, increasing gas solubility and increasing partial pressure difference across the membrane, and decreases with increasing membrane thickness and increasing gas molecular weight (West, 2012). Compression of the lung during gestation may decrease the surface area of the inflated lung that can be utilised for gas exchange and thus may decrease lung diffusion capacity. In addition to the parameters that determine the rate of gas exchange at the alveolar capillary membrane, lung diffusion capacity can also be altered by haemoglobin concentration, pulmonary capillary blood volume or altered temperature (Glass et al., 1981; McAuliffe et al., 2003; Wang et al., 1998; Zavorsky et al., 2010). Lung diffusion capacity is not fixed, and in lizards lung diffusion capacity will increase in response to exercise (Frappell et al., 2002b).

The aims of this experiment were to determine whether lung volume or lung diffusion capacity was significantly decreased during pregnancy in a lizard with high relative litter mass, *T. nigrolutea* (Quoy and Gaimard 1824), and to determine whether exercise-related changes in lung volume or lung diffusion capacity could be maintained in pregnant lizards.

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List of symbols and abbreviations

ACR O_2	air convection requirement for O_2 ($\dot{V}_E/\dot{V}_{O_2}$)
ACR CO_2	air convection requirement for CO_2 ($\dot{V}_E/\dot{V}_{CO_2}$)
BTPS	body temperature and barometric pressure
$D_{L,CO}$	lung diffusing capacity for carbon monoxide
f	breathing frequency
F_{O_2}	fraction of O_2
F_{CO_2}	fraction of CO_2
$F_{E_{CO_2}}$	fraction of expired CO_2
$F_{E_{He}}$	fraction of expired helium
$F_{E_{O_2}}$	fraction of expired O_2
$F_{I_{CO_2}}$	fraction of inspired CO_2
$F_{I_{He}}$	fraction of inspired helium
$F_{I_{O_2}}$	fraction of inspired O_2
MCP	metabolic cost of pregnancy
P_B	barometric pressure
$P_{H_2O}(30)$	pressure water vapour at 30°C
PHS	post-hepatic septum
RER	respiratory exchange ratio
STPD	standard temperature and pressure, dry
T_E	duration of expiration
T_I	duration of inspiration
T_{NVP}	duration of the breath-hold period
$\dot{V}_{CO_2}$	rate of carbon dioxide production
$\dot{V}_E$	minute ventilation
$\dot{V}_{O_2}$	rate of oxygen consumption
$\dot{V}_{O_2,fetal}$	fetal rate of oxygen consumption
$\dot{V}_{O_2,maternal}$	maternal, fetus-free rate of oxygen consumption
$\dot{V}_{O_2,neonatal}$	neonatal rate of oxygen consumption
$\dot{V}_{O_2,non-pregnant}$	non-pregnant female rate of oxygen consumption
$\dot{V}_{O_2,post-partum}$	post-partum rate of oxygen consumption
$\dot{V}_{O_2,total}$	pregnant female rate of oxygen consumption (maternal+fetal+MCP)
V_L	lung volume
V_T	inspired tidal volume
V_T/T_I	rate of inspiration
V_{sys}	volume of the closed breathing system
V_{air}	volume of air

Tiliqua nigrolutea is a large viviparous skink with a distribution in the cool-temperate zones of southeastern Australia (Rawlinson, 1974). In contrast to *T. rugosa*, an armoured skink that gives birth to one to four large young (Munns, 2013), *T. nigrolutea*, has a more compliant, non-armoured body wall and gives birth to five to 17 small young (Edwards et al., 2002). *Tiliqua nigrolutea*'s more compliant body wall and high number of small offspring are more representative of skinks in general compared with *T. rugosa*. Female *T. nigrolutea* display a multiennial reproductive pattern, producing a large gestational load (up to 43%) comprising litters of 8.3±0.68 young (Edwards et al., 2002) after 4–4.5 months gestation every 2–3 years (Edwards et al., 2002; Edwards and Jones, 2001).

RESULTS

The mean relative litter mass of the six pregnancies was conservatively calculated from combined fetal mass (excluding amniotic fluid) at 39±5% of maternal mass, ranging from 20% to 53% with a mean litter number of 5.6 (range 4–8).

Resting maternal breathing patterns during gestation

Hyperventilation [increase in the air convection requirements for O_2 (ACR O_2) and CO_2 (ACR CO_2)] was measured in the later stages of pregnancy (at 2 and 1 week pre-partum) and at 1 week post-partum and was due to decreases in the rate of O_2 consumption ($\dot{V}_{O_2}$) and

CO_2 production ($\dot{V}_{CO_2}$); minute ventilation ($\dot{V}_E$) remained unchanged (Table 1). The only change in breathing pattern during pregnancy was a decrease in the duration of expiration (T_E). Relative to non-pregnant animals, lung volume increased at all stages of pregnancy and during the post-partum period. Lung volume at all pregnancy stages was lower than that at 1 week post-partum. Lung diffusing capacity for carbon monoxide ($D_{L,CO}$) remained unaltered during pregnancy and the post-partum period (Table 1).

Exercise

Non-pregnant lizards increased inspired tidal volume (V_T) by 216% during exercise and, as the duration of inspiration (T_I) remained unchanged, there was a resulting 178% increase in the rate of inspiration (V_T/T_I) (Table 2). The duration of the non-ventilatory period (T_{NVP}) decreased to 60% of resting values, leading to a 170% increase in breathing frequency (f). Together, these alterations resulted in an overall increase in $\dot{V}_E$ (357%) during exercise. $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ increased by 304% and 319%, respectively, and were matched to the increase in $\dot{V}_E$; as a result, there was no change in the ACR for either O_2 or CO_2. $D_{L,CO}$ was not altered during exercise, but lung volume increased (127%).

Similar alterations in ventilation and metabolic rate that accompanied exercise in non-pregnant females were observed in early pregnancy (10–12 weeks pre-partum) along with a significant increase in $D_{L,CO}$.

One week pre-partum, the exercise-induced alterations in ventilation and metabolic rate were no longer observed, with the only alteration being a 338% increase in $\dot{V}_{CO_2}$. The $\dot{V}_E$ elevations induced during exercise 1 week pre-partum were approximately half those induced in non-pregnant lizards (Fig. 1). V_T/T_I and V_T elevations were similarly limited to only 75% of non-pregnant values and the exercise-induced increases in f and T_{NVP} were abolished 1 week pre-partum.

Neonatal metabolic rate

Metabolic rates were calculated from a total of 34 neonates (mass 0.023±0.002 kg) within 24 h of birth. Mean individual $\dot{V}_{O_2}$ was 0.030±0.003 ml min^{-1} (1.331±0.147 ml min^{-1} kg^{-1}) and mean individual $\dot{V}_{CO_2}$ was 0.039±0.005 ml min^{-1} (1.398±0.330 ml min^{-1} kg^{-1}), with a mean respiratory exchange ratio (RER) of 0.81±0.04. Mean clutch $\dot{V}_{O_2}$ was 0.29±0.04 ml min^{-1} and mean clutch $\dot{V}_{CO_2}$ was 0.22±0.03 ml min^{-1}. Neonatal $\dot{V}_{O_2}$ represented 57% of the $\dot{V}_{O_2}$ measured 1 week pre-partum.

DISCUSSION

At rest, during the later stages of pregnancy and in the first week post-partum, females developed a relative hyperventilation, increasing both ACR O_2 and ACR CO_2 (Table 1). This relative hyperventilation was not driven by increases in $\dot{V}_E$ but rather by decreases in $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. A relative hyperventilation was also measured during the later stages of pregnancy in the closely related *T. rugosa* (Munns, 2013). *Tiliqua nigrolutea* reproduces using lecithotrophic viviparity; most, if not all, of the nutrients required for embryonic development are provided in the egg yolk, which is laid down in the vitellogenic period (Bleu et al., 2011), and thus this period is energetically expensive (Bonnett et al., 1994; Stewart, 1989). Young are born with a residual yolk sac (Edwards and Jones, 2004), suggesting that the egg yolk provided ample nutritional support for embryonic development. The vitellogenic period in *T. nigrolutea* begins at emergence in late September–early October and continues until ovulation following mating in November (with parturition occurring in February–March).

Table 1. Resting ventilation and metabolism during pregnancy and post-partum and in non-pregnant females

	Pre-partum (N=6)				Post-partum (N=6)	Non-pregnant (N=12)
	10–12 weeks	3–5 weeks	2 weeks	1 week		
Mass (kg)	**0.48±0.03***	**0.60±0.03***	**0.61±0.03***	**0.60±0.03***	**0.43±0.02‡**	0.54±0.02
T_I (s)	0.96±0.12	0.98±0.14	0.91±0.08	1.14±0.13	0.87±0.14	0.85±0.07
T_E (s)	**0.52±0.05***	**0.49±0.05***	**0.51±0.05***	**0.44±0.05***	0.69±0.08	0.56±0.04
V_T/T_I (ml kg^{-1} min^{-1})	5.58±0.56	5.75±1.01	6.67±0.94	7.27±1.54	5.95±0.90	6.55±0.72
V_T (ml)	11.03±1.34	9.98±1.30	9.73±1.26	13.11±2.35	11.68±2.51	9.90±0.88
T_{NVP} (s)	6.57±0.97	6.87±1.29	5.81±0.56	7.55±1.25	6.70±1.38	6.96±0.96
f (min^{-1})	8.61±0.72	8.26±1.06	8.86±0.71	7.32±0.88	8.65±1.07	7.94±0.69
$\dot{V}_E$ (ml kg^{-1} min^{-1})	91.9±8.8	79.0±12.5	85.1±14.1	90.5±16.0	90.2±12.1	75.1±6.3
$\dot{V}_{O_2}$ (ml kg^{-1} min^{-1})	**1.50±0.29***	**2.06±0.25*‡**	1.03±0.26	**0.74±0.11‡**	**0.77±0.09‡**	1.26±0.14
$\dot{V}_{CO_2}$ (ml kg^{-1} min^{-1})	1.36±0.30	**1.37±0.14*‡**	0.70±0.17	**0.51±0.04*‡**	0.66±0.06	0.94±0.11
RER	0.91±0.10	0.70±0.10	0.74±0.09	0.78±0.15	0.90±0.12	0.77±0.07
ACR O$_2$	**76.5±23.0***	**43.0±11.4***	**138.0±60.0‡**	**129.7±19.5‡**	**133.9±36.0‡**	67.2±8.6
ACR CO$_2$	93.7±31.6	**62.6±14.3***	**175.3±60.4‡**	**179.5±33.0‡**	**139.3±20.4‡**	91.9±14.0
$D_{L,CO}$ (ml kg^{-1} min^{-1} mmHg^{-1})	0.016±0.004	0.020±0.003	0.019±0.006	0.017±0.003	0.017±0.002	0.016±0.002
V_L (ml kg^{-1})	**186.9±25.7***	**207.2±13.7*†**	**226.6±16.4*‡**	**218.5±20.4*‡**	**282.4±22.4‡**	154.8±6.8

Data shown are means±s.e. Bold indicates a significant difference relative to the post-partum period (1 week post-partum; *Dunnett's test) and/or relative to non-pregnant females (‡Dunnett's test).

$D_{L,CO}$ has rarely been measured in reptiles at rest or during activity (Table 3), and has not been measured during gestation in any reptile to our knowledge. $D_{L,CO}$ measured in *T. nigrolutea* in this study was lower than previously measured in lizards (Frappell et al., 2002a,b; Glass et al., 1981) but this result is not surprising considering that the varanid lizards measured to date have multicameral (multi-chambered) lungs (Perry, 1989). These multicameral lungs have increased internal compartmentalisation, and hence surface area (Perry, 1989), and may be capable of much higher rates of gas diffusion than the single-chambered (unicameral) lungs in *T. nigrolutea*. Some unicameral lungs have decreased barrier thickness, as a result of bulging capillaries, which increases rates of gas diffusion; however, it has not been determined whether this anatomical feature is present in *T. nigrolutea* lungs. The low $D_{L,CO}$ measured in the poorly compartmentalised unicameral lungs in this study parallels the low calculated morphometric diffusing capacity found in other unicameral lizard lungs (Perry, 1993; Perry et al., 1994). $D_{L,CO}$ was maintained through pregnancy in *T. nigrolutea*, remained unaltered in the post-partum period and was no different from that of non-pregnant females (Table 1). The maintenance of $D_{L,CO}$ during pregnancy suggests that the relative hyperventilation was not induced by decreases in diffusion at the gas exchange surface.

The lungs of most terrestrial reptiles are 3–5 times larger than the lungs of similarly sized mammals, but have only about 10% of the surface area (Perry, 1993). The lungs in skinks and other squamates are large and unicameral (Perry, 1983; Perry and Duncker, 1978). Branching of the muscular trabeculae and deepening of the associated pulmonary parenchyma creates a honey-comb-like appearance. This parenchyma is termed faveolar, and the associated air spaces, faveoli (Duncker, 1978). The caudal portions of the lung have significantly less partitioning (Perry and Duncker, 1978), larger faveolar units and significantly less vascularisation compared with the cranial portions (Perry, 1983). This pattern of lung architecture is seen in *T. nigrolutea* (Fig. 2). It has been proposed that the caudal, less partitioned regions of the lung in squamates act like bellows, moving air throughout the lungs during non-ventilatory periods, rather than being used as a gas exchange surface (Donnelly and Woolcock, 1977; Spragg et al., 1980). Airflow in reptilian lungs was previously thought to be tidal, but recent evidence from avian, crocodilian, varanid and iguana lungs demonstrates that unidirectional airflow is used and that this trait may be plesiomorphic for Archosauria (Cieri et al., 2014; Farmer and Sanders, 2010; Schachner et al., 2014, 2013) but airflow patterns are yet to be determined in skinks. Large lung volumes in heterogeneously partitioned lungs may also act to reduce the work of breathing, and serve non-respiratory functions such as buoyancy,

Table 2. Pair-wise percentage change in ventilation and metabolism (exercise compared with rest) at each stage of pregnancy

	Pre-partum (N=6)				Post-partum (N=6)	Non-pregnant (N=12)
	10–12 weeks	3–5 weeks	2 weeks	1 week		
T_I	125.9±21.2	106.80±24.1	120.5±8.5	111.2±12.2	157.6±35.5	115.5±11.2
T_E	102.6±42.5	130.0±27.9	144.0±14.3	110.6±8.4	**162.7±24.8**	**152.5±13.4**
V_T/T_I	**269.3±24.8**	**144.6±23.2**	151.6±15.0	144.4±26.5	**205.3±27.4**	**178.2±13.9**
V_T	**566.4±70.0**	127.3±24.2	185.6±25.5	168.2±38.5	**337.7±98.3**	**216.4±42.5**
T_{NVP}	**64.2±13.4**	138.6±33.6	78.8±6.6	109.7±11.7	**53.1±6.5**	**60.8±9.0**
f	118.9±18.7	90.5±21.9	113.5±6.1	91.8±8.9	**138.8±16.2**	**170.3±17.3**
$\dot{V}_E$	**652.8±116.3**	126.8±42.2	216.7±36.1	153.7±36.5	**425.0±90.6**	**357.2±77.3**
$\dot{V}_{O_2}$	**614.0±105.7**	**213.4±45.5**	182.7±49.00	271.2±65.9	**603.9±113.6**	**304.3±33.9**
$\dot{V}_{CO_2}$	**801.6±225.2**	**244.8±54.3**	**232.6±43.8**	**337.9±93.9**	**625.5±150.6**	**319.5±32.1**
RER	122.8±15.9	126.5±27.3	139.3±14.0	151.7±59.4	101.5±13.2	111.9±9.4
ACR O$_2$	107.4±11.1	66.8±24.6	133.5±18.8	60.3±14.7	72.4±10.5	169.3±73.1
ACR CO$_2$	92.3±12.8	51.2±12.1	95.3±9.5	61.5±17.5	**72.6±7.6**	135.4±43.8
$D_{L,CO}$	**240.8±61.6**	130.3±13.6	113.6±17.54	125.3±23.3	112.0±24.7	157.8±28.5
V_L	**177.2±19.9**	137.4±17.2	115.8±7.7	111.2±7.7	105.2±12.9	**127.0±11.0**

Data shown are means±s.e. Bold indicates a significant difference compared with rest (paired *t*-tests).
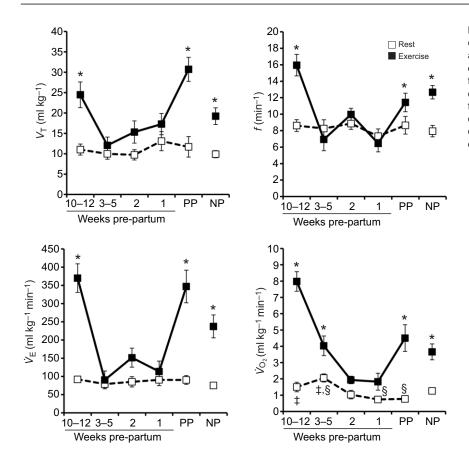
Fig. 1. Changes in ventilation and metabolism during pregnancy (pre-partum), post-partum (PP) and in non-pregnant females (NP) at rest and during exercise. V_T, inspired tidal volume; f, breathing frequency; $\dot{V}_E$, minute ventilation; $\dot{V}_{O_2}$, rate of oxygen consumption. Data are means±s.e., $N=6$ pregnant females, $N=12$ non-pregnant females. *Significant difference between rest and exercise, ‡significant difference relative to the post-partum period, §significant difference relative to non-pregnant females.

locomotory purposes, vocalization and behavioural displays (reviewed in Perry, 1983). Thus, in heterogeneously partitioned lungs, lung volume may not be directly correlated to metabolic demand and $D_{L,CO}$ may not be directly related to lung surface area (Perry, 1983).

During pregnancy in the closely related *T. rugosa*, lung compression is most prominent in radiographs and CT images in the caudal, non-respiratory portions of the lungs, and extends more anteriorly as pregnancy progresses (Munns, 2013). Reductions in surface area in the caudal region of the lung, as a result of compression by the developing embryos, may not have a significant detrimental effect on $D_{L,CO}$ given that this area of the lung is poorly perfused, has faveolar units with significantly lower surface area to volume ratio and is unlikely to be used as a primary gas exchange surface. Compression of the caudal regions of the lung may decrease perfusion to that area (because of the collapse of blood vessels and the resulting increased pulmonary capillary resistance) and thus may act to redirect blood and increase perfusion to the gas exchange

Table 3. $D_{L,CO}$ values for reptiles measured to date

Species	$D_{L,CO}$ (ml kg^{-1} min^{-1} mmHg^{-1})	Temperature (°C)	Reference
Lizards			
Tiliqua nigrolutea (non-pregnant)	0.016±0.002	30	This study
Tiliqua nigrolutea (non-pregnant)*	0.021±0.004	30	This study
Varanus mertensi	0.116±0.027	35	(Frappell et al., 2002a)
*Varanus mertensi**	0.12±0.032	35	(Frappell et al., 2002a)
Varanus gouldi	0.061±0.023	35	(Frappell et al., 2002b)
*Varanus gouldi**	0.133±0.066	35	(Frappell et al., 2002b)
Varanus spenceri	0.026±0.013	35	(Frappell et al., 2002b)
*Varanus spenceri**	0.117±0.052	35	(Frappell et al., 2002b)
Varanus exanthematicus	0.027	17–19	(Glass et al., 1981)
Varanus exanthematicus	0.059±0.006	22–25	(Glass et al., 1981)
Varanus exanthematicus	0.075±0.008	35–37	(Glass et al., 1981)
Tupinambis teguixin	0.04±0.01	22–25	(Glass et al., 1981)
Chelonians			
Caretta caretta	0.11±0.03	25	(Lutcavage et al., 1987)
Pseudemys scripta elegans	0.033	20–23	(Crawford et al., 1976)
Testudo gracea	0.068	20–23	(Crawford et al., 1976)
Chelonia mydas	0.043±0.009	25	(Gatz et al., 1979)

*Measurements taken during exercise.

Fig. 2. Unicameral lungs of adult *Tiliqua nigrolutea*. Dissected lungs were obtained from an injured lizard donated by a member of the public. The cranial portion of the lung (left of image) has smaller faveolar units and increased perfusion relative to the caudal portion of the lung, which has significantly larger faveolar units and is relatively poorly perfused.

surfaces in the cranial portion of the lung. As a result, blood flow may be increased to the smaller faveolar units, which have a higher surface area to volume ratio and are better able to maintain total lung $D_{L,CO}$ in the face of caudal lung compression. Thus, during pregnancy, compression of the caudal lung may redistribute both air and blood in the lung, improving ventilation perfusion matching in the gas exchange regions of the otherwise compressed lung and thus $D_{L,CO}$ may be maintained.

Lung volume was significantly decreased across all stages of pregnancy compared with post-partum females, but was significantly increased relative to non-pregnant females (Table 1). The duration of expiration was decreased during pregnancy compared with post-partum females, suggesting that expiratory braking may, at least in part, be responsible for the increase in lung volume. The caudal non-respiratory regions of the squamate lung are collapsed during normal breathing but can be inflated, for example, in behavioural defence displays (Duncker, 2004). Thus, there may be significant potential to increase functional lung volume by increasing airflow into caudal regions of the lung and expanding the bellows-like folds. Increases in lung volume were measured in *T. nigrolutea* not only during pregnancy but also during exercise in non-pregnant lizards and during the early pregnancy period (Table 2). It is possible that the existing redundancy in lung volume afforded by the bellows-like caudal lung folds was exploited in the mid- to late stages of pregnancy at rest and that during exercise no further increases in lung volume could be produced.

The increases in lung volume measured in *T. nigrolutea* during pregnancy are unlikely to be replicated in lizards with armoured body walls and thus lower body wall compliance, such as the closely related *T. rugosa*. The non-armoured *T. nigrolutea*, like most lizards, has a highly compliant body wall (Perry and Duncker, 1978) and significant thoraco-abdominal expansion can be measured during pregnancy. *Tiliqua nigrolutea* has a similar relative litter mass to *T. rugosa* but produces many small 12–29 g offspring (this study) in comparison to *T. rugosa*'s one to three, 80–110 g offspring (Munns, 2013). These small offspring of *T. nigrolutea* may be better able to disperse around large internal organs compared with the very large offspring produced by *T. rugosa*, and this, combined with the more compliant body wall

that is better able to expand to accommodate the increasing embryonic load, may result in less lung compression during pregnancy in *T. nigrolutea* and thus may have resulted in the smaller number of breathing pattern alterations that were measured during late pregnancy at rest.

Lung compression can also be induced by removal of the post-hepatic septum (PHS) in Tegu lizards. The PHS of teiioids divides the body cavity into a pleurohepatic cavity, containing the lungs and liver, and a peritoneal cavity, containing the remaining visceral organs (Klein et al., 2000). The PHS ensures a minimal space for the lungs and prevents the viscera from moving cranially during exercise or digestion (Klein et al., 2003a,b, 2006). Surgical removal of the PHS in the Tegu *Tupinambis merianae* causes the lungs to be displaced cranially and dorsally by the stomach and intestines (Klein et al., 2003a), resulting in lower resting $\dot{V}_E$ and $\dot{V}_{O_2}$ and an impaired ability to increase V_T during treadmill exercise (Klein et al., 2003b). Skinks, such as *T. nigrolutea* and *T. rugosa*, do not possess a PHS and thus do not have the ability to limit cranial organ displacement during exercise, digestion or the high gestational loads found in late pregnancy. Compression of the lungs during late pregnancy, in this study, shows similar impairments to V_T during exercise compared with the lung compression due to visceral organ displacement in Tegu lizards (Klein et al., 2003b). The lung volume reductions induced by PHS removal in Tegu lizards decreased pulmonary dead space but did not alter the volume and surface area of the gas exchange tissues, and thus it was hypothesised that the $D_{L,CO}$ would be unaffected (Klein et al., 2003b).

Exercise induced significant alterations in a number of breathing pattern parameters (Table 2, Fig. 1). A similar pattern of increasing f, V_T, V_T/T_I, $\dot{V}_E$, $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ and decreasing T_{NVP} in response to exercise has been found in other lizards (Bennett and Hicks, 2001; Frappell et al., 2002a; Hicks et al., 2000; Munns et al., 2004; Wang et al., 1997). Exercise-induced elevations in parameters such as f (1.7-fold) and $\dot{V}_E$ (3.5-fold) were lower in *T. nigrolutea* than those measured (2.5- to 10.5-fold increases in f and 4- to 24-fold increases in $\dot{V}_E$) in other more aerobic lizards such as varanids (Bennett and Hicks, 2001; Frappell et al., 2002b; Munns et al., 2004; Wang et al., 1997), but given the less active lifestyle of the *Tiliqua* genus (Kerr and Bull, 2006), this result is not surprising. The exercise-induced increases in ventilation and metabolic rate measured in non-pregnant females were not maintained in pregnant lizards especially in the mid- to late stages of pregnancy. During late pregnancy, the increase in $\dot{V}_E$ during exercise was only half that achieved by non-pregnant females, and pregnant females were unable to decrease T_{NVP} or increase f in response to exercise. *Tiliqua rugosa* also demonstrated a reduced ability to respond to increased respiratory stimuli (in the form of inhalation of hypoxic hypercapnia or hypercapnic gas) during pregnancy (Munns, 2013; Munns and Daniels, 2007). A reduced ability for pregnant lizards to respond to stimuli that would normally increase ventilation may be one of the main mechanisms that underpins the decrease in sprint speed and locomotor endurance in pregnant lizards (Bauwens and Thoen, 1981; Miles et al., 2000; Olsson et al., 2000; Shine, 1980; Sinervo et al., 1991; van Damme et al., 1989).

$\dot{V}_{O_2}$ measured during gestation ($\dot{V}_{O_2,total}$) represents maternal fetus-free $\dot{V}_{O_2}$ ($\dot{V}_{O_2,maternal}$) plus fetal $\dot{V}_{O_2}$ ($\dot{V}_{O_2,fetal}$) and any metabolic cost associated with maintaining the pregnancy (MCP) (Robert and Thompson, 2000a). In previous studies, it has been assumed that either non-pregnant $\dot{V}_{O_2}$ ($\dot{V}_{O_2,non-pregnant}$) or $\dot{V}_{O_2}$ post-partum ($\dot{V}_{O_2,post-partum}$) can be used as a proxy for $\dot{V}_{O_2,maternal}$, and

that neonatal $\dot{V}_{O_2}$ ($\dot{V}_{O_2,\text{neonatal}}$) can be used as a proxy for $\dot{V}_{O_2,\text{fetal}}$. MCP can then be calculated by:

$$\text{MCP} = \dot{V}_{O_2,\text{total}} - \dot{V}_{O_2,\text{non-pregnant}} - \dot{V}_{O_2,\text{neonatal}} \qquad (1)$$

or

$$\text{MCP} = \dot{V}_{O_2,\text{total}} - \dot{V}_{O_2,\text{post-partum}} - \dot{V}_{O_2,\text{neonatal}}. \qquad (2)$$

In contrast, $\dot{V}_{O_2,\text{total}}$ 1 week pre-partum and $\dot{V}_{O_2,\text{post-partum}}$ were significantly lower than $\dot{V}_{O_2,\text{non-pregnant}}$ in this study, suggesting that $\dot{V}_{O_2,\text{maternal}}$ was depressed from late gestation to shortly after parturition. Thus, for *T. nigrolutea*, the above calculations would yield a MCP less than zero. Assuming that $\dot{V}_{O_2,\text{neonatal}} = \dot{V}_{O_2,\text{fetal}}$, and that MCP=0, then $\dot{V}_{O_2,\text{maternal}}$ can be estimated for *T. nigrolutea* by $\dot{V}_{O_2,\text{total}} - \dot{V}_{O_2,\text{neonatal}}$, yielding an estimated $\dot{V}_{O_2,\text{maternal}}$ of 0.22± 0.05 ml min^{-1} (Fig. 3). This estimated $\dot{V}_{O_2,\text{maternal}}$ represents 42% of $\dot{V}_{O_2,\text{total}}$ for 1 week pre-partum, and a 34% reduction in $\dot{V}_{O_2,\text{maternal}}$ relative to $\dot{V}_{O_2,\text{post-partum}}$ and a 68% reduction in $\dot{V}_{O_2,\text{maternal}}$ relative to $\dot{V}_{O_2,\text{non-pregnant}}$.

However, it is unlikely that the assumptions outlined above (and made in previous studies) are valid for pregnant *T. nigrolutea*. $\dot{V}_{O_2,\text{neonatal}} = \dot{V}_{O_2,\text{fetal}}$ is unlikely to be valid given that fetal oxygen requirements are supplied by diffusion from the maternal blood supply at the placenta whereas neonates exchange gases directly at the pulmonary interface (which is supplied with a higher partial pressure gradient of O_2) and are no longer subject to the diffusion limitations present at the placental interface (Swain and Jones, 1997). Thus $\dot{V}_{O_2,\text{neonatal}}$ is likely to be an overestimate of $\dot{V}_{O_2,\text{fetal}}$ and result in an underestimate of $\dot{V}_{O_2,\text{maternal}}$. Although most, if not all, of the fetal nutritional requirements are provided by the extensive yolk, it is unlikely that MCP=0 given that the maternal system is responsible for providing for fetal gas exchange and nitrogenous waste excretion (Swain and Jones, 1997). Assuming MCP=0 would result in an overestimate of $\dot{V}_{O_2,\text{maternal}}$. In addition, $\dot{V}_{O_2,\text{non-pregnant}} = \dot{V}_{O_2,\text{maternal}}$ is clearly not valid for this species in which a metabolic depression exists during late pregnancy and thus $\dot{V}_{O_2,\text{non-pregnant}}$ would overestimate $\dot{V}_{O_2,\text{maternal}}$.
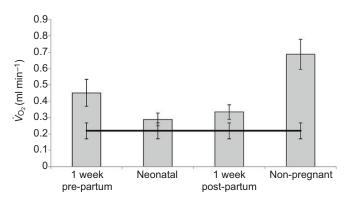

Fig. 3. $\dot{V}_{O_2}$ for non-pregnant (*N*=12), late-stage pregnant (*N*=6), post-partum (*N*=6) and neonatal *T. nigrolutea* (*N*=34). The horizontal line represents calculated estimates of late-stage pregnant maternal (fetus-free) $\dot{V}_{O_2}$. Neonatal $\dot{V}_{O_2}$ (total for clutch) was measured in 34 newborns within 24 h of parturition. Late-stage pregnant maternal (fetus-free) $\dot{V}_{O_2}$ was estimated from 1 week pre-partum $\dot{V}_{O_2}$–neonatal $\dot{V}_{O_2}$ (assuming that there is no change in fetal $\dot{V}_{O_2}$ compared with neonatal $\dot{V}_{O_2}$ and that the maintenance cost of pregnancy is negligible). Late-stage pregnant maternal (fetus-free) $\dot{V}_{O_2}$ represents 42.3% of total $\dot{V}_{O_2}$ (i.e. maternal and fetal) 1 week pre-partum and a 68% reduction compared with non-pregnant $\dot{V}_{O_2}$. Data shown are means±s.e.

A decrease in metabolic rate during late pregnancy was also present in the closely related *T. rugosa* (Munns, 2013). The decrease in metabolic rate during late pregnancy may be due, in part, to a decrease in the energetic cost of gastrointestinal tract tissue maintenance. The gastrointestinal tract organs are very metabolically active and, in mammals, may account for 20–40% of basal metabolic rate (Cant et al., 1996; McBride and Kelly, 1990). Many animals decrease metabolic rate during fasting or limited food availability (for review, see Wang et al., 2006). Progressive and reversible changes in gastrointestinal tract organs occur during fasting in all major groups of ectothermic vertebrates. Some studies have found that up-regulation of the gut is energetically cheap on emergence from fasting in infrequently feeding snakes (Overgaard et al., 2002; Starck and Beese, 2001); however, gut down-regulation may represent significant energy savings relative to fasting metabolic rate (Cox and Secor, 2010; Secor and Diamond, 1998, 2000), particularly in the frequently feeding *T. nigrolutea*. Dramatic decreases in food consumption during the later stages of pregnancy were measured in *T. rugosa* (Munns and Daniels, 2007) and observations of *T. nigrolutea* suggest that decreases in food consumption during pregnancy occur in this species as well. In hibernating mammals, the absence of food intake leads to atrophy of the metabolically expensive gastrointestinal mucosa, and general down-regulation of the gastrointestinal tract, contributing to the reduction of metabolic rate (Carey, 1995). Similarly, the decrease in food consumption during pregnancy in *Tiliqua* is likely to be accompanied by a down-regulation of the gastrointestinal tract and reduction in maternal metabolic rate.

Previous studies have estimated the MCP in viviparous lizards and snakes to be 6.4% (DeMarco and Guillette, 1992), 13.9–14.7% (Dupoué and Lourdais, 2014), 17% (Robert and Thompson, 2000b), 16–29% (Beuchat and Vleck, 1990), 22% (Foucart et al., 2014) and 26.4% (Schultz et al., 2008), and in all these studies maternal metabolic rate increased during gestation. However, in five species of viviparous snakes, the MCP measured was not significantly different from zero and the metabolic cost associated with vitellogenesis was significantly greater at 30% (Van Dyke and Beaupre, 2011). The variation in MCP in previous studies may represent differences due to the techniques used to estimate MCP, variations in the degree of placentotrophy and/or differences between taxa (reviewed by Schultz et al., 2008; Van Dyke and Beaupre, 2011). In the present study, maternal metabolic depression during late pregnancy prevents the calculation of MCP by traditional methods. It is unclear whether maternal metabolic depression during late pregnancy is characteristic of the *Tiliqua* genus or occurs (to varying degrees) in other reptilian viviparous species, and thus the possibility of maternal metabolic depression should be considered in MCP estimates.

In conclusion, breathing patterns at rest were largely unaffected by pregnancy in *T. nigrolutea*, but decreases in metabolic rate in the later stages of pregnancy induced a relative hyperventilation. Lung volume increased during pregnancy compared with that of non-pregnant females, but lung volume was significantly lower during pregnancy compared with post-partum lung volume. A possible redistribution of airflow and blood flow away from the compressed, non-respiratory caudal regions of the lung may help to maintain $D_{L,CO}$ during pregnancy. Pregnant females were unable to affect the breathing pattern alterations induced by exercise in non-pregnant females, and this lack of ability to respond to increased respiratory drive may be one of the mechanisms that underpins the locomotor impairments measured in pregnant lizards.

MATERIALS AND METHODS

Animals

Blotched blue-tongued lizards (*T. nigrolutea*) were collected by hand throughout southeastern Tasmania, and a breeding colony was established at The University of Tasmania, Hobart (42°53′S, 147°19′E), as described in Edwards and Jones (2001). Briefly, animals were held in mixed-sex groups of 10–12 individuals in roofed outdoor enclosures, 1.9×3.4×2.1 m. Enclosures were wire-fronted allowing access to natural photoperiod and ambient temperature: direct sunlight and a supplemental 120 W floodlight globe provided a thermal gradient across which individuals could thermoregulate. Lizards were fed three times a week on meat-flavoured dog food, fresh fruit and frozen mixed vegetables. Vitamin (Herpavite™) and mineral (Multical Dust™) were provided each week. Water was available *ad libitum*. Six gravid and 12 non-gravid females were used in this study. Body mass ranged from 396.4 to 652.6 g (mean±s.e., 540.5±24.5 g) in the non-pregnant females and from 394.7 to 681.0 g in the gravid females (mean±s.e., 600.6±31.8 g) during the late gestational period. Lizards were sexed based on relative head width and the absence of hemipenis musculature. Reproductive condition was assessed based on previous reproductive history and palpation of vitellogenic follicles in the October prior to the mating period. These experiments were performed under University of Tasmania Animal Ethics Committee approval A0012018.

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 previously in similar species (Munns, 2013; Munns and Daniels, 2007; Munns et al., 2004, 2005). A small light-weight mask was constructed from the end of a 20 ml syringe barrel. The mask was fitted over the lizard's nostrils, the mouth sealed, and the mask attached using a non-toxic dental polyether impression material (Impregum F, Henry Schein Halas, Brisbane, QLD, Australia). A pump (Reciprotor AB, Skara, Sweden) pulled fresh room air through the mask at a constant flow set between 0.4 and 0.6 l min^{-1}, depending on the size of the lizard, and controlled with a mass flowmeter (Sierra Instruments Inc., Monterey, CA, 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 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, AD Instruments). 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 desiccant (Drierite, WA Hammond, Xenia, OH, USA) and analysed for fractional concentrations of O_2 (F_{O_2}) and CO_2 (F_{CO_2}) (ML108 gas analyser, AD Instruments). The rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were determined as previously described (Frappell et al., 1992). Briefly:

$$\dot{V}_{O_2} = \text{flow}' \times \left(F'_{I_{O_2}} - F'_{E_{O_2}}\right)/\left(1 - F'_{I_{O_2}}\right), \qquad (3)$$

where I and E represent incurrent and excurrent gas, respectively, and the prime represents dry CO_2-free gas. CO_2 was mathematically scrubbed using $F'_{O_2}=F_{O_2}/(1-F_{CO_2})$:
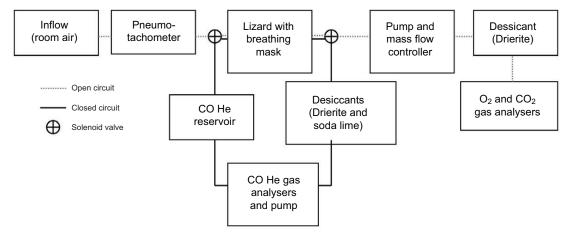
$$\dot{V}_{CO_2} = \text{flow}' \times \left(F'_{E_{CO_2}} - F'_{I_{CO_2}}\right)/\left(1 - F'_{I_{CO_2}}\right), \qquad (4)$$

where the prime represents dry O_2-free gas. Metabolic gas values are reported as dry gases at standard temperature and pressure (STPD).

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 breath-hold period (T_{NVP}) and inspiratory airflow rate (V_T/T_I). An average of 25 consecutive breaths were analysed and ventilatory volumes are reported for saturated gases at body temperature and barometric pressure (BTPS). The air convection requirements for O_2 (ACR $O_2=\dot{V}_E/\dot{V}_{O_2}$) and CO_2 (ACR $CO_2=\dot{V}_E/\dot{V}_{CO_2}$) and respiratory exchange ratio (RER=$\dot{V}_{CO_2}/\dot{V}_{O_2}$) were also calculated.

Measurement of lung volume and lung diffusing capacity

Measurements of lung volume and lung diffusing capacity in adult lizards were obtained using carbon monoxide and helium clearance techniques as previously described for lizards by Frappell et al. (2002a,b). Lizards, with breathing masks attached, were connected to a closed circuit of known volume (V_{sys}), consisting of flexible tubing, carbon monoxide and helium analysers (CO and He Analysers, Morgan, Chatham, Kent, UK), a collapsible reservoir (500 ml paediatric rebreathing bag, Inmed, Malaysia), desiccants Drierite and CO_2 scrubber (soda lime, Dräger, Lübeck, Germany) and a pump that ensured a constant flow (~1000 ml min^{-1}). A series of solenoid valves switched the lizard (during an end-inspiratory pause) from breathing air on the open circuit to breathing the test gas (12.9% He, 0.253% CO in balance of air) on the closed circuit (Fig. 4). When the lizard was connected to the closed circuit, the test gas and the pulmonary air were mixed by the lizard's breathing. CO and He analysers continually measured the concentrations of gases in the closed circuit; the CO continually declined as it diffused across the lung while the He with low diffusibility was diluted to a new level. Lizards were returned to breathing air on the open circuit once He concentrations had reached a new steady-state level (~2–3 min).

Fig. 4. Schematic representation of the experimental set-up used. Breathing patterns and metabolic rates were measured while lizards breathed room air via an open-flow circuit (grey dotted line) consisting of incurrent air being pulled through a pneumotachometer and the lizard's breathing mask via a pump controlled by a mass flow controller. Outflow from the breathing mask was sub-sampled through a desiccant by O_2 and CO_2 gas analysers. Lung diffusing capacity and lung volume were measured via a closed circuit consisting of the lizard with breathing mask, CO He reservoir, desiccants, a pump and CO He gas analysers.

The volume of the system (V_{sys}) was determined by injecting a known volume of air (V_{air}) into the closed circuit containing the test gas and measuring the difference between the initial ($F_{I_{He}}$) and final ($F_{E_{He}}$) fractional concentrations of He in the circuit:

$$V_{sys(BTPS)} = V_{air(BTPS)} \times [F_{E_{He}}/(F_{I_{He}} - F_{E_{He}})]. \quad (5)$$

The volume of the lung (V_L) and the combined circuit ($V_{sys}+V_L$) could then be calculated, given that the solubility of He in blood is very low (0.0095 ml ml^{-1} atm^{-1}) (Muysers and Smidt, 1969):

$$V_{sys(BTPS)} + V_{L(BTPS)} = V_{sys(BTPS)} \times (F_{I_{He}}/F_{E_{He}}). \quad (6)$$

The diffusing capacity of the lung for carbon monoxide ($D_{L,CO}$) was based on methods described previously (Crawford et al., 1976; Depledge, 1985; Frappell et al., 2002a). Briefly, when the lizard was breathing while attached to the closed circuit, the fractional concentration of CO declined continually and thus $D_{L,CO}$ could be calculated by:

$$D_{L,CO} = (V_{sys(STPD)} + V_{L(STPD)})/(P_B - P_{H_2O(30)})$$
$$\times \ln \text{rel-}F_{CO}(t)/t, \quad (7)$$

where t is time and rel-$F_{CO}(t)$ is given by:

$$\text{rel-}F_{CO}(t) = F_{CO}(t) \times F_{He,max}/[F_{CO,max} \times F_{He}(t)]. \quad (8)$$

Experimental protocol

Metabolic rate and breathing patterns were obtained from pregnant and non-pregnant female lizards from December (early pregnancy) to February (late pregnancy) and in the first 24 h after birth. Determining the stage of pregnancy non-invasively in *T. nigrolutea* is difficult and, as a result, data are expressed as weeks prior to birth rather than time post-conception, enabling comparisons to be made between animals without the complication of potentially variable developmental times and unknown conception dates.

Lizards were fasted for a minimum of 72 h prior to commencing experiments to avoid the possible confounding effects 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 wrapped loosely in cotton cloth to discourage movement. After 60 min of breathing air, the lizards were switched to breathing the test gas for 2–3 min. Once He fractional concentrations had reached a steady state, air was returned to the incurrent gas line for a minimum of 30 min and lizards were monitored until normal breathing patterns and levels of $\dot{V}_{O_2}$ were reached. Lizards were then exercised using the righting reflex for 2 min while breathing air and then immediately switched to breathing the test gas on the closed circuit for 2–3 min. Any lizard that failed to right itself over the 2 min exercise period was eliminated from the study. Lizards were returned to breathing air for another 30 min during recovery from exercise.

Measurement of neonatal metabolic rate

In the first 24 h following birth, neonates from each clutch were placed into individual 30°C metabolic chambers with an incurrent air flow rate of 150 ml min^{-1}. For 10 min of each hour, the metabolic chamber was sealed (both incurrent and excurrent air flows from the chamber were stopped) to facilitate the measurement of expired CO_2 and O_2 concentrations. Resting metabolic rates were calculated from the average of periods with low and stable measurements. All individuals from each clutch were measured and average neonate and total clutch metabolic rates were calculated.

Data collection, analysis and statistics

All signals were collected at 1 kHz using a Powerlab data acquisition system (Model 8/30, AD Instruments) and Chart data acquisition software (AD Instruments). For studies using adult lizards, the last 25 consecutive breaths were analysed. All data are presented as means±s.e. Ventilatory volumes are reported at BTPS and $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$ at STPD.

Breathing patterns, metabolic rates, V_L and $D_{L,CO}$ during gestation were analysed using two-way ANOVA ($P<0.05$), followed by paired Dunnett's tests (for comparisons relative to post-partum females) and unpaired Dunnett's tests (for comparisons relative to non-pregnant females).

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