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RESEARCH ARTICLE

The ventilatory response to hypoxia and hypercapnia is absent in the neonatal fat-tailed dunnart

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SUMMARY

At birth, the newborn fat-tailed dunnart relies on cutaneous gas exchange to meet metabolic demands, with continuous lung ventilation emerging several days later. We hypothesised that the delayed expression of lung ventilation ($\dot{V}_E$) in these animals is in part due to a low responsiveness of the respiratory control system to blood gas perturbations. To address this hypothesis, we assessed the ventilatory and metabolic response to hypoxia (10% O_2) and hypercapnia (5% CO_2) using closed-system respirometry from birth to 23 days postpartum (P). Neonatal fat-tailed dunnarts displayed no significant hypoxic or hypercapnic ventilatory responses at any age. Regardless, significant hyperventilation through a suppression of metabolic rate ($\dot{V}_{O_2}$) was observed at birth in response to hypercapnia and in response to hypoxia at all ages, except P12. Therefore, reliance on cutaneous gas exchange during early life may be partially attributed to reduced chemosensitivity or a lack of central integration of chemosensitive afferent information. This may be in part due to the relative immaturity of this species at birth, compared with other mammals.

Key words: chemoresponse, respiration, marsupial, neonatal development.

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INTRODUCTION

In most mammals, the respiratory system is sufficiently developed at birth to function as the sole organ of gas exchange. However, in some marsupials, such as the newborn fat-tailed dunnart (body mass 13 mg), the skin is almost solely responsible for gas exchange, with discernible respiratory efforts generally absent on the day of birth (Mortola et al., 1999; Simpson et al., 2011). A central rhythm generator and sufficiently mature respiratory motoneurones and muscles are essential for coordinated ventilation to occur at birth (Feldman et al., 2003). While afferent information to the respiratory centres from lung and airway mechanoreceptors and peripheral and central chemoreceptors is not required for neuronal rhythmicity, it is important for modulation of the depth, timing and pattern of respiration (Milsom, 1990). The integration of these components is subject to maturational changes and as such neonatal breathing is inherently unstable (Hilaire and Duron, 1999).

The respiratory response to reduced O_2 (hypoxia) or increased CO_2 (hypercapnia) is dependent on the relative size and maturity of the species at birth, and generally increases with postnatal age (Bonora et al., 1994), in part as a result of the increasing sensitivity of both central and peripheral chemoreceptors (Hanson et al., 1989; Davis et al., 2006). In contrast to the adult, the newborn typically does not sustain an increase in minute ventilation ($\dot{V}_E$; hyperpnoea) in response to hypoxia, and in some cases $\dot{V}_E$ during hypoxia falls below pre-hypoxic levels (Neubauer et al., 1990). In general, reductions in $\dot{V}_E$ during hypoxia are associated with decreases in either the mean inspiratory flow (tidal volume V_T/inspiratory time T_I), a correlate of central respiratory drive, or duty cycle (T_I/total breath time T_{TOT}). Hypometabolism also plays an important role in neonates in mitigating the effects of hypoxia (Mortola, 1993). Despite the reduced breathing in newborns subjected to acute hypoxia, they still hyperventilate because of a larger drop in the rate of oxygen consumption ($\dot{V}_{O_2}$) compared with $\dot{V}_E$. While the hypercapnic ventilatory response of most newborn species is reduced compared with that of adults (Carroll et al., 1993; Carroll and Fitzgerald, 1993; Davis et al., 2006), unlike hypoxia, it is sustained over time by a persistent increase in tidal volume (V_T), despite a gradual decline in the initial respiratory frequency (f) increase (Bonora et al., 1994; Cummings and Frappell, 2009). Exceptions to these patterns exist, however, with the neonatal North American opossum (another marsupial) demonstrating an unusually large ventilatory response to hypoxia and a hypometabolic response to hypercapnia (Farber, 1972), which attenuates with age (Farber et al., 1972).

Using an *in vitro* brainstem preparation, it has been demonstrated that the neonatal opossum [postpartum day (P)5], born after a similar gestation to that of the fat-tailed dunnart (13 days), possesses neurons that fire rhythmically at a rate sufficient for normal breathing (Farber, 1993; Eugenín and Nicholls, 2000). In the fat-tailed dunnart, phrenic motoneurons have made contact with the diaphragm at birth, yet continuous breathing does not occur until P3 (Frappell and MacFarlane, 2006; Simpson et al., 2011). If the central pattern generator(s) of the fat-tailed dunnart fires rhythmically, as in the opossum, then there may be an insufficient inspiratory drive, preventing the emergence of normal breathing. In the early neonatal period of the fat-tailed dunnart, reduced inspiratory drive may be due to effective cutaneous exchange and hence a lack of stimulus to the chemoreceptors or may alternatively be a

consequence of reduced chemoreceptor responsiveness. In this study, we therefore aimed to characterise the ventilatory and metabolic response to hypoxia and hypercapnia in the neonatal fat-tailed dunnart to determine the chemoreceptor response during the neonatal period.

MATERIALS AND METHODS

Ventilatory and metabolic responses to inspired gases (air, hypercapnia, hypoxia) were measured in fat-tailed dunnart, *Sminthopsis crassicaudata* (Gould 1844), pouch young in different individuals at P0 (the day of birth) (N=12, 6, 4), P5 (N=7, 4, 4), P12 (N=9, 6, 5) and P23 (N=9, 4, 5) using a closed respirometry system as previously described (Frappell et al., 1989; Simpson et al., 2011). Briefly, an animal was masked and placed into a water-jacketed chamber maintained at a constant temperature of 36°C (pouch temperature) and 100% relative humidity, the chamber effectively separating the head and body. After an equilibration time, the chamber was sealed for a known period of time depending on the mass and, hence, metabolic rate of the animal (5–15 min). Each compartment was then flushed with a known flow (21 ml min^{-1}), and the gas passed through Nafion tubing (dead space 0.6 ml) surrounded by a molecular sieve desiccant (crystalline metal aluminosilicate zeolite) prior to being analysed for fractional concentrations of O_2 and CO_2 by gas analysers (ML205, ADInstruments, Colorado Springs, CO, USA). The output of each gas analyser was recorded at 200 Hz (Chart 4.2 and PowerLab, ADInstruments). The rates of oxygen consumption ($\dot{V}_{O_2}$) and carbon dioxide production ($\dot{V}_{CO_2}$) were calculated from the time integral of the gas concentration curves multiplied by the flow and the reciprocal of the time for which the chamber was sealed (Frappell and Mortola, 2000).

Ventilation ($\dot{V}_E$) was measured when the chamber was sealed *via* a pressure transducer (Spirometer ML141, ADInstruments) connected to the head compartment, and the pressure oscillations were acquired at 200 Hz (Chart 4.2 and PowerLab). The pressure oscillations associated with breathing were calibrated for volume by the injection and withdrawal of 2 µl of air and the stability of the pressure change with each injection was also used to indicate the integrity of the seal for the chamber. When analysing $\dot{V}_E$, at least 50 consecutive breaths were analysed for tidal volume (V_T), inspiratory time (T_I), expiratory time (T_E), post-inspiratory pause (T_P; note that T_P is fractional to T_E because it represents the passive-static component of T_E, achieved with closed glottis), total breath time (T_{TOT}=T_I+T_E), respiratory frequency (f=60/T_{TOT}), mean inspiratory flow (V_T/T_I), duty cycle (T_I/T_{TOT}) and minute ventilation ($\dot{V}_E$=V_Txf). $\dot{V}_E$ in P0 animals displayed marked instability with prolonged apnoea (Simpson et al., 2011); therefore, all recorded breaths were analysed in these animals. Animals in which $\dot{V}_E$ was not discernible were not included in the analysis. $\dot{V}_{O_2}$ is expressed at standard temperature, pressure and humidity (STPD: 1 ml O_2=0.0446 mmol O_2) and volume at body temperature, pressure and humidity (BTPS).

Measurements of $\dot{V}_E$ and $\dot{V}_{O_2}$ were first conducted in room air (~21% O_2, 0.03% CO_2, balance N_2). Animals were then subjected to either hypoxia (10% O_2, balance N_2) or hypercapnia (21% O_2, 5% CO_2, balance N_2) supplied by a Wosthoff gas-mixing pump. In the case of hypercapnia, the animals were exposed to 5% CO_2 for 5 min before the chamber was sealed for measurement, and analysis of the breathing pattern was performed within the first minute (that is, at 5–6 min CO_2 exposure, allowing examination of the total response, i.e. that due to both peripheral and central chemoreceptor activation). When exposed to hypoxia, the chamber was sealed as soon as the gas mix had washed in and oxygen levels had stabilised at 10% O_2 so that we could analyse the early (1 min of hypoxia) and late (6 min of hypoxia) breathing response, thereby enabling the characterisation of a bi-phasic response, should it occur. After measurement of the ventilatory responses to an inspired gas, the chamber was flushed and metabolic rate was determined. The chamber was then flushed for 10 min with room air, prior to a second air measurement, which was used to determine whether the animal preparation had degenerated during the experiment. In the rare event that this occurred, the entire run was rejected.

Student's t-tests were used to determine whether the value obtained during a particular gas challenge was different from the value in normal room air using SPSS (version 19). The effect of age and gas condition, as well as their interaction, was assessed for each metabolic and respiratory variable using a two-way ANOVA. Significance was considered at P<0.05.

RESULTS

Development of the breathing pattern in air

No discernible ventilatory pattern was observed in 2/3 of neonates at P0 (body mass 13 mg). Where a ventilatory pattern was detected, breathing was generally accompanied by extended periods of apnoea, such that breathing may not have been observed again for the remainder of the measurement period. By P5, the continuously expressed breathing pattern was characterised by a post-inspiratory pause, together with frequent augmented breaths, or sighs [see Simpson et al. (Simpson et al., 2011) for representative breathing traces]. There were some periods of instability, as indicated by the coefficients of variation for T_{TOT} and V_T being 11% and 45% respectively. At P12, some animals had a discernible post-inspiratory pause, while others did not, leading to further increased variability in T_{TOT} (37%) and V_T (68%). Thus, P12 is presumably a time of transition from the 'immature' to 'adult' breathing pattern. No discernible post-inspiratory pauses were detected in P23 neonates. Spirograms demonstrating a characteristic breath, in terms of timing and amplitude, are shown for each age in Fig. 1.

The effects of hypercapnia on fat-tailed dunnart neonates

While there was a tendency for convective requirement $\dot{V}_E/\dot{V}_{O_2}$ to increase at all ages in response to 5% CO_2, a significant effect was

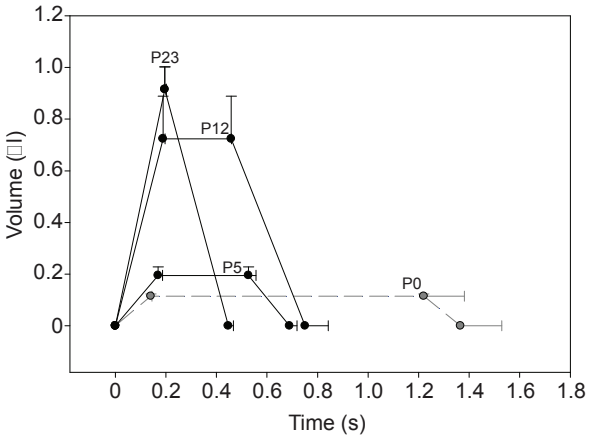

Fig. 1. Spirogram demonstrating a characteristic breath for fat-tailed dunnarts at postpartum days (P)0, 5, 12 and 23 under normoxic/normocapnic conditions. Respiratory timing variables and tidal volumes are shown for neonatal fat-tailed dunnarts at P5, 12 and 23 in black. Data for P0 (grey) include all measurable breaths.

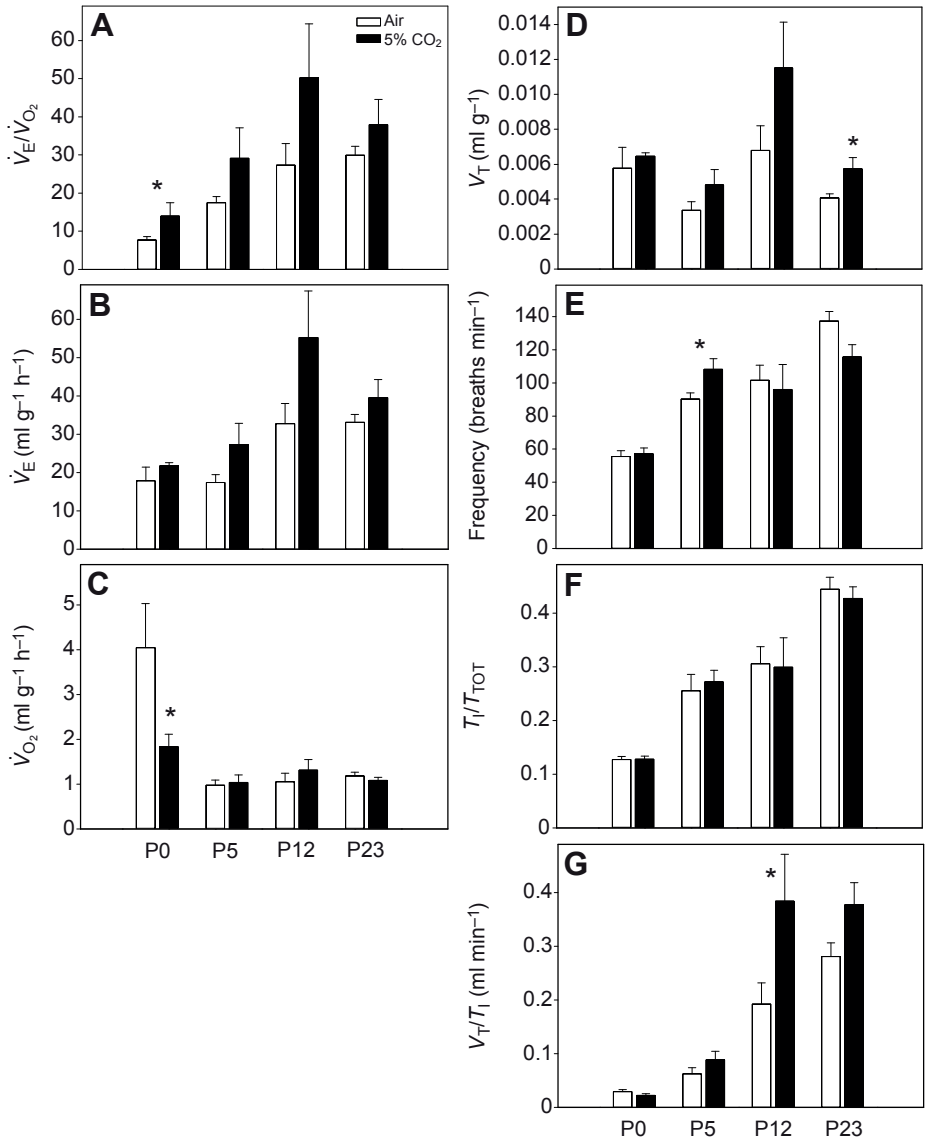

Fig. 2. The effects of hypercapnia on breathing and metabolism in the neonatal fat-tailed dunnart. Changes in convective requirement ($\dot{V}_E/\dot{V}_{O_2}$; A), mass-specific minute ventilation ($\dot{V}_E$; B), total mass-specific rate of oxygen consumption (skin + pulmonary, $\dot{V}_{O_2}$; C), tidal volume (V_T; D), respiratory frequency (E), duty cycle (T_I/T_{TOT}; F) and respiratory drive (V_T/T_I; G) are shown after 5 min exposure to 5% CO_2 in P0, 5, 12 and 23 fat-tailed dunnarts. For definitions, see List of symbols and abbreviations. Data are presented as means ± 1 s.e.m. *Significant difference from air.

only observed at P0, owing to an unexpected but significant decrease in $\dot{V}_{O_2}$ (P<0.05; Fig. 2A,C). Increasing the CO_2 level decreased $\dot{V}_{O_2}$ only at P0 (P<0.05), and not in older animals (Fig. 2C). There was no significant hypercapnic ventilatory response at any age; the response approached significance in animals older than P0 (P<0.075; Fig. 2B). The attempted hyperpnoeic effort observed in P5 animals exposed to 5% CO_2 was due mainly to a significant increase in f, while at P23 the attempted response was dominated by a significant increase in V_T (Fig. 2D,E). The decrease in f experienced by P23 animals, however, negated any increase in $\dot{V}_E$. There were no significant changes in duty cycle in response to 5% CO_2 exposure (Fig. 2F). There was a significant increase in mean inspiratory flow V_T/T_I in response to CO_2 at P12, while the effect at P23 was marginal (P=0.06; Fig. 2G).

The effects of hypoxia through development in the fat-tailed dunnart

Across all ages, except P12, there was a significant increase in $\dot{V}_E/\dot{V}_{O_2}$ after 1 min of hypoxia (P<0.05; Fig. 3A). This effect was achieved predominantly by a decrease in $\dot{V}_{O_2}$ (P<0.05, Fig. 3C). There was no increase in $\dot{V}_E$ in response to hypoxia, regardless of the duration of exposure. Hypoxia actually suppressed $\dot{V}_E$ in P5 and P12 animals; at P12 this suppression occurred after only 1 min (Fig. 3B). The decrease in $\dot{V}_E$ in response to hypoxia was mainly due to a decrease in V_T at all ages except P23 (Fig. 3D). At P0, the fall in V_T was offset by an increase in f, thus maintaining $\dot{V}_E$ during hypoxia. The increase in f was reflected in an increase in T_I/T_{TOT} (i.e. reduced expiratory time) (Fig. 3E,F). The absence of a ventilatory response at any age precluded any increase in respiratory drive (V_T/T_I) (Fig. 3G).

In addition, analysis with two-factor ANOVA demonstrated that increasing age contributed to alterations in all respiratory and metabolic variables over the course of the neonatal period (P<0.05), while the evolution of the response to the gas challenge was evident in all respiratory and metabolic outcomes except f and T_I/T_{TOT} during the developmental period studied. Convective requirement ($\dot{V}_E/\dot{V}_{O_2}$) was the only variable to demonstrate a significant interaction between age and the gas condition (P=0.025).

In summary, no significant ventilatory response to hypoxia and hypercapnia was observed in the neonatal fat-tailed dunnart (Fig. 4). Regardless, a hyperventilatory response (increase in $\dot{V}_E/\dot{V}_{O_2}$) was observed in response to hypoxia (and hypercapnia at P0) owing to a suppression of $\dot{V}_{O_2}$.

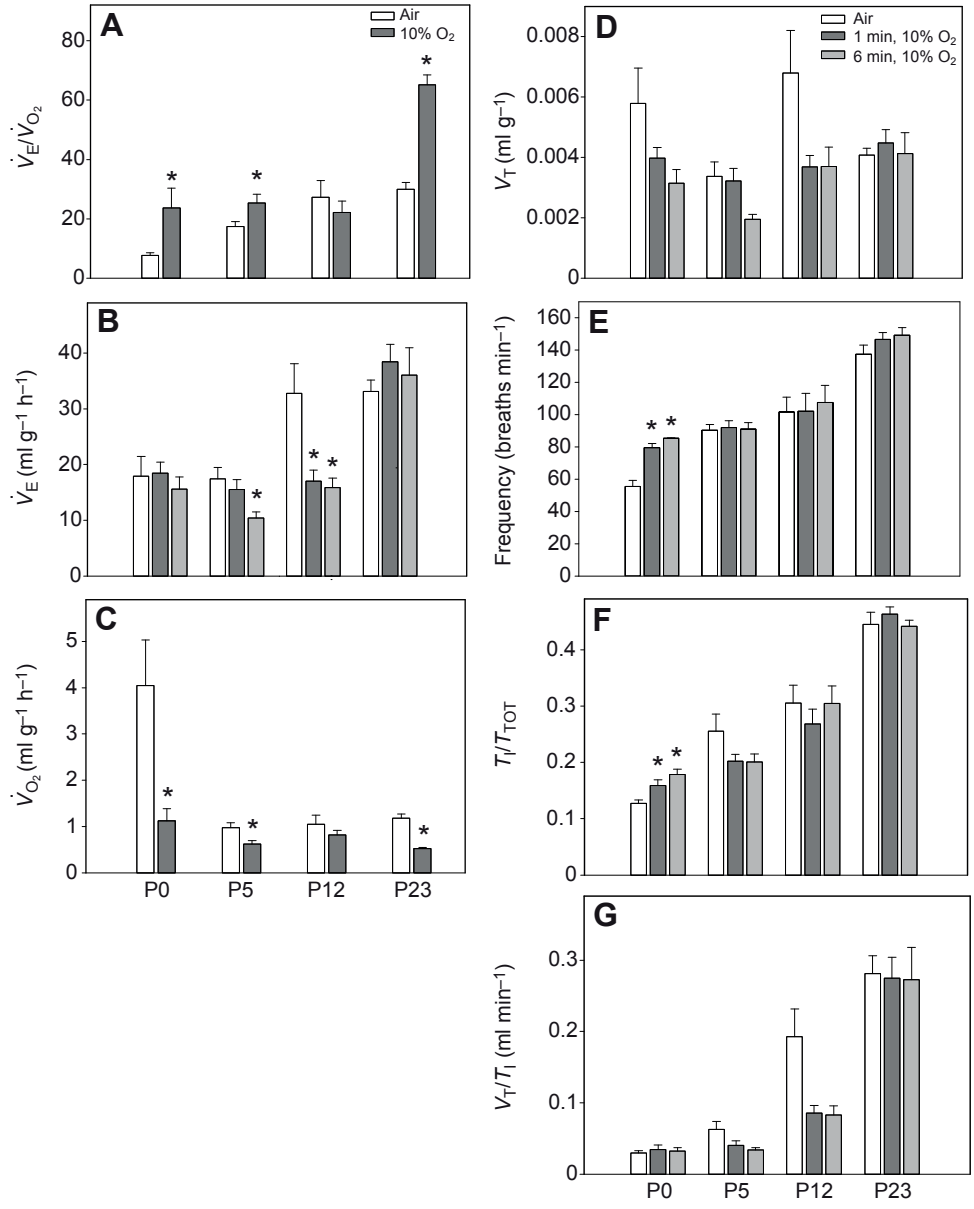

Fig. 3. The effects of hypoxia on breathing and metabolism in the neonatal fat-tailed dunnart. Changes in convective requirement ($\dot{V}_E/\dot{V}_{O_2}$; A), mass-specific minute ventilation ($\dot{V}_E$; B), total mass-specific rate of oxygen consumption (skin + pulmonary, $\dot{V}_{O_2}$; C), tidal volume (V_T; D), respiratory frequency (E), duty cycle (T_I/T_{TOT}; F) and respiratory drive (V_T/T_I; G) are shown for breathing variables after 1 and 6 min exposure to 10% O_2 and for metabolic rate after the total time the chamber was sealed. For definitions, see List of symbols and abbreviations. Data are presented as means ± 1 s.e.m. *Significant difference from air.

DISCUSSION

To summarise, a very limited ventilatory response to hypoxia and hypercapnia was evident in the neonatal fat-tailed dunnart. Despite this, suppression of $\dot{V}_{O_2}$ resulted in a hyperventilatory response (increase in $\dot{V}_E/\dot{V}_{O_2}$) to hypoxia at all ages, except P12, and hypercapnia at P0.

Development of the breathing pattern

The breathing pattern of the neonatal fat-tailed dunnart undergoes considerable postnatal development, with the post-inspiratory pause diminishing, and V_T, T_I and f increasing. Breathing was not continuously expressed in P0 neonates, and when present f was low. We have previously postulated that these movements are more likely to be akin to fetal breathing movements, preparing the musculature for breathing rather than participating in alveolar gas exchange (Simpson et al., 2011). The respiratory network of the opossum, a species born after a similar gestation to the dunnart, is capable of generating activity sufficient for normal breathing. If this is also the case in the dunnart, it may be that reduced responsiveness of respiration to changing blood gases, possibly *via* reduced chemoreceptor activity, contributes to their relatively reduced $\dot{V}_E/\dot{V}_{O_2}$ compared with that of other newborn mammals. Regardless, the ability of the dunnart to exchange gas across the skin perhaps renders inconsequential the relative immaturity of the respiratory control system (Simpson et al., 2011).

Hypometabolism during hypercapnia: a novel strategy

We have shown that, unlike other newborn mammals (Bonora et al., 1994; Mortola and Lanthier, 1996; Saiki and Mortola, 1996) the newborn fat-tailed dunnart utilises a hypometabolic strategy to mitigate the effects of increasing CO_2. While the newborns of other species exhibit an attenuated ventilatory response to hypercapnia (and hypoxia) in comparison to adults, there is nearly always a discernible ventilatory response, even at birth (Mortola and Lanthier, 1996). While there was some evidence of an increase in respiratory f (P5), V_T (P23) and V_T/T_I (P12), overall, we were unable to detect any significant increase in $\dot{V}_E$ in response to hypercapnia throughout the first 23 days of life. Unlike the majority of newborn mammals,

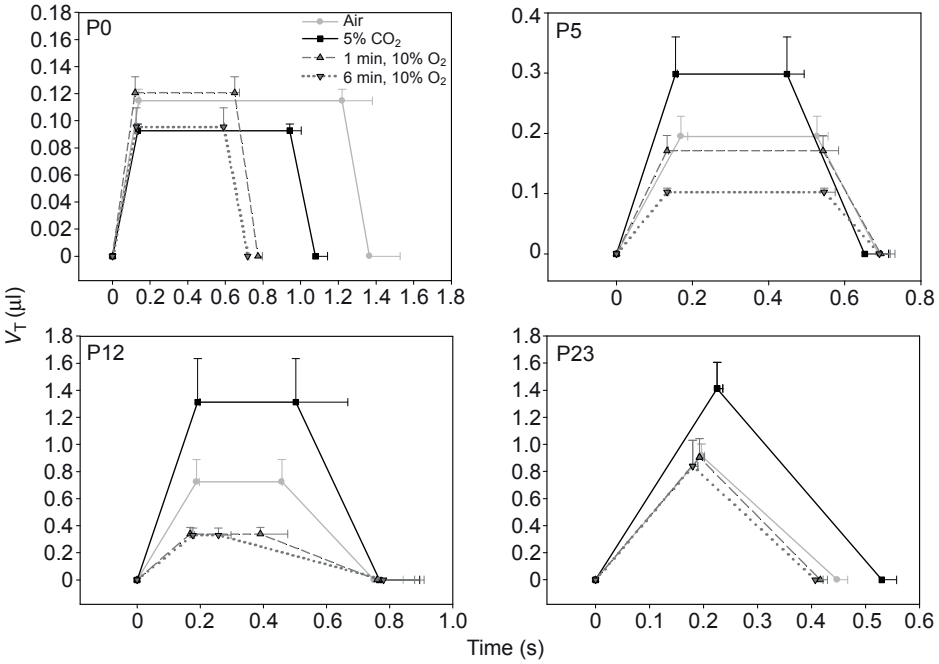

Fig. 4. Spirograms demonstrating the change in breath timing and tidal volume (V_T) in response to hypoxia and hypercapnia in developing fat-tailed dunnart neonates. Shown are mean control values (air; light grey) at P0, 5, 12 and 23, the effects of hypercapnia (solid black) at 5 min and hypoxia at 1 min (dash dark grey) and 6 min (dash-dot dark grey). Error bars represent 1 s.e.m.

our newborn dunnarts utilised a hypometabolic strategy in response to hypercapnia; hypometabolism is observed in most other newborn mammals during hypoxia. While a higher mass-specific metabolic rate is observed in the control (room air) P0 dunnarts when compared with the older neonates, this is not unexpected. Birth is a tumultuous event associated with drastic changes in environmental conditions. The surge of catecholamines associated with the stress of parturition generally results in newborns having a higher mass-specific $\dot{V}_{O_2}$ (Bartlett and Areson, 1977; Mortola, 2001), with $\dot{V}_{O_2}$ decreasing markedly within the first 24 h after birth (Noblet and Le Dividich, 1981; Stewart et al., 1984). While previous studies have demonstrated the absence of this increased mass-specific metabolic rate in neonatal marsupials (Singer et al., 1995; MacFarlane and Frappell, 2001; Szdzuy et al., 2008), these studies were not concentrated on young in the first hours of life, where the newborn may have just recently attached to a teat and not yet fully settled.

In addition to our newborn dunnarts, a hypometabolism during hypercapnia has also been described in the P5 opossum, albeit in conjunction with a hyperpnoea (Farber et al., 1972). Hypometabolism may be the preferred strategy for the opossum and dunnart because of mechanical constraints on the respiratory system related to their relative immaturity (MacFarlane et al., 2002). But it is equally plausible that marsupials generally have reduced chemosensitivity or increased blood-buffering capacity owing to their pouch development and exposure to elevated CO_2 (Frappell and Baudinette, 1995; Frappell et al., 2002). As our analysis of the hypercapnic response was limited to the period following 5 min exposure to CO_2 (representing the integrated response of both the peripheral and central chemoreceptors), it is possible that the ventilatory response in the dunnart peaks before 5 min of exposure. In neonatal rats, $\dot{V}_E$ has been shown to decrease slightly between 3 and 5 min though it was still significantly elevated at 5 min with respect to baseline (Cummings and Frappell, 2009). Alternatively, it may be that the reduced response we observed was due to the small size of the dunnarts. Others have described a size dependency of the $\dot{V}_E$ response to CO_2, with larger newborns possessing a greater ventilatory response than smaller ones (Mortola and Lanthier, 1996).

The effects of hypoxia on breathing and metabolism

Hypoxia did not lead to an increase in $\dot{V}_E$ at any age. In fact, hypoxia caused a sustained decrease in $\dot{V}_E$, similar to newborns of other species, including preterm humans (Alvaro et al., 1992; Mortola et al., 1992). Regardless, other than at P12, our dunnarts still hyperventilated, as the fall in $\dot{V}_{O_2}$ was greater than the fall in $\dot{V}_E$. While the decrease in $\dot{V}_E$ during hypoxia could be due to the immaturity of the peripheral chemoreceptors, adult fat-tailed dunnarts also have a relatively weak hypoxic ventilatory response, with $\dot{V}_E$ dropping below normoxic levels (Frappell et al., 1992). Thus, the carotid body of the dunnart may be generally less responsive to hypoxia compared with that of other species. Alternatively, there could be an active, centrally mediated suppression of $\dot{V}_E$ (Blanco et al., 1984; Coles and Dick, 1996).

Despite the lack of ventilatory response, the hypometabolic response observed in the fat-tailed dunnarts is sufficient to increase $\dot{V}_E/\dot{V}_{O_2}$ for the preservation of tissue oxygenation and acid–base balance. The decreased ventilatory response to hypoxia may drive the hypometabolism *via* extreme hypoxaemia, or the hypometabolism may be an adaptive response that negates the need for an increase in $\dot{V}_E$ for energy conservation. Why animals did not hyperventilate at P12 is unknown, but it may reflect either the high tidal volume observed in control animals (probably a result of the highly variable breathing pattern and the switch from neonatal to adult breathing in this age group), which stabilised during CO_2 exposure, or a window of development where these animals are more vulnerable to hypoxia. Such a window of vulnerability has been reported in P12 rats with an absence of increasing $\dot{V}_E/\dot{V}_{O_2}$, due to a decrease in tidal volume and a comparatively weak metabolic response to hypoxia; a response that was different from that in the rest of the first 3 postnatal weeks (Liu et al., 2006). In addition, a reduction in excitatory and an increase in inhibitory neurotransmitters (Wong-Riley and Liu, 2005) as well as a significant decline in tryptophan hydroxylase and serotonin transporter immunoreactivity (Liu and Wong-Riley, 2010b; Liu and Wong-Riley, 2010a) further suggests that normal development can contribute to a narrow window of vulnerability in rats at this age (P12), though lack of proper controls limits the interpretation of

these developmental studies. Developmentally, the P12 dunnart (eye opening at P49; weaning at P70) is markedly more immature when compared with the P12 rat (eye opening at P14.5; weaning at P21), with lungs that are yet to commence alveolarisation (Simpson et al., 2011). However, P12 marks the period where the breathing pattern changes from the neonatal pattern (with the presence of a post-inspiratory pause) to a typical adult mammalian pattern, indicating that this is an important time for respiratory system development in the neonatal fat-tailed dunnart.

In summary, the reduced $\dot{V}_E$ and $\dot{V}_E/\dot{V}_{O_2}$ exhibited by the fat-tailed dunnart in early postnatal life may be at least in part due to severely reduced sensitivity of the respiratory control system to hypoxia and hypercapnia. In turn, this may be due to the relative immaturity of this species, with central and peripheral chemoreceptors being underdeveloped. Despite this, these animals probably maintain tissue oxygenation and pH *via* a hypometabolic response. Reduced chemoresponses and low $\dot{V}_E/\dot{V}_{O_2}$ are most likely to be the response to an underdeveloped lung and respiratory system, hence the heavy reliance on skin gas exchange during early postnatal life in the dunnart.

LIST OF SYMBOLS AND ABBREVIATIONS

f	frequency of breathing
P	days postpartum
T_E	expiratory time
T_I	inspiratory time
T_I/T_{TOT}	duty cycle
T_{TOT}	total breath time
$\dot{V}_E$	minute ventilation
$\dot{V}_E/\dot{V}_{O_2}$	convective requirement
$\dot{V}_{O_2}$	rate of oxygen consumption
V_T	tidal volume
V_T/T_I	mean inspiratory flow

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