Brief hypoxia in late gestation sheep causes prolonged disruption of fetal electrographic, breathing behaviours and can result in early labour

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Key points

- Brief episodes of severe fetal hypoxia can arise in late gestation as a result of interruption of normal umbilical blood flow
- Systemic parameters and blood chemistry indicate complete recovery within 1–2 hours, although the long-term effects on fetal brain functions are unknown
- Fetal sheep were subjected to umbilical cord occlusion (UCO) for 10 min at 131 days of gestation, and then monitored intensively until onset of labour or delivery (<145 days of gestation)
- Normal patterns of fetal behaviour, including breathing movements, episodes of high and low voltage electorocortical activity, eye movements and postural (neck) muscle activity, were disrupted for 3–10 days after the UCO
- Preterm labour and delivery occurred in a significant number of the pregnancies after UCO compared to the control (sham-UCO) cohort.

Abstract  Complications arising from antepartum events such as impaired umbilical blood flow can cause significant fetal hypoxia. These complications can be unpredictable, as well as difficult to detect, and thus we lack a detailed understanding of the (patho)physiological changes that occur between the antenatal in utero event and birth. In the present study, we assessed the consequences of brief (~10 min) umbilical cord occlusion (UCO) in fetal sheep at ~0.88 gestation on fetal plasma cortisol concentrations and fetal behaviour [electroocortical (EcoG), electro-oculogram (EOG), nuchal muscle electromyography (EMG) and breathing activities] in the days following UCO. UCO caused a rapid onset of fetal hypoxaemia, hypercapnia, and acidosis; however, by 6 h, all blood parameters and cardiovascular status were normalized and not different from the control (Sham-UCO) cohort. Subsequently, the incidence of fetal breathing movements decreased compared...
to the control group, and abnormal behavioural patterns developed over the days following UCO and leading up to the onset of labour, which included increased high voltage and sub-low voltage ECoG and EOG activities, as well as decreased nuchal EMG activity. Fetuses subjected to UCO went into labour 7.9 ± 3.6 days post-UCO (139.5 ± 3.2 days of gestation) compared to the control group fetuses at 13.6 ± 3.3 days post-sham UCO (144 ± 2.2 days of gestation; $P < 0.05$), despite comparable increases in fetal plasma cortisol and a similar body weight at birth. Thus, a single transient episode of complete UCO late in gestation in fetal sheep can result in prolonged effects on fetal brain activity and premature labour, suggesting persisting effects on fetal cerebral metabolism.

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**Introduction**

Perinatal hypoxia-ischaemia that occurs late in gestation (>37 weeks of gestational age) is associated with adverse outcomes such as cognitive and motor impairments, developmental delays, epilepsy and cerebral palsy (Cowan *et al.* 2003; Ferriero, 2004; Volpe, 2012). Promising clinical markers that aid in diagnosis and predicting neonatal outcome include APGAR scores, serum cortisol, arterial base deficit and plasma lactate collected from neonates in the hours following intrapartum asphyxia (Wayenberg *et al.* 1994; da Silva *et al.* 2000; Merchant & Azzopardi, 2015; Scaramuzzo *et al.* 2015; Ahearne *et al.* 2016). Following birth, electroencephalogram (EEG) monitoring is widely used in term neonates with evidence of encephalopathy, with EEG abnormalities also shown to be a risk factor for neurological deficits in the newborn (Biagioni *et al.* 2001; Ahearne *et al.* 2016; Jain *et al.* 2017). Complications arising from antepartum events such as impaired umbilical blood flow or placental abruption can result in significant hypoxia and/or ischaemia in the perinatal brain. These antepartum complications are random and variable in nature, making them especially difficult to detect prior to the sentinel event, and may only be identifiable once brain injury has been established. Currently, we still lack a detailed description of the physiological and functional fetal responses to *in utero* events that may damage the brain. Further understanding of the pathophysiological responses and subsequent fetal adaptations can help identify at-risk newborns and thereby inform and aid in predicting neurological outcomes in those infants.

Brain injury evolves over the hours, days and weeks following hypoxia-ischaemia, with the severity and timing of injury strongly associated with neurological outcome (Wassink *et al.* 2014). Thus, early detection, via fetal monitoring appears to be paramount in identifying infants at risk of developing brain injury so that appropriate management can be initiated in a timely fashion. The assessment of fetal distress *in utero* by monitoring fetal body and breathing movements and heart rate (HR) variability has shown some success in predicting the severity of hypoxia-ischaemia and neonatal outcomes in both clinical and experimental models (Martinez-Biarge *et al.* 2013; Frasch *et al.* 2014; Luo *et al.* 2014; Kasai *et al.* 2019). Fetal measurements of EEG and HR have provided additional details regarding hypoxia-acidaemia status during labour (Wang *et al.* 2014; Frasch *et al.* 2015).

Pregnant sheep allow for continuous monitoring of physiological and neurodevelopmental parameters to better understand the aetiology of injury *in utero* (Gunn & Bennet, 2009; Volpe, 2012; Abbasi & Unsworth, 2020). By 120 days of gestation (term is ~145 days), electrocortical activity has developed so that two distinct states [high voltage (HV) and low voltage (LV)] can be readily identified, and fetal activity shows cyclical changes with time, similar to the human fetus (Clewlow *et al.* 1983; Lamblin *et al.* 1999; Vanhatalo & Kaila, 2006; Andre *et al.* 2010). Seizure activity and changes in electrocorticogram (ECoG) occur in the hours following *in utero* asphyxia, caused by reversible interruption of umbilical cord blood flow in late gestational fetal sheep (Mallard *et al.* 1994; Kawagoe *et al.* 1999; Wassink *et al.* 2007; Yan *et al.* 2009; Drury *et al.* 2014). We have also found that, after birth following an *in utero* umbilical cord occlusion (UCO) in fetal sheep, there are significant behavioural delays in newborn lambs in achieving a stable standing posture and finding the udder, as well as structural alterations to grey and white matter at 24 h of age (Castillo-Melendez *et al.* 2013).

The current literature has extensively reported the immediate electrophographic, cardiovascular and fetal behavioural responses following a single severe episode of *in utero* hypoxia-ischaemia. However, the long-term effects, especially those occurring in the days leading up to birth, have not yet been assessed. The importance of establishing the progressive effects of a brief fetal hypoxic stress is to determine whether persisting abnormal fetal
behaviour is an identifiable consequence, and whether its presence can be used clinically to identify fetuses that are recovering from a sentinel event such as acute hypoxic-acidaemia. Thus, the present study aimed to assess the consequences of UCO in fetal sheep at ~0.88 gestation on fetal cortisol and behavioural parameters during the time that remained before the onset of labour. We hypothesized that a single severe bout of fetal asphyxia would produce abnormal changes of fetal ECoG activity, eye movements and other behavioural parameters that persist until the end of gestation, signifying that the trajectory of functional brain development can be altered by a transient change of umbilical–placental perfusion late in gestation.

Methods

Animal ethical approval

The use of these animals and all procedures received approval from the School of Biomedical Science Animal Ethics Committee of Monash University, Australia (MMCA2005/52, MMCA2007/24) under guidelines established by the National Health and Medical Research Council of Australia Code of practice for the care and use of animals for scientific purposes.

Animal surgery

Time-mated pregnant Border-Leicester ewes carrying a singleton fetus at 123–128 (125.3 ± 1.3; mean ± SD) days of gestational age were sourced from a private supplier who used induced ovulation and time mating procedures to allow calculation of gestation to ± 1 day; term in this flock was ~147 days of gestation. Ewes were fed once a day with Lucerne-chaff mix with access to water available to house induction of ovulation and time mating parameters during the time that remained before the onset of labour. We hypothesized that a single severe bout of fetal asphyxia would produce abnormal changes of fetal ECoG activity, eye movements and other behavioural parameters that persist until the end of gestation, signifying that the trajectory of functional brain development can be altered by a transient change of umbilical–placental perfusion late in gestation.

Measurement of fetal physiological parameters and fetal behaviour

Fetal blood pressure, tracheal pressure and amniotic pressure were measured continuously from 1 day prior to experimentation using solid-state pressure transducers (DTX Plus; Becton Dickinson Medical Systems, Singapore). Fetal arterial pressure and changes of tracheal pressure were recorded after subtraction of amniotic pressure to remove the effect of changes in amniotic pressure and movements of the ewe. Fetal HR was calculated online from the arterial pressure pulse. ECoG, EOG and nuchal EMG activity were recorded using high common-mode rejection amplifiers, and appropriate band-pass filters. All signals were digitized at 100 Hz and data recorded and analysed using LabChart, version 7 (PowerLab; ADInstruments, Castle Hill, NSW, Australia).

Experimental procedures

At 129–133 (131.2 ± 1.1) days of gestation, the umbilical cord was occluded by inflating the cuff with a predetermined volume to induce complete cessation of
Fetal arterial blood pressure and HR. Physiological parameters were divided into successive 10 min epochs beginning 6 h prior to onset of UCO and continuing until the end of the recording period (death of the ewe). The average amplitude of each parameter was determined for each 10 min epoch for the entire period of recording.

Fetal breathing movements. Fetal breathing movements were assessed for amplitude and incidence in 10 min epochs. Tracheal pressure was averaged and the incidence of fetal breathing movements was calculated by detecting the number of negative deflections of tracheal pressure (>2 mmHg) (Clewlow et al. 1983).

ECoG. ECoG was scored as either high or low based on the average amplitude of each type of activity present in the 6 h of control recording that occurred prior to the onset of UCO (−6h to 0h). Very low amplitude ECoG activity (hereafter referred to as sub-low voltage) was defined as an amplitude sustained for at least 1 min that was <25% of the amplitude of normal low-voltage ECoG activity (Yan et al. 2009; Yawno et al. 2010).

EOG and nuchal EMG activity for each 10 min epoch was manually scored as either 0 (predominantly absent) or 1 (predominantly present), based on the mean amplitude for each signal during the 6 h pre-UCO control period.

Fetal behavioural states. The assessment of fetal behavioural states as ‘normal’ was assessed in accordance with previously described criteria (Clewlow et al. 1983; Yan et al. 2009) and as ‘abnormal’ by assessing the incidence of nuchal EMG activity during LV-ECoG and, in addition, the incidence of EOG activity or fetal breathing movements during HV-ECoG, for each of the 10 min epochs throughout the entire record. Baseline (pre-UCO) was calculated from −4 h to 0 h.

Cortisol radioimmunoassay

Fetal plasma cortisol was determined after dichloromethane extraction as previously described by Bocking et al. (1986). The intra-assay and inter-assay coefficients of the assay were 15.4% and 24.6%, respectively.

Statistical analysis

Data are presented as the mean ± SD and graphs are presented as the mean ± 95% confidence interval (CI), unless otherwise stated. As a result of ewes going into labour during the monitoring period following UCO, a few parameters could not be recorded and thus statistical analysis was only conducted when group numbers were greater ≥3. A D’Agostino and Pearson test for normality was conducted prior to parametric or non-parametric statistical tests being conducted. Gestational age in labour was analysed with an unpaired t test (control vs. UCO). Blood gas parameters, pH, glucose, lactate, plasma cortisol (−1 h to 48 h post-UCO) and measures of fetal behaviour [ECoG, EOG, nuchal EMG, LV ECoG + nuchal EMG, HV ECoG-EOG, HV ECoG-FBM] and fetal breathing movements (FBM) (amplitude and incidence) were assessed using a two-way (mixed model) ANOVA for treatment factor (between groups) and time (within subject, repeated measure). If a significant interaction and/or main effect was obtained, further post hoc analyses using Sidak’s correction for multiple comparisons were conducted. Sphericity was not assumed and a Geisser–Greenhouse correction was applied. Linear regression was calculated for analysis of fetal behavioural disorganization trajectory from 3 days (72 h) post-UCO to 10 days (252 h) post-UCO (HV ECoG-EOG, HV ECoG-FBM, LV ECoG + nuchal EMG), with the difference between groups being assessed.
using analysis of covariance. For all fetal physiological parameters, baseline values were calculated as an average value over $-6$ h to 0 h, as well as disorganization of fetal behavioural states from $-4$ h to 0 h, all relative to onset of the UCO (0 h). Statistical analysis and data visualization was conducted using Prism 8, version 8.2.1 (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$ was considered statistically significant.

Results

Pregnancy outcomes and post-mortem data

Figure 1A shows a plot for the gestational age at which ewes went into labour. One control ewe went into labour at 140 days. The remaining four control fetuses continued to develop until 145 days, two of which clearly went into active labour, and two of which were electively killed at 145 days. Ewes with fetuses that underwent UCO went into labour significantly earlier (mean: $139.5 \pm 3.2$ days; range: $136–144$ days; $n = 11$) than control fetuses (mean: $144.0 \pm 2.2$ days; range: $140–144$ days; $n = 5$) (Table 1) ($P = 0.0135$). On average, UCO sheep went into labour $7.9 \pm 3.6$ days post-UCO compared to the control group fetuses at $13.6 \pm 3.3$ days post-UCO ($P = 0.0091$). One fetus from the UCO group died in utero when the ewe was in early labour at 136 days (included in analysis). All remaining fetuses ($n = 10$) were alive during labour prior to death and post-mortem. In the days prior to labour onset, both the control and UCO group fetuses had comparable increases in fetal plasma cortisol (Fig. 1B).

At post-mortem, there were no significant differences in fetal size (crown–rump length; $P = 0.2208$), fetal weight ($P = 0.1598$) or the absolute and relative weights of major organs (brain, heart, liver, brown fat, adrenal glands, kidneys) (Table 1). Significant differences between groups were found in the actual weights of the cerebellum ($P = 0.0019$) and the lung ($P = 0.0382$), as well as brown fat:body weight ratio ($P = 0.0124$).

Immediate and short-term physiological effects of UCO

Animals in the control and UCO groups presented with similar baseline parameters (Table 2). The effects of the 10 min UCO on fetal MAP, HR, blood gases, pH, whole blood lactate and glucose concentrations, and plasma cortisol concentrations from 1 h prior to and up to 48 h after the UCO ($N = 11$) or control ($N = 5$) are shown in Table 2. As expected, UCO caused a rapid onset of fetal hypoxemia, hypercapnia and acidosis, as reported previously (Yan et al. 2009; Castillo-Melendez et al. 2013). One fetus had the occlusion stopped at 9 min 45 s and another at 9 min 56 s as a result of a rapid decrease in MAP below 15 mmHg. All other fetuses ($n = 9$) tolerated 10 min of UCO and all the fetuses survived until the onset of spontaneous labour. Blood lactate was significantly increased for up to 2 h post-UCO ($P = 0.0015$; +5 min, $P = 0.0031$; +9 min, $P = 0.0016$; +30 min, $P = 0.0004$; +1 h, $P = 0.011$; +2 h, $P = 0.0059$), whereas glucose concentrations were only significantly increased at +9 min ($P = 0.0059$), +30 min ($P = 0.0458$) and +2 h ($P = 0.0073$) with respect to the onset of the UCO. Fetal plasma cortisol was significantly increased at +30 min ($P = 0.0201$) and 1 h ($P = 0.0057$) in response to UCO compared to controls (significant interaction time and group ($F_{9,121} = 2.55$, $P = 0.0344$, group $P = 0.0115$) (Table 2). However, by 6 h after the 10 min UCO, all of the blood parameters, as well as MAP and HR, were normalized and were not different from the control (sham-UCO) cohort. Figure 2 shows an example of the fetal behavioural and blood flow responses to UCO. 
Table 1. Fetal outcomes after 10 min UCO at 130–132 days of gestation

|                          | Control          | UCO              |
|--------------------------|------------------|------------------|
| n                        | 5                | 11               |
| Male/female              | 3/2              | 4/7              |
| Gestational age in labour (days) | 144 ± 2.24       | 139.5 ± 3.24*    |
| In labour (days post-UCO) | 13.6 ± 3.29      | 7.9 ± 3.56**     |
| Body weight (kg)         | 5.6 ± 0.91       | 4.8 ± 1.1        |
| Crown–rump length (cm)   | 65.5 ± 4.1 (n = 3) | 61.7 ± 4.6      |

Organ weights

|                      | Control         | UCO             |
|----------------------|-----------------|-----------------|
| Brain (g)            | 51.2 ± 5.0      | 49.4 ± 5.7      |
| Brain:body weight (g kg\(^{-1}\)) | 9.3 ± 1.2      | 10.7 ± 1.8      |
| Cerebellum (g)       | 5.7 ± 0.6       | 4.6 ± 0.5*      |
| Cerebellum:body weight (g kg\(^{-1}\)) | 1.0 ± 0.1      | 1.0 ± 0.2      |
| Heart (g)            | 39.1 ± 5.4      | 33.1 ± 7.4      |
| Heart:body weight (g kg\(^{-1}\)) | 7.1 ± 0.8       | 7.0 ± 1.4          |
| Brown fat (g)        | 19.3 ± 3.5      | 23.9 ± 6.8      |
| Brown fat:body weight (g kg\(^{-1}\)) | 3.6 ± 1.3      | 5.0 ± 0.6*      |
| Liver (g)            | 153.6 ± 39.7    | 146.7 ± 32.4    |
| Liver:body weight (g kg\(^{-1}\)) | 27.3 ± 5.0      | 31.4 ± 7.3      |
| Lungs (g)            | 170.4 ± 37.0    | 114.4 ± 48.4*   |
| Lungs:body weight (g kg\(^{-1}\)) | 30.3 ± 2.1      | 24.1 ± 8.9      |
| Adrenals combined (g) | 1.0 ± 0.2       | 0.9 ± 0.3       |
| Adrenals:body weight (g kg\(^{-1}\)) | 0.2 ± 0.0       | 0.2 ± 0.1       |
| Kidneys combined (g) | 28.7 ± 6.4      | 25.9 ± 5.3      |
| Kidneys:body weight (g kg\(^{-1}\)) | 5.1 ± 0.7      | 5.5 ± 0.7       |

Data are shown as the mean ±SD.

*P < 0.05.

**P < 0.01, UCO compared to control.

Effect of UCO on fetal breathing movements

All fetuses displayed episodic fetal breathing movements, occurring mainly during LV ECoG activity. The abrupt interruption of umbilical blood flow resulted in a temporary cessation of the normal low amplitude (2–4 mmHg) breathing movements, as well as the onset of occasional fetal ‘gasping’, described as single deep breaths (Fig. 2D). There was a significant interaction between time and group for the amplitude of fetal breathing movements (\(F_{23,215} = 1.914, P = 0.0092\)). Fetal breathing movements of normal incidence and amplitude resumed shortly after umbilical blood flow was reinstated, although there was a notable >3-fold increase in amplitude of the breathing movements from 12 to 36 h post-UCO (Fig. 3A) before recovery of normal amplitude movements, which then persisted until the end of the experiment 10–12 days later. The was a significant time effect for the incidence of fetal breathing movements (\(F_{4,74,44,33} = 4.894, P = 0.0014\)), although there was no difference between the control and UCO fetuses. From 54 h post-UCO, it appeared that the incidence began to fall and was less than that of control fetuses at various times until the end of the experiment (Fig. 3B).

Effect of UCO on fetal ECoG, EOG and muscle EMG

All fetal sheep exhibited a highly co-ordinated pattern of ECoG, EOG and nuchal muscle EMG activities prior to UCO (Fig. 2). The electrical activity recorded from the surface of the cerebral cortex in fetal sheep manifests as episodes of either high amplitude (generally >100 μV) activity or lower amplitude (≈50 μV) activity, with the episodes succeeding each other and having a duration of 10–20 min. Associated with these high and low voltage ECoG episodes was the presence of increased nuchal muscle EMG and EOG activities, respectively. There was a clear co-ordinated pattern of fetal behaviour associated with high and low voltage ECoG activity for all fetuses, including the fetal breathing movements described above, during the pre-UCO baseline (Fig. 2).

We found that UCO caused a brief cessation of eye, muscle and breathing activities (Fig. 2). In the present study, we observed the gradual development of abnormal behavioural patterns over the days following UCO and leading up to labour and/or birth. The relative amount of high voltage ECoG activity increased from 8 days after the UCO (Fig. 4A) and, statistically, there was a significant interaction of time and group (\(F_{23,215} = 2.297, \quad \_\_\_\_\_\_\_\_\_\_\_\_\_\_)
Table 2. Blood gases, pH, blood glucose and lactate, plasma cortisol, MAP, and HR prior to, during and after UCO in control (n = 5) and UCO (n = 11) sheep

|                  | −1 h       | +5 min     | +9 min     | +30 min    | +1 h       | +2 h       | +6 h       | +12 h      | +24 h      | +48 h      |
|------------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|------------|
| **P**<sub>co2</sub> (mmHg) | Control    | 22.5±2.3   | 22.8±2.1   | 24.1±2.8   | 23.4±2.5   | 22.5±2.0   | 22.3±2.2   | 22.2±2.1   | 22.6±2.0   | 22.1±2.0   | 21.4±2.2   |
|                 | UCO        | 22.4±3.2   | 8.3±5.4*** | 9.5±5.8*** | 22.8±4.6   | 22.4±3.3   | 21.8±3.2   | 21.3±3.7   | 21.7±3.5   | 22.9±2.3   | 21.6±3.4   |
| **P**<sub>co2</sub> (mmHg) | Control    | 50.3±2.2   | 50.9±0.6   | 51.5±2.1   | 50.9±0.9   | 50.1±1.2   | 49.7±1.5   | 49.3±1.2   | 51.7±0.8   | 50.6±0.8   | 51.9±1.8   |
|                 | UCO        | 51.2±2.3   | 90±17.8*** | 103±30.3** | 54.1±3.7   | 49.2±2.6   | 48.8±2     | 49.7±2.9   | 49.3±2.5   | 49.6±3.8   | 50±3.1     |
| pH               | Control    | 7.36±0.01  | 7.35±0.02  | 7.35±0.03  | 7.35±0.02  | 7.36±0.01  | 7.36±0.01  | 7.36±0.01  | 7.35±0.01  | 7.35±0.01  | 7.35±0.01  |
|                 | UCO        | 7.36±0.02  | 7.12±0.11** | 7.03±0.15** | 7.23±0.06*** | 7.31±0.04** | 7.35±0.02  | 7.37±0.02  | 7.37±0.02* | 7.37±0.02  | 7.38±0.02  |
| Glucose (mmol L<sup>−1</sup>) | Control    | 0.8±0.2    | 0.8±0.2    | 0.8±0.2    | 0.8±0.3    | 0.9±0.2    | 0.9±0.2    | 1±0.3      | 1±0.2      | 1±0.2      | 0.9±0.1    |
|                 | UCO        | 1±0.2      | 0.5±0.2    | 1.4±0.7    | 1.8±0.3**  | 1.5±0.2*   | 1.6±0.2**  | 1.5±0.3    | 1.2±0.2    | 1.4±0.2    | 1.2±0.3    |
| Lactate (mmol L<sup>−1</sup>) | Control    | 1.3±0.1    | 1.3±0.1    | 1.2±0.1    | 1.3±0.2    | 1.3±0.2    | 1.2±0.2    | 1.4±0.2    | 1.5±0.4    | 1.4±0.2    | 1.6±0.7    |
|                 | UCO        | 1.6±0.2*   | 5.3±2.5**  | 7.0±3.3**  | 6.3±2.4*** | 5.1±2.1**  | 4.0±1.8**  | 2.9±1.4    | 1.9±0.5    | 1.8±0.3    | 1.6±0.3    |
| Plasma Cortisol (ng mL<sup>−1</sup>) | Control    | 12.98±11.37 | 6.03±5.05  | 9.66±12.22 | 11.03±12.76 | 5.33±3.36 | 10.43±10.31 | 14.51±20.95 | 13±15.7    | 12.15±8.67 | 8.41±8.09 |
|                 | UCO        | 8.83±5.83  | 21.61±19.5 | 27.25±24.94 | 46.18±23.96* | 38.01±22.11** | 24.9±16.12 | 24.1±9.25 | 20.16±13.73 | 32.02±18.73 | 13.72±6.58 |
| Mean Arterial Pressure (mmHg) | Control    | 42.28±1.61 | 41.07±0.85 | 40.55±1.82 | 41.03±2.17 | 42.16±1.95 | 41.33±1.38 | 42.10±1.04 | 41.49±1.21 | 41.42±0.88 | 40.64±2.45 |
|                 | UCO        | 42.90±2.33 | 50.21±7.23 | 36.11±11.76 | 44.52±3.35 | 46.99±2.11 | 44.55±2.97 | 46.64±2.09 | 42.10±2.25 | 42.74±3.05 | 43.59±1.23 |
| Heart rate (beats min<sup>−1</sup>) | Control    | 162.54±13.55 | 171.83±26.79 | 159.14±15.78 | 159.50±21.38 | 170.46±37.01 | 163.58±23.32 | 163.76±28.65 | 164.55±20.38 | 161.25±15.65 | 151.98±8.05 |
|                 | UCO        | 171.73±20.55 | 169.15±62.38 | 206.56±73.65 | 181.60±22.35 | 185.11±24.94 | 171.79±12.82 | 157.90±3.42 | 156.98±6.69 | 175.31±4.04 | 152.11±11.76 |

Data are shown as the mean ± SD.
* P < 0.05.
** P < 0.01.
*** P < 0.001.
**** P < 0.0001 compared to controls.
and a main effect with time ($P = 0.0010$). In addition, episodes of very low amplitude ECoG, which were sometimes isoelectric, were observed in six of 11 fetuses following UCO, although these were not present at these gestational ages in all five of five control fetuses (Fig. 4B). In the six of 11 UCO fetuses that exhibited sub-low voltage ECoG activity, all had reached a blood pH nadir <7.00 (range 6.89–6.97) and $P_{CO_2}$ maximum >115 mmHg (range 115–134 mmHg) at 9 min during UCO. The incidence of eye movements (EOG activity) was not significantly different between UCO and control (Fig. 4C). The average incidence of EOG activity in the control group was between 40% and 60% until the end of the experiment at term, whereas UCO fetuses appeared to have increased EOG incidence from 5 days post-UCO (Fig. 4C). No consistent changes were seen in the overall incidence of nuchal EMG activity in either group after the UCO (Fig. 4D).

Typical co-ordinated fetal behaviour prior to UCO is indicated as increased nuchal muscle activity predominantly during periods of HV-ECoG activity, whereas breathing and eye movements occurred mainly during LV-ECoG activity (Fig. 2). Consequently, we measured the percentage incidence of disorganized fetal behaviour as the percentage incidence of HV-ECoG episodes along with EOG activity (Fig. 5A), incidence of fetal breathing (Fig. 5B) and the occurrence of nuchal EMG activity during LV-ECoG episodes (Fig. 5C). Prior to UCO, EOG activity was present during high voltage ECoG activity for 11.5% ± 15.7% and 11.3% ± 10.9% of the time in control and UCO fetuses respectively. In the control fetuses, EOG activity during high voltage ECoG persisted at this level until ~2 days before the onset of labour when it then increased to occupy ~20% of high voltage ECoG episodes (Fig. 5A). However, following UCO and despite a large variability in response, the incidence of EOG activity during high voltage ECoG episodes increased progressively from 19% to 48% (Fig. 5A), in which the percentage incidence became more variable in individual animals from day 8, the same time during which control animals displayed the increased incidence of EOG-high voltage ECoG episodes. Linear regression of the incidence of HV ECoG with EOG activity over from 3 days post-UCO showed that there was a significant difference ($F_{1,203} = 8.059$, $P = 0.005$) between UCO fetuses ($y = 0.1949x + 1.308$) and
controls ($y = 0.07991x + 1.308$) (Fig. 5Aa). The incidence of fetal breathing during high voltage ECoG remained consistent between fetuses that experienced a UCO and controls for the duration of the recording (Fig. 5B). Linear regression analysis of the incidence of HV ECoG along with fetal breathing from 3 days (72 h) post-UCO showed that there was no difference between UCO fetuses ($y = 0.06396x + 10.11$) and controls ($y = 0.06333x + 4.809$) (Fig. 5Ba).

Prior to UCO, the incidence of nuchal muscle EMG activity during low voltage ECoG episodes was 27.1% ± 17.2% in control and 32.7% ± 16.6% in UCO fetuses. In the days leading up to labour, EMG activity continued to occupy between 18% and 34% of low voltage ECoG episodes in control fetuses (Fig. 5C). By contrast, UCO resulted in a progressive decrease of EMG activity over time. There was less variability in EMG activity observed in UCO fetuses compared to EOG activity. The development of disorganized EMG fetal behaviour over time was statistically greater in UCO fetuses as indicated by the reduced EMG activity during LV-ECoG nearing labour compared to control animals ($P = 0.0378$). Linear regression analysis of the incidence of LV ECoG with nuchal EMG activity from 3 days post-UCO showed that there was a significant difference ($F_{1,219} = 24.74$, $P = 0.0030$) between UCO fetuses ($y = 0.06236x + 23.16$) and controls ($y = −0.03188x + 12.73$) (Fig. 5Ca).

**Discussion**

The present study has shown that a single transient episode of complete UCO late in gestation in fetal sheep results in persisting effects on fetal behaviour, despite fetal blood gases and cardiovascular haemodynamics returning to pre-UCO levels soon after the UCO event. There was evidence of increased sub-low ECoG, increased amplitude of fetal breathing and disordered fetal behavioural states in the days following UCO. We found that all of the ewes, where fetal UCO occurred, entered labour earlier.

We observed a persistent increase in the incidence of suppressed (sub-low) ECoG activity following UCO consistent with our previous study (Yan et al. 2009). This occurred in six of 11 of the UCO fetuses, each of which had fetal blood pH below 7 and $P_{CO_2}$ above 115 mmHg measured just before the end of the UCO period. Previous studies have found this ECoG activity response to be related to gestational age, with the degree of suppression increasing with advancing gestational age (Reddy et al. 1998; Wassink et al. 2007) and duration
of cerebral ischaemia (Williams et al. 1992). Although it was beyond the scope of the present study to assess seizure-like activity, it has been shown that prolonged UCO at 0.85 gestation is strongly associated with a high incidence of status epilepticus and subsequent neural injury (Castillo-Melendez et al. 2013; Drury et al. 2014). Clinically, persistent disruption of EEG patterns has been associated with the severity of hypoxic-ischaemic encephalopathy (Sarnat & Sarnat, 1976; Horn et al. 2013) and correlates with brain injury detected by magnetic

Figure 4. ECoG HV, sub-low, EOG and nuchal EMG activity responses in late gestation fetal sheep following UCO
The percentage of time spent in high voltage (HV) (A), sub-low isoelectric voltage activity (B), active eye movements (C) and active nuchal EMG (D) in fetuses subjected to sham (control) (grey, n = 4–5) or UCO (black, ECoG, nuchal EMG, n = 3–11; EOG, n = 3–9) prior to UCO until 12 days post-UCO. Note: baseline: −6 h to 0 h. 0 indicates the start of UCO. x-axis scales from hours (incidence per hour) to days (incidence per 12 h). A, two-way mixed model ANOVA, Time × Group: P < 0.01; Time: P < 0.0001; post hoc: Sidak, not significant. Data are shown as the mean ± 95% CI
resonance imaging (Toet et al. 1999; van Laerhoven et al. 2013; Merchant & Azzopardi, 2015; Jain et al. 2017; Kota et al. 2020). We observed fetal gasping (single, deep breaths) during and following UCO in the present study consistent with our previous study (Yan et al. 2009). We also found that, in the hours following UCO, some fetuses displayed persistent large amplitude breathing movements, and there was also a significant effect on decreasing the incidence of breathing movements over days post-UCO (Fig. 2). Fetal breathing movements are both functionally important for lung maturation (Jansen & Chernick, 2009).

**Figure 5. Disorganization of fetal behaviour in late gestation fetal sheep**
Percentage incidence (per 12 h) of the occurrence of HV ECoG and EOG activity (A), HV ECoG and incidence of fetal breathing movements (FBM) (B) and nuchal EMG activity with LV ECoG (C) in control (grey, n = 3–5) and fetal sheep that experienced UCO, (black, n = 3–9). Inset: individual points and linear regression from 3 days post-UCO for HV ECoG with EOG (Aa), FBM (Ba) and LV ECoG with nuchal EMG (Ca). As a result of early onset labour, data are shown until UCO group, n = 3. Data are shown as the mean ± 95% CI. **P < 0.01.
1991; Harding & Hooper, 1996) and are neurologically co-ordinated with LV ECoG activity and eye movements (Dawes et al. 1972; Koos et al. 1987). With advancing gestation, episodes of fetal breathing movements become longer in duration, with breathing activity accounting for a measurable fraction of fetal oxygen consumption in fetal sheep (Rurak & Gruber, 1983). The present study is the first to demonstrate reduced fetal breathing movements following a single, transient hypoxic event in a physically intact fetal brain. We also found decreased lung weight in UCO fetuses, although it is not known whether alterations to fetal breathing or early labour contributed to this.

A decreased incidence of normal low amplitude, intermittent breathing movements, and sometimes also onset of gasping activity have been seen following single or repeated episodes of hypoxia in fetal sheep (Clewlow et al. 1983; Bocking & Harding, 1986; Bissonnette et al. 1989; Bocking, 1992; Kawagoe et al. 1999), although, in the present study the fetal arterial oxygenation and pH were normal from ~6 h after the UCO.

We observed that, in some of the UCO fetuses, there were more profound and persistent changes in disorganized and abnormal behavioural states. There was a decreased incidence of nuchal EMG activity during LV-ECoG and increased EOG movements during HV-ECoG in the days following UCO. However, we did not observe any difference in the incidence of fetal breathing and the co-ordination with HV-ECoG following UCO. Neural pathways above and at the level of the mid-brain have been suggested to co-ordinate motor (eye and nuchal activity) and breathing activity (Clewlow et al. 1983). Large brainstem transection (Dawes et al. 1983) and punctate brain lesions (Gluckman & Johnston, 1987; Johnston & Gluckman, 1989; Koos et al. 2000) have resulted in loss of the fundamental connection between fetal breathing movements and ECoG activity. It is possible that the networks responsible for descending inhibition in the pontine parabrachial nucleus (Walker, 1995; Breen et al. 1997; Nitos & Walker, 1999), or from the thalamic nuclei (Koos et al. 2000) of late gestation fetal sheep, are severely disrupted by hypoxic-ischaemic conditions. Clinically, brainstem lesions have been well documented following severe asphyxia (Leech & Alvord, 1977; Roland et al. 1988; Sugama & Eto, 2003), with the severity of brainstem lesions being associated with severity of motor impairments (Martinez-Biarge et al. 2011).

However, the present results suggest that brief in utero hypoxia-ischaemia could make these neural pathways dysfunctional for a considerable time, and further suggest that close surveillance of fetal activity in late gestation might identify the consequences of fetal hypoxia before the onset of labour.

We used fetal plasma to measure cortisol as one indicator of fetal stress and, as expected, we found that cortisol production increased following UCO. Cortisol secretion in response to acute or chronic hypoxia, or to a sudden decrease of uterine blood flow is well known to occur in fetal sheep, piglets and goats (Bocking et al. 1986; Giussani et al. 1994; Unno et al. 1997; Supramaniam et al. 2004; Roelfsema et al. 2005; Fujimori et al. 2008; Harris et al. 2009). It is assumed this occurs primarily as the result of increased adrenocorticotropic hormone (ACTH) release following stimulation of the hypothalamic-pituitary-adrenal axis by arterial chemoreceptor activation (Challis & Brooks, 1989). However, the initial rapid release of cortisol could also be the result of splanchic stimulation of the adrenal gland because this is known to result not only in increased release of medullary catecholamines (hence, the increase of MAP), but also the release of steroids from the adrenal cortex (Edwards & Jones, 1993; Ehrhart-Bornstein et al. 1998), as well as reflect a paracrine interaction close apposed medullary and cortical tissue (Bornstein et al. 1991). The cortisol response following hypoxia has been used as an indicator of neurological outcome in a neonatal piglet model (Harris et al. 2009) and, clinically, during therapeutic hypothermia (Scaramuzzo et al. 2015).

The increase of fetal plasma cortisol that precedes and accompanies labour in sheep has been fully described (Magyar et al. 1980; Wood & Keller-Wood, 1991). Interestingly, in the present study, we found that, after the 10 min UCO, labour occurred significantly earlier for these fetuses, and when the increase in plasma cortisol occurred prior to labour, it was comparable to that in the control fetuses. An explanation may be that the sympathetic stimulation of the adrenal gland discussed above also increased the sensitivity of the cortex to ACTH, as discussed elsewhere in relation to adrenal function in conscious newborn calves (Jones & Edwards, 1990). Also to be considered is the possibility that the fetal compartment of the placenta was metabolically changed after the UCO, leading to either myometrial or fetal changes (or both) that provoked the early onset of labour. These are necessarily speculative arguments, although they point to where future research should lead.

There are a few important limitations to highlight in the present study. We observed a large variability in the fetal response to UCO, despite inflating the cuff sufficiently to completely occlude umbilical blood flow. The small sample size of the control group could have also contributed to the non-significant trends observed. HR variability and subsequent cardiovascular responses are strongly related to the behavioural differences seen long term, both experimentally in fetal sheep and clinical studies (Gunn et al. 1992; Mallard et al. 1992; Harris et al. 2009; Andersen et al. 2019). It is also important to highlight that sex may influence both the response to UCO and the outcomes observed, although the low number of animals studied here precluded any sex-influenced differences to be identified. In addition, although we
did not assess neuropathology in the present study, previous studies have shown that a single, transient UCO late in gestation (126–136 days) results in alterations to the hippocampus, cortex, striatum, white matter and cerebellum, with evidence of cell death, inflammation and lipid peroxidation (Mallard et al. 1992; Ikeda et al. 1998; Duncan et al. 2004; Yawno et al. 2012; Castillo-Melendez et al. 2013). Future studies measuring real-time chemical changes in the brain using microdialysis in the days following UCO could provide additional insight. In the present study, we noted a significant 20% reduction in the absolute weight of the cerebellum, although no change in the weight of the brain as a whole.

We have shown that a single, relatively brief episode of hypoxic-acidaemia leads to gradual changes in fetal breathing and other brain activities that are not normally present in the weeks leading up to labour and delivery in sheep. This may indicate that the chemical environment in the fetal brain may have been changed for some time after UCO, even though systemic blood chemistry does not provide evidence of persisting effects on global fetal metabolism. The highly integrated patterns of fetal activity that resemble ‘quiet’ and ‘rapid eye movement’ sleep are considered to arise from neural networks comprising the reticular activating system in the pons and diencephalon. It is possible that a legacy of brief severe hypoxia is to alter the activity in the ascending (i.e. thalamo-cortical) and descending (i.e. medullary, spinal cord) projections of this network so that the highly co-ordinated behavioural patterns are significantly changed and do not recover before birth. If so, this does not appear to alter HPA activity because the late gestation trajectory of cortisol secretion was not different in the UCO and control groups. Notwithstanding these speculative considerations, the present study suggests that closer attention to fetal activity in utero in late gestation could reveal useful clinical signs of the effects of hypoxia on the fetal brain.

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Additional information
Data availability statement
The data that support the findings in this study are available from the corresponding author upon reasonable request.

Competing interests
The authors declare that they have no competing interests.

Author contributions
AAB, MCM and DW contributed to the experiments. AAB and DW acquired and analysed the data. AAB, NTT, MCM, TY and DW contributed to data interpretation and drafting of the manuscript. All authors have approved the final version of the manuscript and agree to be accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.
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Keywords
asphyxia, cortisol, fetal behaviour, fetal sheep, perinatal encephalopathy

Supporting information
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Statistical Summary Document