Altered autonomic control of heart rate variability in the chronically hypoxic fetus

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Abstract

Although fetal heart rate variability (FHRV) has long been recognised as a powerful predictor of fetal wellbeing, the mechanisms by which it is reduced in the chronically hypoxic fetus have yet to be established. In this study, we present a longitudinal study of the development of autonomic control of FHRV, assessed by time domain and power spectral analysis, in normoxic and chronically hypoxic, chronically instrumented, singleton fetal sheep over the last third of gestation. We used isobaric chambers able to maintain pregnancy sheep for prolonged periods in hypoxic conditions, and a customised wireless data acquisition system to record beat-to-beat variation in the fetal heart rate. We determined in vivo longitudinal changes in overall FHRV and the sympathetic and parasympathetic contribution to FHRV in chronically hypoxic (n=6) and normoxic (n=6) ovine fetuses with advancing gestational age. Normoxic fetuses show gestational age-related increases in overall indices of FHRV, and in the sympathetic nervous system contribution to FHRV. Conversely, gestational-age related increases in overall FHRV were impaired by exposure to chronic hypoxia, and there was evidence of suppression of the sympathetic nervous system control of FHRV in the chronically hypoxic fetus. The data show that exposure to late gestation isolated chronic fetal hypoxia alters the development of the autonomic nervous system control of FHRV in sheep. Reduction in overall FHRV, or STV, may therefore provide an important biomarker in obstetric medicine to highlight that autonomic dysregulation of FHR control has taken place in a fetus carried by a pregnancy where uteroplacental dysfunction is suspected.
Introduction

Antenatal electronic monitoring of fetal heart rate variability (FHRV) is an important clinical tool to assess the fetal condition, as authoritatively described over many years by Parer (Fox et al., 2000). It is routinely used to assess fetal wellbeing in pregnancies affected by utero-placental dysfunction and profound, prolonged reductions in FHRV are thought to represent acute fetal compromise (Turan et al., 2007; Serra et al., 2008). An increasingly commonly used non-invasive clinical measure, short term variation (STV), is a statistical summary measure of interpolated beat-to-beat variation in the fetal heart rate, excluding periods of pronounced accelerations and decelerations. A persistent reduction in STV below 3 ms in the antenatal period, within 24 hours of delivery, is predictive of an increased risk of metabolic acidosis in the neonate at birth, and early neonatal death (Serra et al., 2008). As such, STV, as part of computerised CTG (cCTG) examination, is recommended for antenatal surveillance of human fetuses with suspected utero-placental dysfunction and chronic fetal hypoxia to detect acute fetal distress (Royal College of Obstetricians and Gynaecologists (2013)). However, the physiological basis underlying this reduction in FHRV remains unclear.

The fetal heart rate fluctuates under the influence of basal sympathetic and parasympathetic tone, different fetal sleep states (Nijhuis et al., 1982), and has some intrinsic variability (Kimura et al., 1996). The sympathetic and parasympathetic influences on FHRV mature at different rates throughout gestation (Walker et al., 1979), and their relative contributions can be assessed by time domain and power spectral analysis. However, unlike cCTG, these methods require the FHR to be acquired on an actual, not interpolated, beat-to-beat basis for reliable results, so that the time R-R interval between each heart beat can be measured. This mandates the insertion of a fetal electrode, arterial catheter or flow probe, which has significantly limited human studies in this field and thereby clinical application (Peters et al.,
Recently, fetal MRI has been used to acquire FHR on a sufficiently accurate beat-to-beat basis to allow such analysis, termed a fetal magnetocardiogram (fMCG) (Ferrario et al., 2009; Sriram et al., 2013).

Time domain analyses, encompassing the standard deviation of normal to normal R-R intervals (SDNN) and the root mean square of successive differences (RMSSD), are statistical summary measures of the successive time differences between each heart pulsation. SDNN represents overall FHRV and RMSSD reflects the parasympathetic control of FHRV (Malik et al., 1996). Power spectral analysis of the heart rate variability determines the energy in specific frequency components of heart rate variability and allows a more precise evaluation of the sympathovagal balance than time domain measures. The low frequency component (LF) reflects predominantly sympathetic control of heart rate variability (Kimura et al., 1996); the high frequency component (HF) reflects parasympathetic control of heart rate variability (Akselrod et al., 1981). Total power reflects overall FHRV, but is affected by the absolute heart rate (Malliani et al., 1994). Therefore, in longitudinal studies, where fetal heart rate changes with advancing gestation, normalised LF and HF (LF or HF as a proportion of total power) should be presented to detect changes in relative LF and HF power, which can otherwise be masked by changes in total power (Van Laar et al., 2008).

Time domain and power spectral indices of FHRV need to be separated by fetal behavioural state. Fetal behavioural states (1F-4F) relate to quiet and active sleep (1F = quiet sleep, 2F = active sleep). The correlation between oscillation bandwidth of heart rate variability and frequency of accelerations of fetal heart rate is sufficiently strong that different states can be determined by visual identification of heart rate patterns alone (Schaffer et al., 2009), an established method previously used in this field (van Laar et al., 2009). In ovine pregnancy, fetal electrocortical (ECoG) recordings have been used to differentiate between quiet (low
voltage ECoG) and active sleep (high voltage ECoG) (Koome et al., 2014a). Intrauterine acute hypoxaemia is known to switch fetal ECoG activity predominantly to high voltage (Boddy et al., 1974; Clewlow et al., 1983; Bamford & Dawes, 1990). However, ultrasound-CTG studies (Henson et al., 1984), fMCG studies (Sriram et al., 2013) as well as ECoG studies in chronically instrumented sheep (Keen et al., 2011) demonstrate that the relative time spent in each behavioural state is unchanged between healthy and chronically hypoxic fetuses. This not only suggests some resilience in the control of fetal behavioural states during adverse intrauterine conditions, but also that fetal behavioural states may be successfully identified by visual inspection of the fetal heart rate.

A recent elegant study investigated the normal evolution of frequency power spectra over the last third of gestation in chronically instrumented, healthy singleton fetal sheep at 0.6, 0.7 and 0.8 of gestation. The study reported that total spectral power increased with advancing gestational age, and that sympathetic dominance of FHRV was more evident in active than quiet sleep (Koome et al., 2014a). Similar studies in chronically hypoxic sheep fetuses have not been reported to date. Progress in this field has been hampered in part by the inability to record continuous cardiovascular function in the fetus during the period of chronic fetal hypoxia. We have now designed and created isobaric hypoxic chambers able to maintain pregnant sheep for prolonged periods of gestation under controlled, long-term hypoxia (Brain et al., 2015). We have also established a wireless data acquisition system, able to record fetal cardiovascular signals from free moving ewes as the hypoxic pregnancy is developing (Brain et al., 2015; Allison et al., 2016). In this study, we have used these novel technologies to determine a longitudinal study of the development of FHRV, assessed by time domain and power spectral analysis, in normoxic and chronically hypoxic, chronically instrumented, singleton fetal sheep over the last third of gestation.
The study tested the hypothesis that hypoxic pregnancy affects the ontogeny of the fetal heart rate power spectrum in the last third of gestation, and contributes to the reduction in FHRV measured in chronically hypoxic fetuses.

**Methods**

All procedures were performed in accordance with the UK Animals (Scientific Procedures) Act 1986 and were approved by the Ethical Review Committee of the University of Cambridge.

**Surgical preparation and post-operative care.** Twelve pregnant Welsh mountain sheep carrying singleton fetuses were used in the study. In brief, using the protocol described in (Allison et al., 2016), at 117±1 days of gestation (0.8 of gestation; term ~150 days), under isoflurane general anaesthesia, a midline laparotomy and hysterotomy were performed to allow insertion of arterial catheters into the fetal carotid and femoral arteries unilaterally. The femoral artery catheter was advanced into descending aorta. Transonic flow probes were placed around the contralateral fetal carotid and femoral arteries (2mm aperture, S-series, Transonic Systems Inc., Ithaca, USA) and the data used in separate studies. The fetus was returned to the amniotic cavity, amitotic membranes were sealed and uterine and abdominal incisions were closed. An arterial catheter was introduced into the maternal femoral artery and advanced into the descending aorta. Catheters and flow probes were exteriorised via the maternal flank. Catheters were connected to pressure transducers (ArgoTrans®, Argon Medical Devices Inc. Texa, USA). Pressure transducers and flow probe cables were connected to the Cambridge Data Acquisition System (CamDAS, Figure 1).
Following surgery, ewes were housed in individual floor pens with a 12h light and dark cycle with *ad libitum* access to hay, nuts and water. After 5 days post-operative recovery, ewes and fetuses were randomly allocated to chronic normoxia (n=6) or chronic hypoxia (n=6). Chronically hypoxic animals were housed in bespoke isobaric hypoxic chambers for 2 days prior to the initiation of hypoxia, then a further 10 days before being returned to the individual floor pens. Hypoxia was induced incrementally over the first 24 h, then maintained at 10% maternal inspired oxygen for the remainder of the experimental protocol, the full details of which have been previously described in (Brain *et al.*, 2015; Allison *et al.*, 2016). Pregnancies allocated to the chronic normoxia group were housed in a barn in floor pens with the same floor area as that of the hypoxic chambers. Both the chronic normoxia and chronic hypoxia groups of ewes were fed daily the same bespoke maintenance diet made up of concentrate pellets and hay (40 g nuts kg\(^{-1}\) and 3 g hay kg\(^{-1}\); Manor Farm Feeds Ltd, Oakham, UK) to facilitate the monitoring of food intake.

**Fetal Heart Rate recording and identification of sleep state.** Continuous physiological values of fetal heart rate were recorded using a customised data acquisition system, CamDAS (Figure 1), from the immediately post-operative period to the end of the experiment (Allison *et al.*, 2016). These data were converted into absolute physiological values by IDEEQ data recording software at a sampling rate of 500 kHz (IDEEQ, Maastricht Instruments, Maastricht, The Netherlands), and was available thereafter for offline data analysis. Fetal heart rate was sampled daily in 6 x 5 min blocks between midnight and 0600. Mean values for sequential 2.5 s epochs throughout the recording period were generated for cardiovascular data using Labchart 7 Pro (AD Instruments Pty Ltd., NSW, Australia) and used to create a CTG of fetal heart rate (Figure 2). Segments were visually identified as FHR patterns A-D, and quiet sleep was defined as FHR pattern A and active sleep as FHR pattern B. If no
suitable FHR pattern was acquired, re-sampling was performed within the same time period for that day.

**Fetal heart rate variability analysis.** Time domain and power spectral analysis was performed in Labchart 7 Pro (AD Instruments Pty Ltd., NSW, Australia). Based on previous published literature the frequency boundaries used were: VLF 0-0.04 Hz, LF 0.04-0.15 Hz, HF 0.15-0.4 Hz (Min et al., 2002; van Laar et al., 2009; Koome et al., 2014b). Mean daily values for average heart rate, SDNN, RMSSD, absolute and normalised LF and HF, LF/HF ratio, absolute VLF and total power were calculated daily from the categorised 5 min samples. The baseline value quoted represents an average of 72 h. STV was calculated using R-R intervals: first average heart rate was used to calculate the baseline R-R value; second the baseline R-R value was used to exclude values representing accelerations or decelerations as these are excluded by STV calculation software (Pardey et al., 2002). The remaining values were averaged in 3.75 s (1/16th minute) epochs and the mean difference between 16 sequential periods (1 minute mean difference) was calculated as per published STV calculation algorithms (Street et al., 1991). Final STV values are the mean of the 5 min mean difference values. All these values were compared between chronically normoxic and chronically hypoxic pregnancies in quiet and active sleep states.

**Blood sampling regime and analysis.** Samples of descending aortic fetal (0.3 mL) and maternal (1 mL) blood were taken daily. These were used to determine acid-base status, partial pressures of oxygen and carbon dioxide (ABL5 Blood Gas Analyser, Radiometer, Copenhagen, Denmark); haemoglobin and oxygen saturation of the blood (OSM3, Radiometer).
**Determination of hormone concentrations.** An additional 1 ml of fetal arterial blood was taken after 5 days postoperative recovery (baseline) and 2 days after removal from chronic hypoxia, or equivalent time in normoxic controls (day 13) for determination of plasma cortisol and catecholamine concentrations before and after chronic hypoxia exposure. Blood was collected into EDTA, centrifuged for plasma extraction and frozen at -80°C. The concentration of fetal cortisol in plasma was quantified using a commercially available cortisol indirect enzyme-linked immunosorbent assay (ELISA) kit according to the product instructions (RE52061, IBL International, Hamburg, Germany). This assay has previously been validated for use in fetal ovine plasma (Kabaroff et al., 2006). Duplicate 20 µl plasma aliquots (undiluted) were taken from previously unthawed samples. The inter-assay and intra-assay coefficients of variation were 5.2% and 5.0% respectively. The lower limit of detection was 3.2 ng.ml⁻¹. The concentration of fetal noradrenaline and adrenaline in plasma was quantified using a commercially available ELISA kit according to product instructions (KA1877, Abnova, Taipei, Germany) which has been previously optimised and validated for ovine plasma in our laboratory (Brain et al. 2016). Duplicate 300 µl of previously unthawed plasma was used to extract noradrenaline and adrenaline. For noradrenaline, the inter-assay and intra-assay coefficients of variation were 12.8% and 14.6%, respectively, and the lower limit of detection was 0.05 ng.ml⁻¹. For adrenaline, the inter-assay and intra-assay coefficients of variation were 9.7% and 15.7%, respectively, and the lower limit of detection was 0.01 ng.ml⁻¹.

**Statistical analyses.** Values are expressed as mean ± SEM. Statistical analysis was performed in SPSS 22.0 (SPSS Inc., Chicago, IL, USA). Repeated measures two-way ANOVA was performed. If a significant interaction between gestational age and oxygenation was identified, the post hoc Holm-Sidak and Tukey tests were performed to
isolate the effect of oxygenation and the change from baseline with advancing gestational age. Normality was assessed with Shapiro-Wilkes test and for normally distributed data means were compared with Student’s t-test. For all comparisons, statistical significance was accepted when \( P<0.05 \).

**Results**

*Maternal and fetal oxygenation, acid-base and endocrine status*

Baseline values for maternal and fetal pH, partial pressure of arterial oxygen (PaO\(_2\)) and carbon dioxide (PaCO\(_2\)) and the saturation of oxyhaemoglobin (Sat.Hb) were not different between group prior to randomisation to chronic hypoxia or normoxia (Figure 3), and were within normal ranges for Welsh mountain ewes at this gestational age (Fletcher et al., 2003, 2006). Daily maternal food intake at baseline was not different between groups: 1.3 ± 0.9 kg.day\(^{-1}\) (to be assigned normoxia) and 1.1 ± 0.4 kg.day\(^{-1}\) (to be assigned hypoxia) and maternal food intake was not affected by exposure to chronic hypoxia (Brain et al. 2016).

In this cohort of animals, there was a significant fall in maternal and fetal PaO\(_2\) and Sat.Hb during the period in which the fraction of maternal inspired oxygen was reduced to 10% in the chronic hypoxia group; maternal and fetal oxygenation levels remained stable in the normoxic control group. There was no effect of time or oxygenation on maternal or fetal pH and maternal PaCO\(_2\) was unaffected by time or treatment. However, fetal PaCO\(_2\) was reduced by the effect of chronic hypoxia (Figure 3).

Baseline (122 ± 1d GA) fetal cortisol, noradrenaline and adrenaline concentrations in plasma were not different between animals to be assigned to either chronic normoxic or chronic
hypoxia pregnancies (Table 1). There was a gestational age-dependent rise in fetal plasma cortisol concentration by the time of the second sample (136 ± 1d GA). However, the magnitude of the ontogenic increase in fetal plasma cortisol was not affected by whether the fetus had previously been exposed to chronic hypoxia or chronic normoxia. There was no change in concentration of fetal plasma catecholamines related to either gestational age or treatment group (Table 1).

**Effects on FHRV of gestational age and of chronic hypoxia**

A total of 882 FHR records were analysed in this study (381 normoxic, 501 hypoxic) 107 (12%) during quiet sleep and 477 (54%) during active sleep which is expected from the observed distribution of fetal behavioural states at these gestational ages (van Woerden & van Geijn, 1992). In the baseline state, prior to randomisation to chronic normoxia or chronic hypoxia, there were no differences between the fetal absolute heart rate or any indices of fetal heart rate variability based on their future treatment group. There was no effect of sleep state on fetal heart rate between quiet and active sleep. However, there was an effect of sleep state at 0.8 of gestation observed in SDNN, STV, total power, LF, HF, VLF, LF/HF ratio, but not RMSSD (Table 2).

In both quiet and active sleep states, there was a decrease in average fetal heart rate with increasing gestational age. However, this fall was greater in the chronically hypoxic group than in normoxic pregnancies (Figure 4). There was a corresponding gestational age dependent increase in FHRV in the normoxic group. Measured by SDNN and STV, this reached significance nine days after baseline in active sleep. In normoxic pregnancy in quiet sleep, STV first showed a significant rise 7 days after baseline, and was consistently elevated.
10 days after baseline. However, the trend to increase observed in SDNN did not reach significance over the period assessed. This increase in overall FHRV was not observed in the chronic hypoxia group, in either quiet or active sleep, measured by SDNN or STV. There was an oxygenation-dependent difference between groups in STV and SDNN observed, although this occurred earlier in active sleep than quiet sleep (Figure 4). In normoxic pregnancies in active sleep there was an increase of total power with gestational age which related temporally to changes in STV and SDNN, reaching significance at nine days after baseline. Again, this increase was not observed in chronically hypoxic pregnancies, and there was an oxygen-dependent difference between groups observed consistently from nine days after baseline measurements (Figure 4). There were no sustained changes of either gestational age or chronic hypoxia relating to total power in quiet sleep.

In active sleep in normoxic pregnancy, there was a progressive increase in LF with increasing gestational age, which reached significance eleven days after baseline. However, when these values were related to increases in total power, there was no increase in normalised LF with gestation. In chronic hypoxia, after the initial response to the fall in oxygenation, there was a trend for a decrease in both LF and nLF in active sleep, such that there was a significant oxygenation-dependent contribution on both LF and nLF to FHRV from seven days after baseline (Figure 5). There were no sustained differences seen in LF or nLF with gestational age or oxygenation in quiet sleep. Conversely, in quiet sleep there was a gestational age dependent increase in both HF and normalised HF observed from nine and ten days after baseline, respectively, in both normoxic and chronically hypoxic pregnancies, with no effect of treatment. This increase was not mirrored in RMSSD. There were no changes observed in parasympathetic indices of control of FHRV in active sleep (Figure 6).
In active sleep in normoxic pregnancy there was a gestational-age related increase in the LF/HF ratio, which reached significance eleven days after baseline. Conversely, in hypoxic pregnancy there was a gestational-age related fall in the LF/HF ratio which reached significance at ten days after baseline. Accordingly, there was an oxygenation-dependent significant difference between groups evident twelve days after baseline. In quiet sleep, there was a significant shift to sympathetic dominance at the time of onset of hypoxia compared to normoxic pregnancies, which shifts towards sympathetic suppression as the chronic hypoxia persisted, and an oxygenation effect becoming significant from days five to eight after baseline (Figure 7).

**Discussion**

This longitudinal study in chronically instrumented sheep preparations shows that induction of late gestation chronic hypoxia has the potential to alter the development of autonomic nervous system control of FHRV, supporting the hypothesis tested. Exposure of pregnant ewes to a reduced fraction of inspired oxygen produced a sustained reduction in fetal femoral arterial PO$_2$ to 10-12 mmHg between 0.8 and 0.9 of gestation. This represents an estimated 50% reduction in the normal oxygenation values of the fetal umbilical artery, ca. 20 mmHg (Longo, 2013). Reductions of a similar or greater magnitude have been measured through sampling the umbilical vein in growth-restricted human fetuses at comparable points in gestation (Soothill et al., 1987; Nicolaides et al., 1989). Therefore, the degree of chronic fetal hypoxia achieved in this study is relevant to the human clinical situation in pregnancy complicated by significant fetal growth restriction.
In our study, in normoxic fetuses there was an ontogenic increase in FHRV indices of overall variability (total power, SDNN) as well as an increase in STV with advancing gestation. These changes were more pronounced in active sleep, but were still evident in quiet sleep. Total power has previously been shown to increase with advancing gestational age in fetal sheep (Koome et al., 2014a) and STV, measured by computerised CTG, has been observed to increase towards term in the human fetus (Serra et al., 2009). The mean STV values for our normoxic sheep fetuses fall between the 10-50th centile of the published ranges for human fetuses at an equivalent gestational age, despite not being measured by computerised CTG in our fetuses (Serra et al., 2009). Therefore, our control group show the expected ontogenic changes over the period studied, which represents approximately 10% of the total gestational period in sheep.

In the present study, exposure to chronic hypoxia prevented the ontogenic increase in FHRV indices of overall variability and STV, in both active and quiet sleep states. Compared to their normoxic counterparts at an equivalent gestational age, STV, SDNN and total power were lower in chronically hypoxic fetuses, even after their return to normoxia. Despite this, the STV remained above 3 ms in hypoxic fetuses in active sleep states, which correlated well with the normal fetal arterial pH values observed throughout, and fetal survival to the end of the experimental procedure in all cases. In human fetuses, impaired increases in overall heart rate variability with increasing gestational age, measured by cCTG, have been described in fetuses affected by uteroplacental dysfunction (Lobmaier et al., 2012). Similarly, fMCG studies have demonstrated a lower SDNN in human fetuses affected by uteroplacental dysfunction than was calculated in gestation age-matched, normally grown controls (Sriram et al., 2013). Therefore, the data in our study highlight that changes in FHRV measured in...
human pregnancy affected by uteroplacental dysfunction are likely due to isolated chronic fetal hypoxia.

Both SDNN and STV are decreased in sympathectomised sheep compared to controls (Lear et al., 2016), as is total power (Koome et al., 2014a). Although resting sympathetic tone is low in the fetus (Assali et al., 1977), the sympathectomy studies suggests a significant role for the sympathetic nervous system in contributing to the ontogenic changes in FHRV. Symathetic innervation is completed by the second half of pregnancy and increases gradually towards term in fetal sheep (Lebowitz et al., 1972). Conversely, the parasympathetic system does not begin to increase tone until term (van Laar 2009). Consistent with the literature, in our normoxic fetuses, in active sleep, LF power but not HF, normalised LF but not normalised HF power, showed an increase with gestational age. The LF/HF ratio also increased with gestational age, suggesting a ontogenic shift towards sympathetic dominance as the healthy fetus approached term. Compared to normoxic fetuses, the LF and normalised LF power was significantly lower in chronically hypoxic fetuses in active sleep, while HF and normalised HF power remained unchanged. This resulted in an ontogenic decrease rather than increase in the LF/HF ratio, and a marked difference in the LF/HF ratio between normoxic and chronically hypoxic fetuses by the end of the experimental protocol. This appeared to be due to a reduction in the influence of the sympathetic nervous system on the control of FHRV, rather than due to changes in the parasympathetic nervous system in the chronically hypoxic fetus.

The chronically hypoxic fetuses were, at the start of the experimental protocol, capable of increasing the sympathetic control of FHRV in response to an acute challenge. There was an increase in LF, normalised LF and in the LF/HF ratio, seen in quiet sleep, during the first 48h of exposure to hypoxia, representing a prolonged period of sympathetic dominance in the
control of FHRV. This increase in sympathetic nervous output is a characteristic response to acute hypoxia, and forms part of the fetal cardiovascular defence mechanism to hypoxic episodes (Giussani, 2016). However, the increased sympathetic tone cannot be maintained indefinitely, and blunting of fetal heart rate and peripheral vascular responses to sympathetic activity has been reported after around 72 hours (Bennet & Gunn, 2009). In our animals, it is only after this time that the reduction in sympathetic contribution of FHRV becomes evident. This likely underlies the delay in onset of the fall in basal FHR seen in chronically hypoxic fetuses. Therefore, unlike the normoxic fetuses, in which the trend was to develop sympathetic dominant control of FHRV approaching term, the trend in the chronically hypoxic fetus was towards overall sympathetic suppression, despite a normal ontogenic rise in fetal plasma cortisol levels. The fetal endocrine data are in keeping with studies of ovine pregnancy at high altitude. Such studies have reported that ovine fetuses exposed to chronic hypoxia have unaltered levels of basal cortisol and catecholamines compared to normoxic controls at corresponding gestational ages (Newby et al., 2015).

The findings reported in the present study agree with fMCG studies, which show a progressive increase in LF power in normoxic human pregnancy (Ferrario et al., 2009; Schneider et al., 2010). The LF/HF ratio in human pregnancies also shows an ontogenic increase and a change towards sympathetic dominance during the third trimester (Schneider 2009, Fukushima 2011), while HF power remains unchanged (Ferrario et al., 2009). Lower values of the LF/HF ratio are associated with decreased overall FHRV (Ferrario et al., 2009) and STV (Schneider et al., 2010). In human fetuses in pregnancy complicated by uteroplacental dysfunction, overall FHRV is reduced due to a reduction in the sympathetic contribution to the control of the fetal heart, while the parasympathetic contribution remains unchanged (Ferrario et al., 2009; Sriram et al., 2013). A decrease in LF has been linked to
umbilical artery PO₂ < 20 mmHg at delivery (Ohta et al., 1999; Suzuki et al., 2000).

Therefore, our studies again highlight that sympathetic suppression in the control of FHRV in the late gestation human fetus in complicated pregnancy is likely due to isolated chronic hypoxia.

Collectively, past and present data suggest that at reduction in overall FHRV in the human fetus, as in fetal sheep, may be due to sympathetic suppression rather than an increase in parasympathetic tone. Appropriate activation of the sympathetic nervous system in the late gestation fetus is an indispensable component of the fetal homeostatic compensatory response to acute stress, such as in response to acute hypoxia or acute asphyxia (Giussani et al. 1993; Giussani, 2016; Bennet & Gunn, 2009; Lear et al., 2016). Therefore, dysregulation of sympathetic control of the cardiovascular system would predict that the chronically hypoxic fetus may be more vulnerable to acute insults in late gestation, such as birth hypoxia or asphyxia. Reduction in overall FHRV, or STV, may therefore provide an important biomarker in obstetric medicine to highlight that autonomic dysregulation of FHR control has taken place in a fetus carried by a pregnancy where uteroplacental dysfunction is suspected.
Akselrod S, Gordon D, Ubel FA, Shannon DC, Berger AC & Cohen RJ. (1981). Power spectrum analysis of heart rate fluctuation: a quantitative probe of heart-to-heart cardiovascular control. *Science* **213**, 220-222.

Allison BJ, Brain KL, Niu Y, Kane AD, Herrera EA, Thakor AS, Botting KJ, Cross CM, Itani N, Skeffington KL, Beck C & Giussani DA. (2016). Fetal in vivo continuous cardiovascular function during chronic hypoxia. *J Physiol* **594**, 1247-1264.

Assali NS, Brinkman CR, 3rd, Woods JR, Jr., Dandavino A & Nuwayhid B. (1977). Development of neurohumoral control of fetal, neonatal, and adult cardiovascular functions. *Am J Obstet Gynecol* **129**, 748-759.

Bamford OS & Dawes GS. (1990). Hypoxia and electrocortical activity in the fetal lamb: effects of brainstem transection and chemoreceptor denervation. *J Dev Physiol* **13**, 271-276.

Bennet L & Gunn AJ. (2009). The fetal heart rate response to hypoxia: insights from animal models. *Clin Perinatol* **36**, 655-672.

Boddy K, Dawes GS, Fisher R, Pinter S & Robinson JS. (1974). Foetal respiratory movements, electrocortical and cardiovascular responses to hypoxaemia and hypercapnia in sheep. *J Physiol* **243**, 599-618.

Brain KL, Allison BJ, Niu Y, Cross CM, Itani N, Kane AD, Herrera EA & Giussani DA. (2015). Induction of controlled hypoxic pregnancy in large mammalian species. *Physiol Rep* **3**.

Clewlow F, Dawes GS, Johnston BM & Walker DW. (1983). Changes in breathing, electrocortical and muscle activity in unanaesthetized fetal lambs with age. *J Physiol* **341**, 463-476.

Ferrario M, Signorini MG & Magenes G. (2009). Complexity analysis of the fetal heart rate variability: early identification of severe intrauterine growth-restricted fetuses. *Med Biol Eng Comput* **47**, 911-919.

Fletcher AJ, Gardner DS, Edwards CM, Fowden AL & Giussani DA. (2003). Cardiovascular and endocrine responses to acute hypoxaemia during and following dexamethasone infusion in the ovine fetus. *J Physiol* **549**, 271-287.
Fletcher AJ, Gardner DS, Edwards CM, Fowden AL & Giussani DA. (2006). Development of the ovine fetal cardiovascular defense to hypoxemia towards full term. *Am J Physiol Heart Circ Physiol* **291**, H3023-3034.

Fox M, Kilpatrick S, King T & Parer JT. (2000). Fetal heart rate monitoring: interpretation and collaborative management. *J Midwifery Womens Health* **45**, 498-507.

Giussani DA. (2016). The fetal brain sparing response to hypoxia: physiological mechanisms. *J Physiol* **594**, 1215-1230.

Henson G, Dawes GS & Redman CW. (1984). Characterization of the reduced heart rate variation in growth-retarded fetuses. *Br J Obstet Gynaecol* **91**, 751-755.

Kabaroff L, Boermans H & Karrow NA. (2006). Changes in ovine maternal temperature, and serum cortisol and interleukin-6 concentrations after challenge with Escherichia coli lipopolysaccharide during pregnancy and early lactation. *J Anim Sci* **84**, 2083-2088.

Keen AE, Frasch MG, Sheehan MA, Matushewski B & Richardson BS. (2011). Maturational changes and effects of chronic hypoxemia on electrocortical activity in the ovine fetus. *Brain Res* **1402**, 38-45.

Kimura Y, Okamura K, Watanabe T, Murotsuki J, Suzuki T, Yano M & Yajima A. (1996). Power spectral analysis for autonomic influences in heart rate and blood pressure variability in fetal lambs. *Am J Physiol* **271**, 1333-1339.

Koome ME, Bennet L, Booth LC, Davidson JO, Wassink G & Gunn AJ. (2014a). Ontogeny and control of the heart rate power spectrum in the last third of gestation in fetal sheep. *Exp Physiol* **99**, 80-88.

Koome ME, Bennet L, Booth LC, Wassink G, Davidson JO, Gunning M & Gunn AJ. (2014b). Quantifying the power spectrum of fetal heart rate variability. *Exp Physiol* **99**, 468.

Lear CA, Galinsky R, Wassink G, Mitchell CJ, Davidson JO, Westgate JA, Bennet L & Gunn AJ. (2016). Sympathetic neural activation does not mediate heart rate variability during repeated brief umbilical cord occlusions in near-term fetal sheep. *J Physiol* **594**, 1265-1277.

Lebowitz EA, Novick JS & Rudolph AM. (1972). Development of myocardial sympathetic innervation in the fetal lamb. *Pediatr Res* **6**, 887-893.
Lobmaier SM, Huhn EA, Pildner von Steinburg S, Muller A, Schuster T, Ortiz JU, Schmidt G & Schneider KT. (2012). Phase-rectified signal averaging as a new method for surveillance of growth restricted fetuses. *J Matern Fetal Neonatal Med* **25**, 2523-2528.

Longo LD. (2013). *The rise of fetal and neonatal physiology: basic science to clinical care*. Published on behalf of the American Physiological Society. Springer. New York, USA.

Malik M, Camm J, Bigger JT, Breithardt G, Cerutti S, Cohen R, Coumel P, Fallen E, Kennedy HL, Kleiger RE, Lombardi F, Malliani A, Moss AJ, Rottman JN, Schmidt G, Schwartz PJ & Singer DH. (1996). Heart rate variability: standards of measurement, physiological interpretation and clinical use. Task Force of the European Society of Cardiology and the North American Society of Pacing and Electrophysiology. *Circulation* **93**, 1043-1065.

Malliani A, Lombardi F & Pagani M. (1994). Power spectrum analysis of heart rate variability: a tool to explore neural regulatory mechanisms. *Br Heart J* **71**, 1-2.

Min SW, Ko H & Kim CS. (2002). Power spectral analysis of heart rate variability during acute hypoxia in fetal lambs. *Acta Obstet Gynecol Scand* **81**, 1001-1005.

Newby EA, Myers DA & Ducsay CA. (2015). Fetal endocrine and metabolic adaptations to hypoxia: the role of the hypothalamic-pituitary-adrenal axis. *Am J Physiol Endocrinol Metab* **309**, E429-439.

Nicolaides KH, Economides DL & Soothill PW. (1989). Blood gases, pH, and lactate in appropriate- and small-for-gestational-age fetuses. *Am J Obstet Gynecol* **161**, 996-1001.

Nijhuis JG, Prechtl HF, Martin CB, Jr. & Bots RS. (1982). Are there behavioural states in the human fetus? *Early Hum Dev* **6**, 177-195.

Ohta T, Okamura K, Kimura Y, Suzuki T, Watanabe T, Yasui T, Yaegashi N & Yajima A. (1999). Alteration in the low-frequency domain in power spectral analysis of fetal heart beat fluctuations. *Fetal Diagn Ther* **14**, 92-97.

Pardey J, Moulden M & Redman CW. (2002). A computer system for the numerical analysis of nonstress tests. *Am J Obstet Gynecol* **186**, 1095-1103.

Peters CH, ten Broeke ED, Andriessen P, Vermeulen B, Berendsen RC, Wijn PF & Oei SG. (2004). Beat-to-beat detection of fetal heart rate: Doppler ultrasound
cardiotocography compared to direct ECG cardiotocography in time and frequency domain. *Physiol Meas* **25**, 585-593.

Royal College of Obstetricians and Gynaecologists (2013). The Investigation and Management of the Small-for-Gestational-Age Fetus (published March 2013). RCOG Guidance (Green-top Guideline No.31).

Schaffer L, Luzi F, Burkhardt T, Rauh M & Beinder E. (2009). Antenatal betamethasone administration alters stress physiology in healthy neonates. *Obstet Gynecol* **113**, 1082-1088.

Schneider U, Fiedler A, Schroder B, Jaekel S, Stacke A, Hoyer D & Schleussner E. (2010). The effect of antenatal steroid treatment on fetal autonomic heart rate regulation revealed by fetal magnetocardiography (fMCG). *Early Hum Dev* **86**, 319-325.

Serra V, Bellver J, Moulden M & Redman CW. (2009). Computerized analysis of normal fetal heart rate pattern throughout gestation. *Ultrasound Obstet Gynecol* **34**, 74-79.

Serra V, Moulden M, Bellver J & Redman CW. (2008). The value of the short-term fetal heart rate variation for timing the delivery of growth-retarded fetuses. *Br J Obstet Gynecol* **115**, 1101-1107.

Soothill PW, Nicolaides KH & Campbell S. (1987). Prenatal asphyxia, hyperlacticaemia, hypoglycaemia, and erythroblastosis in growth retarded fetuses. *Br Med J (Clin Res Ed)* **294**, 1051-1053.

Sriram B, Mencer MA, McKelvey S, Siegel ER, Vairavan S, Wilson JD, Preissl H, Eswaran H & Govindan RB. (2013). Differences in the sleep states of IUGR and low-risk fetuses: An MCG study. *Early Hum Dev* **89**, 815-819.

Street P, Dawes GS, Moulden M & Redman CW. (1991). Short-term variation in abnormal antenatal fetal heart rate records. *Am J Obstet Gynecol* **165**, 515-523.

Suzuki T, Okamura K, Kimura Y, Watanabe T, Yaegashi N, Murotsuki J, Uehara S & Yajima A. (2000). Power spectral analysis of R-R interval variability before and during the sinusoidal heart rate pattern in fetal lambs. *Am J Obstet Gynecol* **182**, 1227-1232.

Turan S, Turan OM, Berg C, Moyano D, Bhide A, Bower S, Thilaganathan B, Gembruch U, Nicolaides K, Harman C & Baschat AA. (2007). Computerized fetal heart rate analysis, Doppler ultrasound and biophysical profile score in the prediction of acid-base status of growth-restricted fetuses. *Ultrasound Obstet Gynecol* **30**, 750-756.
van Laar JO, Peters CH, Vullings R, Houterman S & Oei SG. (2009). Power spectrum analysis of fetal heart rate variability at near term and post term gestation during active sleep and quiet sleep. *Early Hum Dev* **85**, 795-798.

Van Laar JO, Porath MM, Peters CH & Oei SG. (2008). Spectral analysis of fetal heart rate variability for fetal surveillance: review of the literature. *Acta Obstet Gynecol Scand* **87**, 300-306.

van Woerden EE & van Geijn HP. (1992). Heart-rate patterns and fetal movements. In *Fetal behaviour: developmental and perinatal aspects*, ed. Nijhuis JG, pp. 41-56. Oxford University Press, Oxford.

Walker AM, Cannata JP, Dowling MH, Ritchie BC & Maloney JE. (1979). Age-dependent pattern of autonomic heart rate control during hypoxia in fetal and newborn lambs. *Biol Neonate* **35**, 198-208.

### Additional Information

**Competing interests:** DG worked with Maastrict Instruments to design and create the data acquisition system and with Telstar ACE to design the hypoxic chambers. No other authors have competing interests.

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**Author Contributions:** The experiments in this study were performed in the Department of Physiology, Development and Neuroscience, University of Cambridge. DG, CJS, BJA and
YN, conceived and designed the experiments. DG, CJS, BJA, YN, KJB and NI collected, analysed and interpreted the experimental data. CJS, DG and CCL drafted the article and revised it for important intellectual content.
Table 1: Ontogenic changes in fetal plasma cortisol and catecholamines during chronic hypoxia and chronic normoxia

Values represent the mean ± SEM of plasma concentrations of fetal cortisol, noradrenaline and adrenaline at baseline (122 ± 1 d GA) and 2 days after the end of chronic hypoxia/normoxia (day 13, 136 ± 1 d GA). Significant differences (P < 0.05): * significant effect of gestational age (RM two-way ANOVA with post hoc Tukey’s test).

|                      | Normoxia | Hypoxia |
|----------------------|----------|---------|
|                      | Baseline | Day 13  | Baseline | Day 13  |
| Cortisol (ng.ml⁻¹)   | 11.9 ± 1.4 | 24.1 ± 1.8 * | 13.8 ± 1.8 | 32.2 ± 5.2 * |
| Noradrenaline (ng.ml⁻¹) | 1.1 ± 0.3 | 0.8 ± 0.1 | 0.7 ± 0.1 | 0.6 ± 0.1 |
| Adrenaline (ng.ml⁻¹)  | 0.09 ± 0.02 | 0.07 ± 0.02 | 0.06 ± 0.01 | 0.07 ± 0.01 |

Table 2: Effect of sleep state on FHRV indices before onset of chronic normoxia or chronic hypoxia

Values represent the mean ± SEM (n=12) for fetal heart rate and indicies of fetal heart rate variability in quiet and active sleep. Significant differences (p <0.05): * significant effect of sleep state (Student’s t-test).
Figure 1. Isobaric hypoxic chambers and the CamDAS system.

Each chamber was equipped with an electronic servo-controlled humidity cool steam injection system to return the appropriate humidity to the Inspirate (i). Ambient $pO_2$, $pCO_2$, humidity and temperature within each chamber were monitored via sensors (ii). For experimental procedures, each chamber had a double transfer port (iii) to internalise material and a manually-operated sliding panel (iv) to bring the ewe into a position where daily sampling of blood could be achieved through glove compartments (v). Each chamber incorporated a drinking bowl on continuous water supply and a rotating food compartment (vi) for determining food intake. A sealed transfer isolation cart could be attached to a side exit (vii) to couple chambers together for cleaning. The CamDAS system was contained in a custom-made sheep jacket able to hold the data acquisition system box (ix) in one side pouch and a box containing the Transonic flow probe and pressure connectors (x) in the other. Cables (xi) connected the two boxes together and also to two battery packs able to power the system for 24 hours. Measurements made using the data acquisition were transmitted wirelessly via Bluetooth (xiii) to a laptop kept outside the chamber room (xii) on which it was possible to view continuous recordings of the maternal and fetal cardiovascular data (Reprinted with permission from Allison et al. 2016)
Figure 2: Fetal heart rate variability patterns.

A representative example of active sleep (left) and quiet sleep (right) categorised by visual identification.
Figure 3. Maternal and fetal blood gas, acid base status during chronic normoxia and chronic hypoxia.

Values represent the mean ± SEM (n=12) for arterial pH, partial pressure of arterial carbon dioxide ($p_{a}CO_2$) and oxygen ($p_{a}O_2$) and percentage oxygen saturation of haemoglobin (Sat.Hb). Chronic hypoxia closed circles; chronic normoxia open circles. Significant differences (P < 0.05): *
significant main effect of hypoxia compared with normoxia; ‡ significant main effect of time in hypoxic pregnancy (RM two-way ANOVA with post hoc Holm-Sidak and Tukey’s tests).

Figure 4. Ontogenic changes in fetal heart rate and variability during chronic normoxia and chronic hypoxia.

Values represent the mean ± SEM (n=12) for absolute fetal heart rate, SDNN, short term variation (STV) and total power in quiet and active sleep. Period of chronic hypoxia (closed circles) or normoxia (open circles) indicated by dashed box. “B” represents an average of values taken in the 72 h period -2 to 0. Significant differences (p <0.05): * significant effect of oxygenation between treatment groups; † significant effect of gestational age in normoxic pregnancy; ‡ significant effect of
gestational age in hypoxic pregnancy (RM two-way ANOVA with post hoc Tukey and Holm-Sidak tests).

Figure 5. Ontogenic changes in indices of sympathetic contribution to fetal heart rate variability during chronic normoxia and chronic hypoxia.

Values represent the mean ± SEM (n=12) for absolute and normalised LF in quiet and active sleep. Period of chronic hypoxia (closed circles) or normoxia (open circles) indicated by dashed box. “B” represents an average of values taken in the 72 h period -2 to 0. Significant differences (p <0.05): * significant effect of oxygenation between treatment groups; † significant effect of gestational age in normoxic pregnancy; ‡ significant effect of gestational age in hypoxic pregnancy (RM two-way ANOVA with post hoc Tukey and Holm-Sidak tests).
Figure 6. Ontogenic changes in indices of parasympathetic contribution to fetal heart rate variability during chronic normoxia and chronic hypoxia.

Values represent the mean ± SEM (n=12) for RMSSD, absolute and normalised HF in quiet and active sleep. Period of chronic hypoxia (closed circles) or normoxia (open circles) indicated by dashed box. “B” represents an average of values taken in the 72 h period -2 to 0. Significant differences (p <0.05): * significant effect of oxygenation between treatment groups; † significant effect of gestational age in normoxic pregnancy; ‡ significant effect of gestational age in hypoxic pregnancy (RM two-way ANOVA with post hoc Tukey and Holm-Sidak tests).
Figure 7. Ontogenic changes in sympathovagal balance during chronic normoxia and chronic hypoxia.

Values represent the mean ± SEM (n=12) for the LF to HF ratio in quiet and active sleep. Period of chronic hypoxia (closed circles) or normoxia (open circles) indicated by dashed box. “B” represents an average of values taken in the 72 h period -2 to 0. Significant differences (p <0.05): * significant effect of oxygenation between treatment groups; † significant effect of gestational age in normoxic pregnancy; ‡ significant effect of gestational age in hypoxic pregnancy (RM two-way ANOVA with post hoc Tukey and Holm-Sidak tests).