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Placental restriction in multi-fetal pregnancies increases spontaneous ambulatory activity during daylight hours in young adult female sheep.

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Running head: Developmental programming of physical activity

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Abstract

Intrauterine growth restriction (IUGR) has adverse effects on metabolic health and early life, while physical activity is protective against later development of metabolic disease. Relationships between birth weight and physical activity in humans, and effects of IUGR on voluntary activity in rodents, are mixed and few studies have measured physical activity in a free-ranging environment. We hypothesized that induced restriction of placental growth and function (PR) in sheep would decrease spontaneous ambulatory activity (SAA) in free-ranging adolescent and young adult progeny from multi-fetal pregnancies. To test this hypothesis, we used Global Positioning System watches to continuously record SAA between 1800h and 1200h the following day, twice during a 16-day recording period, in progeny of control (CON, n=5M, 9F) and PR pregnancies (n=9M, 10F) as adolescents (30 weeks) and as young adults (43 weeks). PR reduced size at birth overall, but not in survivors included in SAA studies. In adolescents, SAA did not differ between treatments and females were more active than males overall and during the day (each $P < 0.001$). In adults, daytime SAA was greater in PR than CON females ($P = 0.020$), with a similar trend in males ($P = 0.053$) and was greater in females than males ($P = 0.016$). Adult SAA was negatively correlated with birth weight in females only. Contrary to our hypothesis, restricted placental function and small size at birth did not reduce progeny SAA. The mechanisms for increased daytime SAA in adult female PR and low birth weight sheep require further investigation.

Keywords: Placental Insufficiency; Physical activity; Behavior; Sex differences; Sheep

Introduction

Intrauterine growth restriction (IUGR) arises from maternal, fetal and/or placental factors that prevent the fetus from achieving its genetic potential for growth[1, 2]. In developed countries IUGR, which is most commonly caused by placental insufficiency, affects 6-12% of births[3, 4]. Placental insufficiency progressively restricts transfer of nutrients and oxygen to the developing fetus, reducing growth particularly in late gestation[5, 6]. In human studies, low birth weight or small size at birth for gestational age (SGA) are often used as surrogate markers of IUGR [7]. There is conflicting evidence from human cohorts that voluntary levels and intensity of physical activity are altered in low birth weight compared with normal birth weight adolescents and adults[8-12]. In human cohorts, physical activity throughout life, and in childhood or adolescence is associated with decreased risk of developing metabolic disease in adult life[13-16]. Decreased physical activity after IUGR may therefore contribute to the increased risk of metabolic disease in this population[17-19].

A meta-analysis categorizing adolescents and adults as active or inactive by self-report, showed an inverse U-shaped relationship between birth weight and levels of leisure time physical activity (LTPA)[8]. Other studies confirm these findings with low and high birth weight individuals self-reporting lower levels of LTPA compared to those born of average birth weight[8, 10, 11]. In contrast, LTPA measured objectively through accelerometer data during adolescence was either not related[12] or positively related[9] to birth weight. Similarly, reduced birth weight due to maternal famine exposure in mid- or late-gestation did not significantly alter self-reported physical activity[20]. Variable gender differences in physical activity have also been reported in humans, with LTPA either not differing between genders in adolescents and adults[8, 12] or females being more sedentary than males in adolescence[9]. In addition to the variable effects of birth weight and sex, it is difficult to infer causality from human studies due to confounding by environmental factors that affect growth before birth and activity during postnatal life. For example, the risk of a SGA birth increases with lower socioeconomic status[21, 22] and socioeconomic status is positively correlated with levels of physical activity in both adolescents and adults[23, 24]. Animal models of IUGR where progeny are delivered at term may be useful in separating out these effects of prenatal and postnatal environment on postnatal voluntary physical activity, whilst evaluating outcomes in both sexes is important given evidence for sex-specific effects of prenatal exposures.

Data from animal studies support the concept that prenatal exposure to maternal undernutrition may program voluntary physical activity. In rats, IUGR induced by maternal food restriction to 30% of ad libitum intakes decreased the activity of male and female progeny within a test arena over a recording period of fifteen minutes, measured during the peri-pubertal period at 35 d of age and in adulthood at 14 months of age[25]. Similarly, young adult (60 d old) IUGR male rat progeny of dams whose food intake was restricted by 50% from d 10 of pregnancy and throughout lactation, ran a shorter total distance over 7 d when provided with continuous running wheel access compared to control progeny from dams with ad libitum access to feed[26]. Interestingly, female progeny from food-restricted dams ran more than female progeny from control dams in the same experiment[26]. In a separate study, locomotor activity measured over half an hour during daylight hours in adult rats at 91 d of age was reduced in male progeny when mothers were protein-restricted during early pregnancy but not mid- or late-pregnancy[27]. In female progeny, activity was reduced by maternal protein restriction regardless of whether restriction was imposed during early-, mid-, or late-pregnancy[27]. This provides further evidence that effects of some perinatal exposures on later activity may be sex-specific, and reinforces the need to include progeny of both sexes when evaluating impact. Also consistent with the hypothesis that prenatal exposures program physical activity, periconceptional maternal undernutrition in sheep decreased the distance walked voluntarily by adult male and female progeny over a period of 48 h in a paddock environment[28]. Interestingly, in this cohort comprising twin and singleton progeny, litter size did not affect activity in adulthood[28]. Maternal undernutrition of sheep throughout early-mid gestation did not alter physical activity of progeny, but this may reflect the fact that in this study the progeny were barn-housed and therefore had restricted opportunity for activity, compared to paddock-housed sheep[29]. These studies do not however evaluate effects of IUGR on activity, since periconceptional maternal undernutrition does not reduce size at birth in sheep[30], while early-mid gestation nutrient restriction actually increased birth weight of progeny[31]. Each are also likely to affect fetal growth at different times than occurs in IUGR, where restricted placental function restricts fetal growth mostly in late gestation[6]. As yet, effects of restricted placental growth and/or function on spontaneous ambulatory activity (SAA) have not been reported; nor has the effect of IUGR or restricted placental function on progeny physical activity been assessed in a free-ranging environment.

Restricted placental growth and function (placental restriction, PR) resulting in IUGR can be induced experimentally in sheep by surgical removal of the majority of placental attachment sites from the non-pregnant endometrium prior to mating[32, 33]. This induces similar fetal and postnatal consequences as seen in human IUGR, by decreasing placental blood flow and oxygen and nutrient supply to the fetus[34-37]. In previous studies, average birth weight in PR lambs at term was reduced by 20-31%[38-40]. Postnatally these lambs experience catch-up growth[41, 42] and develop insulin resistance in early postnatal life[40], whilst males but not females have impaired insulin action which persists to adulthood[43]. We therefore utilized this experimental paradigm to test the hypothesis that restriction of placental growth and small size at birth would reduce levels of SAA in adolescent and young adult sheep in a free-ranging environment, and that effects would be greater in female than male progeny.

Methods

All procedures were approved by the University of Adelaide Animal Ethics Committee (approval M-2013-231B) and conducted in accordance with Australian guidelines[44].

Animal Cohort

Placental growth of Merino × Border Leicester ewes was restricted by surgical removal of all but four visible endometrial placental attachment sites (caruncles) from each uterine horn, at least 10 weeks before timed mating of PR and un-operated control (CON) ewes[32, 33]. Because surgery and recovery occur before pregnancy in this model such that the fetus is not exposed to maternal surgery in PR pregnancies, and initial studies in this model established that sham surgery did not reduce size at birth[32], we did not perform sham surgery on CON ewes in the present study. Pregnancy was confirmed by ultrasound at 48-55 d after mating. Only ewes scanned as pregnant with twins (12 CON, 24 PR ewes) were selected for the study, due to limited availability of singleton control pregnancies. Ewes were housed indoors from day 110 of gestation until their spontaneously born lambs were weaned at 97.0 ± 0.4 days of age. Throughout late gestation and lactation ewes were fed 1 kg Rumevite pellets daily (10.6 MJ metabolisable energy/kg dry matter; 12.3% crude protein, Ridley AgriProducts, St Arnaud, Australia), with ad libitum access to lucerne chaff and water. Gestational ages, lamb weights, and litter sizes were recorded at birth. Only lambs born from litters with two or three lambs were included in the present study; not all litter sizes from ultrasound corresponded to litter size at delivery (Figure 1). A total of 23 CON lambs (1 still born and 22 live born) from 10 CON ewes and 39 PR lambs (26 live born and 13 still born) from 19 PR ewes were delivered between 12 and 27 July 2014 (Figure 1). Due to deaths of some non-viable lambs and removal of triplet siblings, surviving lambs included in the spontaneous activity study [5 CON males (2 twins, 2 triplets), 9 CON females (6 twins, 3 triplets), 9 PR males (9 twins), and 10 PR females (10 twins)] were reared as twins or singletons during lactation. The litter size during lactation (number of lambs suckling the ewe) was therefore included in statistical models to account for neonatal nutritional environment.

Lambs were weighed daily until 30 d after birth, when catch-up growth usually occurs in PR lambs, based on our previous study in mixed singletons and twins[41] and then weekly until weaning. Absolute and fractional growth rates from birth to 30 d postnatal age, were calculated by linear regression[42]. After weaning, progeny were housed in adjacent paddocks in same sex groups at the Roseworthy campus of the University of Adelaide and group fed daily at a rate of 0.5 kg Rumevite pellets per sheep, with ad libitum access to oaten hay, seasonal pasture, and water, and were weighed at monthly intervals.

Spontaneous Ambulatory Activity

Spontaneous ambulatory activity studies were performed under natural light and temperature conditions in the paddocks where animals were held throughout the study. Animals remained in their same-sex groups, each with access to paddocks of the same size (~0.25 ha) and shape, throughout both series of activity studies. Each animal was studied as an adolescent (204 ± 1 d of age, during summer in January-February 2015) and as a young adult (294 ± 1 d of age, during autumn in May 2015). Two recordings of 18 h duration were taken on each animal at each age. At each age, 5-6 animals were randomly allocated to each study day, with one recording of each animal completed before the second block of recordings, and different randomized orders used in each block to allow correction for day effects. All studies were completed within a 16-d period at each age. Garmin Forerunner 910XT GPS devices (Garmin Limited, Lenexa, Kansas, United States) were attached to a collar placed on individual sheep at 1800h, and removed at 1200h on the following day. Recording duration was determined by battery life and timed to capture periods of peak and changing activity seen in the evening and morning in free-ranging sheep[45]. Data were uploaded to the Garmin Website using Garmin Connect software (Garmin Ltd, v 15.7.4.1), and distance in 5-second intervals was downloaded for subsequent data cleaning (to remove satellite artefacts). Distance travelled was used as the measure of spontaneous ambulatory activity and was summed for each 10 minute period between 1800h and 1200h the following day for each animal for the analysis of activity patterns. Average distance travelled per hour was calculated for the whole recording period, during daylight hours (before sunset and after sunrise), during night hours (between sunset and sunrise), and for hourly blocks from 2 h before sunrise to 2 h after sunrise. Average distance travelled per hour was also summed for hourly blocks from 1 h before to 2 h after sunset in adolescent animals only, when the recordings consistently started over an hour prior to sunset; pre-sunset data was not available in adults due to season. Average times of sunrise and sunset were 0642 h and 2015 h, respectively during adolescence in summer, and 0656 h and 1725 h, respectively during adulthood in autumn. Half-hourly temperature data for the Roseworthy campus weather recording station throughout each recording period were downloaded from the Australian Government Bureau of Meteorology server (http://www.bom.gov.au/climate/dwo/IDCJDW5062.latest.shtml).

Statistical Analysis

Size at birth and gestational age at birth were analyzed by mixed models ANOVA, for effects of treatment (CON compared with PR) and lamb sex as main factors, and including the dam as a random factor to correct for maternal effects. Neonatal growth rates were analyzed by mixed models ANOVA, including treatment, lamb sex and lactation litter size (one or two lambs suckling the ewe) as main factors, and including the dam as a random factor to correct for maternal effects. Effects of treatment and sex on proportions of lambs born alive were analyzed by χ^2 test. Repeated measures of lamb body weights from birth to 30 d of age (during catch up growth), from 30 d of age until weaning, and from weaning until the end of the study were analyzed by mixed models ANOVA, for effects treatment, lamb sex, and lactation litter size as main factors, age as a within-animal factor and including the dam as a random factor in each model to correct for maternal effects. At each age, distances travelled per hour during the whole recording period, and during daylight and night hours of the recording period, were analyzed using a repeated measures ANOVA, including treatment, sex, lactation litter size and recording block (1st or 2nd replicate) as main factors, dam and recording date as random factors, recording block as a within-animal factor and maximum temperature during the sampling period as a covariate. Spline analysis of behavioral patterns was conducted used 10 minute interval data across the recording period, with 7037 distance records included. These were analyzed using a linear mixed model with a cubic spline that had 18 knot points, which fits a very flexible polynomial regression as previously detailed[46]. Fixed effects included: treatment, sex, recording block, maximum temperature, time*treatment (linear treatment effect) and time*sex (linear sex effect). Random effects included: dam, lamb, spline (time)*treatment (test for treatment differences in activity patterns), spline(time)*sex, and factor(time) to allow for non-smooth departures in activity due to things like human disturbances. Pairwise comparisons between male and female activity at specific times based on predictions of activity every half-hour were analyzed by t-test. Associations between total, daylight and night activity as adolescents and adults and birth weight were assessed by Pearson's correlation. Excluding lambs born in triplet litters limited between-sex comparisons and did not change effects of treatment on size at birth, neonatal growth or activity totals (Supplementary Table 1); data reported below therefore includes progeny born to twin and triplet litters. All analyses were performed using IBM SPSS v 22 (SPSS, Chicago, IL), and data are presented as estimated means ± SEM unless otherwise stated.

Results

Size at Birth and Perinatal Survival

In the subset of live born lambs, PR lambs were 26% lighter at birth than CON lambs (CON: 4.28 ± 0.79 kg; PR: 3.17 ± 1.29 kg; $P < 0.001$), and birth weight did not differ between males and females ($P > 0.5$). Still born lambs were 44% lighter than live born lambs (live born: 3.98 ± 0.98 kg; still born: 2.22 ± 1.10 kg; $P < 0.001$). Gestational age was lower in PR lambs compared to CON lambs (CON: 146.0 ± 2.6 days; PR: 143.5 ± 2.0 days; $P < 0.001$), although the majority were still within the term range, based on our previous observations of gestation length at spontaneous delivery in a larger cohort of CON pregnancies in this strain of sheep (mean: 147.0 ± 0.3 days; range 143-150 days). Overall, PR lambs were less likely to be born alive than CON lambs (Control: 22 of 23 born alive; PR: 26 of 39 born alive; $P = 0.008$).

In the lambs that survived and were included in spontaneous ambulatory activity studies, birth weight did not differ between treatments or sexes overall (Table 1), or in twin-born progeny only (Supplementary Table 1). Within CON lambs included in spontaneous ambulatory activity studies, birth weights did not differ between those born in twin and triplet litters (CON twin: 4.64 ± 0.24 kg; CON triplet: 4.36 ± 0.26 kg; P > 0.1), and birth weights of triplets all fell within the range of birth weights observed in twins (CON twin: 3.5 - 5.7 kg; CON triplet: 43.7 – 5.2 kg). For ewes that had at least one lamb survive to be included in the spontaneous activity study, gestational age did not differ between CON and PR lambs (CON: 145.5 ± 2.7 days; PR: 143.9 ± 1.6 days; P > 0.1).

Postnatal Growth

In the first month of life, absolute growth rate (Table 1) did not differ between CON and PR lambs (P > 0.9), and was higher in males than females (P = 0.012), and fractional growth rate (Table 1) did not differ between treatments (P > 0.1) or sexes (P > 0.1). Similar effects were observed in twin-born progeny analysed separately (Supplementary Table 1). Absolute and fractional growth rates from birth to day 30 did not differ between lactation litter sizes (each P > 0.3). Body weight during the first month of life (Figures 2A and 2D) increased with age (P < 0.001), tended to be higher in CON than PR overall (P = 0.054), and was higher in males than females (P = 0.005). Lambs reared as singletons due to perinatal death of a sibling were heavier overall (P = 0.001) and grew faster (lactation litter size*age interaction P < 0.001) than lambs reared as twins. From the end of the neonatal period until weaning at 14 weeks of age (Figures 2B and 2E), body weight increased with age (P < 0.001), did not differ between treatments (P > 0.4) or lactation litter sizes (P > 0.1), and was higher in males than females (P = 0.040). Similarly, body weight after weaning (Figures 2C and 2F) increased with age (P < 0.001), did not differ between treatments (P > 0.5) or lactation litter sizes (P > 0.6), and was higher in males than females (P = 0.001).

Spontaneous Ambulatory Activity in Adolescence

In adolescent sheep, the distance travelled over each 30-minute interval changed throughout the recording period, following a typical diurnal pattern of greater activity during daylight than night times (Figures 3A and 3B). Over the total 18-h recording period distance travelled per hour did not differ between treatments ($P > 0.1$, Figure 4A), females travelled 17% further than males ($P < 0.001$, Figure 4A), and distance travelled did not differ between recording blocks, lactation litter sizes or with maximum temperature ($P > 0.1$, data not shown). During daylight, distance travelled per hour did not differ between treatments ($P > 0.1$, Figure 4B), females travelled 25% further than males ($P < 0.001$, Figure 4B), and distance travelled did not differ between recording blocks, lactation litter sizes or with maximum temperature ($P = 0.09$, data not shown). During night, distance travelled per hour did not differ between CON and PR progeny ($P = 0.082$, Figure 4C), or sexes (each $P > 0.1$, Figure 4C), tended to be greater during the first recording block than during the second recording block ($P = 0.063$, data not shown), and did not differ between lactation litter sizes or vary with maximum temperature ($P > 0.1$, data not shown). Similar treatment and sex effects were observed in analyses restricted to twin-born progeny (Supplementary Table 1).

Analysis of hourly ambulatory activity during the peak activity period two hours before and after sunrise[45, 47, 48], showed no treatment differences in distance travelled in any hour (each $P > 0.1$, Figures 4D-4G). In the hour leading up to sunrise (Figure 4E), distance travelled by females was greater than males ($P = 0.012$), with a similar trend for the preceding hour ($P = 0.099$, Figure 4D), and no sex differences in activity in the two hours after sunrise (Figures 4F and 4G, each $P > 0.1$). In the hour leading up to sunset (Figure 4H), effects of treatment on distance travelled differed between sexes (treatment*sex interaction, $P = 0.043$). Distance travelled in the hour leading up to sunset was higher in PR than CON males ($P = 0.025$, Figure 4H) and did not differ between treatments in females ($P > 0.1$, Figure 4H). During the remaining hourly blocks, from sunset to one h after sunset and from 1 – 2 h after sunset, distance travelled did not differ between treatments or sexes (each P > 0.1, Figures 4I, 4J).

Spline analysis of activity in adolescents showed treatment differences in linear activity pattern (P < 0.001) but no sex effects on the linear trend (P > 0.05), and no effects of temperature (P > 0.05). In pairwise comparison of predicted activity at specific times (Figure 5) female activity was greater than that of males during periods of peak activity (each P < 0.05).

Spontaneous Ambulatory Activity in Adulthood

In adult sheep, similar to the pattern observed in adolescents, the distance travelled over each 30-minute interval changed throughout the recording period, following a typical diurnal pattern of greater activity during daylight than night times (Figures 6A and 6B). Over the total 18 hour recording period, distance travelled per hour did not differ between treatments (P > 0.1, Figure 7A), sexes (P > 0.1, Figure 7A) or lactation litter sizes (P > 0.1, data not shown), was higher during the first recording block than during the second recording block (P < 0.001, data not shown) and was positively correlated with maximum temperature (P < 0.001, data not shown). In analyses restricted to twin-born progeny only, although sex differences were observed, treatment similarly did not affect distance travelled per hour (Supplementary Table 1).During daylight, distance travelled by PR progeny tended to be greater than CON overall (P = 0.092, Figure 7B), females travelled 8% further than males overall (P = 0.016, Figure 7B), distance travelled was greater during the first recording block compared to the second recording block (P = 0.025, data not shown), lambs raised as twins during lactation travelled 18% further than lambs raised as singletons (P = 0.039, data not shown) and distance travelled did not correlate with maximum temperature (P > 0.1, data not shown). Outcomes differed between sexes, such that in males distance during daylight tended to be higher in PR than CON progeny (+8%, P = 0.053, Figure 7B), was higher during recording block one than block two (P < 0.001, data not shown), did not differ between lactation litter sizes (P > 0.1, data not shown) and distance did not correlate with maximum temperature (P > 0.1, data not shown). In females, distance travelled during daylight was 29% higher in PR than CON progeny (P = 0.020, Figure 7B), was not different between recording blocks (P > 0.1, data not shown), lambs raised as singletons during lactation tended to be less active than lambs raised as twins (P = 0.09, data not shown) and distance travelled did not correlate with maximum temperature (P > 0.1, data not shown). During the night, there were no differences in distance travelled between treatments (P > 0.1, Figure 7C), sexes (P > 0.1, Figure 7C) or lactation litter sizes (P > 0.1, data not shown). Distance travelled during the first recording block was higher than during the second recording block (P = 0.02, data not shown) and tended to be positively correlated with maximum temperature (P = 0.064, data not shown). In adults, average distance travelled across the total recording period correlated negatively with birth weight in females (Figure 8B), r = -0.644, P = 0.003, n = 19) but not in males (Figure 8A) (r = 0.021, P > 0.1, n = 14). Similarly, daylight activity correlated negatively with birth weight in females (Figure 8D) (r = -0.586, P = 0.008, n = 19) but not in males (Figure 8C) (r = 0.092, P > 0.9, n = 14). Night activity did not correlate with birth weight in either sex (data not shown)

Hourly activity during the periods from two hours before to two hours after sunrise (Figures 7D, 7E, 7F, 7G), did not differ between treatments or sexes (each P > 0.1). Spline analysis of activity in adults (data not shown) found no treatment differences in linear activity pattern (P > 0.05), and a negative effect of maximum temperature (P < 0.001). Although the linear trend differed between sexes (P < 0.05), predicted activity did not differ between sexes at any time point (P > 0.05, data not shown).

Discussion

In this study we report for the first time the effects of placental insufficiency and variable size at birth in an animal model on spontaneous levels of physical activity in later life, specifically ambulatory activity in a free-ranging environment. Contrary to our hypothesis, in the present cohort of progeny from multi-fetal pregnancies, PR increased spontaneous ambulatory activity in adult female sheep during daylight hours, with a similar trend in males, and low birth weight was similarly associated with greater spontaneous ambulatory activity overall as well as during daylight in females. PR did not affect spontaneous ambulatory activity in adolescent sheep. Consistent with previous findings, spontaneous ambulatory activity levels were higher in females than males, particularly as adolescents. This suggests that decreased spontaneous ambulatory activity in adolescence and adulthood is not a primary driver in the postnatal development of metabolic disease after restricted placental function.

In the present study of sheep from multi-fetal litters, both PR and low birth weight female adult progeny were more active than CON and higher birth weight females during daylight, with similar diurnal activity patterns in both sexes. In males, although PR also tended to have greater adult daylight activity than CON, this effect was much smaller in magnitude and activity was not correlated with birth weight. This result is consistent with the sex-specific effects of experimental IUGR in a rodent study, in which dams were subjected to 50% global food restriction from d 10 of gestation until weaning, which increased activity in female but not male progeny[26]. Sex-specific effects of PR and associations with birth weight may reflect sex-specific fetal adaptations to adverse environments, similar to patterns observed in maternal asthma in humans where growth is reduced in females but not males[49]. Whether PR or IUGR have sex-specific effects on fetal growth trajectories in these animal models is not yet known. Due to limited numbers of progeny, it was not possible to subdivide groups according to gestation litter size, although we included only progeny from multi-fetal litters in the present study. All surviving CON triplets had birth weights within the range of birth weights seen in CON twins, suggesting a similar degree of restriction. Litter size during lactation had very limited effects on activity in the present study, consistent with findings in a previous study including twins and singletons, where activity did not differ between singleton and twin litter size groups[28]. Furthermore, when we analysed activity outcomes only for lambs born in twin litters (Supplementary Table 1), effects of treatment were similar to those reported in overall analyses including twins and triplets.

The mechanisms underlying this greater activity in low birth weight and PR adult females compared to CON and high birth weight females have not yet been identified. One brain region which is an important driver of spontaneous physical activity is the dorsal medial habenula[50]. In mice, genetic elimination of neuronal development in this region reduced motivation-based locomotor activity, such as voluntary wheel running, with no abnormalities in gait and balance and the same physiological capacity for exercise seen in control progeny[50]. Induced activation of neurons in this region in normally-developed mice increased voluntary locomotor activity[50], further confirming the importance of this region as a driver of spontaneous physical activity. Effects of PR or IUGR on this region, or on biological messengers implicated in modifying voluntary activity including dopamine, noradrenaline and serotonin[51, 52] are yet to be investigated. Confounding postnatal factors such as body weight, which is negatively correlated with physical activity[53], can potentially affect activity. However, in the present study when adolescent and adult spontaneous activity measures were taken, body weight did not differ between treatments, and is therefore unlikely to have contributed to the greater levels of activity observed in PR and low birth weight adult female progeny. Similarly, although a systematic review in humans reported variable and generally negative effects of psychological stress on physical activity[54], because the sheep in the present study were habituated to human contact by regular handling as lambs and frequent weight measures from birth throughout the study, stress is unlikely to have affected activity. A possible mechanism that might contribute to effects of PR and IUGR on postnatal activity is appetite. Movement in adult sheep is predominately driven by grazing, and sheep increase grazing time when hungry[55]. Feeding frequency, an indicator of appetite, is increased in PR compared to CON lambs during catch-up growth; effects of litter size were not reported in that study[41]. The increased ambulatory activity in PR and low birth weight adult females might therefore suggest hyperphagia in adult life, which has been reported in adult IUGR rat progeny whose mothers were globally food-restricted to 30% of the intake of ad libitum-fed animals during gestation[56]. Effects of PR on adult appetite have not as yet been reported, although the similar body weights between PR and CON sheep in the present study suggests that PR might affect grazing behavior via altered food type preference rather than increased drive for total nutrient intake. Interestingly, adult human data suggest that positive feedback occurs between spontaneous physical activity and hyperphagia, such that hyperphagia stimulates the desire for increased spontaneous activity which in turn stimulates hyperphagia, due to an inherent desire to maintain homeostatic body weight[52]. This suggests that increased ambulatory activity in our adult female PR and low birth weight females might reflect increased appetite or altered food preference.

Activity patterns across the recording period were sexually dimorphic, with greater activity in females than males overall and in daylight in adolescent sheep, and also during daylight in adults. Our results are consistent with the greater physical activity in females than males reported in mice[52], rats[25, 27] and sheep[28]. Estrogen is a positive driver of activity and possibly underlies these sex-differences in activity, since in female mice ovariectomy reduces voluntary activity to levels similar to males, and 17β-estradiol treatment in ovariectomized mice increase activity[57]. Interestingly, despite variable sex-differences in activity in humans, where activity is either similar between genders[8, 12], or greater in males than females[9], estrogen also appears to be a positive driver of activity in humans, with loss of ovarian function in women during menopause correlating with a marked fall in physical activity[58]. Our sheep were likely post-puberty during measures of SPA, particularly at 43 weeks of age, given the *ad libitum* nutrition and because Merino ewes enter puberty at an average age of 31 weeks (23 – 43 weeks old)[59]. In order to minimize the likely impact and confounding by stress, we did not collect blood or track cycles in our cohort, and it was therefore not possible to match the ewes for estrus cycle. Interactions between PR and estrous cycle stage, and whether effects of PR change further with ageing beyond young adulthood, are yet to be investigated. Further characterization of sex effects on voluntary activity and understanding of underlying mechanisms including pathways for estrogen responses are required.

This is the first study showing the effect of surgical pre-mating removal of placental attachments sites, which restricts placental growth and function[32, 33], on spontaneous physical activity, where all progeny shared a common post-weaning environment, maternal age and similar genetic background. Additional strengths of the study were inclusion of both sexes, and that since all lambs delivered within 4 days of average term, this model is not confounded by prematurity, which is associated with reduced physical activity in adolescent and adult humans[60]. Effects of PR on activity may, however, have been diluted by the flocking behavior that occurs in this herd species, where groups of sheep tend to move together[61], making it even more significant that differences were observed between groups. Dilution of group differences in activity was seen in studies of circadian patterns of activity in transgenic Huntington's disease (HD) sheep. HD sheep have relatively mild behavioral changes when kept in a mixed flock including individuals of normal genotype, but circadian abnormalities were far more evident in sheep living in flocks comprising only HD sheep[45]. Therefore, dilution of treatment effects on activity and/or activity patterns may potentially have occurred in the present study and should be kept in mind when interpreting the magnitude of difference that was observed. An additional limitation in interpreting the results of the present study is that, unlike previous studies in CON and PR sheep[39-41], PR lambs within the cohort of animals that survived to be included in the spontaneous physical activity study were not lighter at birth and did not experience accelerated growth rates in early life compared to CON lambs. In part, we suspect this reflects restriction of fetal growth in all lambs, including CON lambs, within the present cohort, as a consequence of studying outcomes in offspring of multi-fetal pregnancies. Use of multiple-birth litters was chosen on the basis of ultrasound results in order to achieve similar litter sizes between treatments, due to insufficient availability of CON singleton pregnancies, but nutrient supply in late gestation is restricted in twins compared to that of singletons[62]. This may have reduced the magnitude of effects of PR on size at birth compared to these previous studies, since likely all progeny were subject to a degree of growth restriction *in utero*. Growth curves of twins and singletons diverge in sheep by ~d100 of gestation[63]. Reduction of litter size by death of one fetus of twin litters in early gestation (d42 after mating) does not fully normalise birth weight[64], possibly because the number of placental attachments to the endometrium is already fixed with adhesion occurring by d16 of development[65], and hence prior to reduction in litter size. The surgical reduction in numbers of placental attachment sites prior to pregnancy in PR ewes may thus mirror some of the effects of multi-fetal litter size in ovine pregnancy, since both reduce the numbers of placental attachments and cotyledons formed. Whether triplets suffer additional growth-restriction compared to twins is less clear, with similar fetal and placental weights reported in twin and triplet ovine fetuses in late gestation[66]. In the overall cohort of live born lambs, PR were 26% lighter than CON. The lack of birth weight difference in lambs included in the SAA studies (seen also when analysis was restricted to twin-born animals), therefore also reflects poorer survival of PR lambs, particularly the more restricted animals, including twins. An additional limitation of the study design is that, due to perinatal deaths and removal of some non-viable lambs, our study included lambs gestated in multi-fetal litters but that were raised as singletons or twins during lactation, which may have added variation in neonatal nutrition. We found however, that lactation litter size had little effect on activity measures in adolescents or adults, consistent with reports in another cohort including lambs gestated and raised as singletons and twins[28]. In human cohorts exposed to severe maternal malnutrition at different times before, during and after pregnancy, maternal exposures in early pregnancy induced adverse changes in progeny health without changes in birth weight[67], although self-reported activity was not affected by in utero famine exposure[20]. Similarly, periconceptional maternal undernutrition in sheep decreased activity in adult progeny without altering birth weight[28]. Our findings of PR effects even in the absence of differences in birth weight in females are thus consistent with the concept that periconceptual and gestational insults can affect postnatal outcomes without changes in birth weight.

IUGR is associated with increased burden of metabolic disease risk in later life, and understanding the determinants of this association may help to identify potential preventative interventions. In the present study, spontaneous ambulatory activity during adolescence and adulthood was not reduced by PR or associated with low birth weight in progeny of multi-fetal pregnancies. This may suggest that decreased physical activity does not explain the increased risk of metabolic disease after IUGR, if similar findings hold true in singleton cohorts not subjected to a level of restriction in controls as well as the PR group. In fact, contrary to the hypothesis, in the present study, PR females were more active than CON females, particularly as adults. Further studies are needed to explain why the effects of PR that we observed were sex-specific, to determine whether similar effects of PR are seen in comparisons within singleton cohorts, and to identify the mechanisms underlying this greater spontaneous ambulatory activity after IUGR in adult female sheep from multi-fetal pregnancies.

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Conflicts of Interest

None.

Figure legends

Figure 1. Animal Cohort. *Lost to study: 8 CON and 7 PR lambs were lost to study due to removal of triplet siblings to control for litter size (n = 3 CON lambs, n=1 PR lambs), maternal removal from the study for health reasons (n=3 CON lambs, n=6 PR lambs), or lamb birth defects (n=2 CON lambs from 1 pregnancy).

Figure 2. Effects of PR on postnatal weight in male (A, B, C), and female (D, E, F) sheep. Body weight of CON (white circles) and PR (black circles) are shown daily from birth to d 27 (A, D), weekly from d 27 to weaning (B, E), and monthly from weaning to d 320 (C, F). Data are estimated means ± SEM.

Figure 3. Activity patterns in male (A) and female (B) adolescent sheep at 204 ± 1 d of age. Distance travelled, in CON (white circles) and PR (black circles) progeny are shown as actual means ± SEM, averaged for 30-minute blocks between 1800 h and 1200 h. Time from sunset to sunrise (night) is shaded grey.

Figure 4. Effects of treatment and sex on average distance travelled during specific periods in adolescent sheep at 204 ± 1 d of age. Average distance travelled per hour was calculated across the entire recording period (A), during daylight (B), and night (C), and in blocks of time relative to sunrise: -2 to -1 (D), -1 to 0 (E), 0 to +1 (F), +1 to +2 (G) hours from sunrise, and in blocks of time relative to sunset: -1 to 0 (H), 0 to +1 (I), +1 to +2 (J) hours from sunset, in CON (white bars) and PR (black bars) adolescent sheep. Data are estimated means ± SEM; * P<0.05, ** P<0.01, *** P<0.001.

Figure 5. Predicted activity patterns for male (closed squares) and female (open squares) adolescent sheep at 204 ± 1 d of age. Distance travelled per hour was predicted by spline analysis, utilizing 18 spline points, and estimated means ± SEM are shown for

males (black squares) and females (white squares) across the 18-h recording period. Differences in estimated means between male and females are indicated: * P<0.05.

Figure 6. Activity patterns in male (A) and female (B) adult sheep at 294 ± 1 d of age. Distance travelled, in CON (white circles) and PR (black circles) progeny are shown as actual means ± SEM, averaged for 30-minute blocks between 1800 h and 1200 h. Time from sunset to sunrise (night) is shaded grey.

Figure 7. Effects of treatment and sex on average distance travelled during specific periods in adult sheep at 294 ± 1 d of age. Average distance travelled per hour was calculated across the entire recording period (A), during daylight (B), night (C), and in blocks of time relative to sunrise: -2 to -1 (D), -1 to 0 (E), 0 to +1 (F), +1 to +2 (G) hours from sunrise, in CON (white bars) and PR (black bars) young adult sheep. Data are estimated means ± SEM; #, P=0.053, * P<0.05, ** P<0.01, *** P<0.001.

Figure 8. Adult ambulatory activity correlates negatively with birth weight in females (B) but not males (A). Average distance travelled per hour across the entire recording period (A, B) or during daylight (C, D) as adults, correlated negatively with birth weight in females (B, D) but not in males (A, C).

Figure 1.

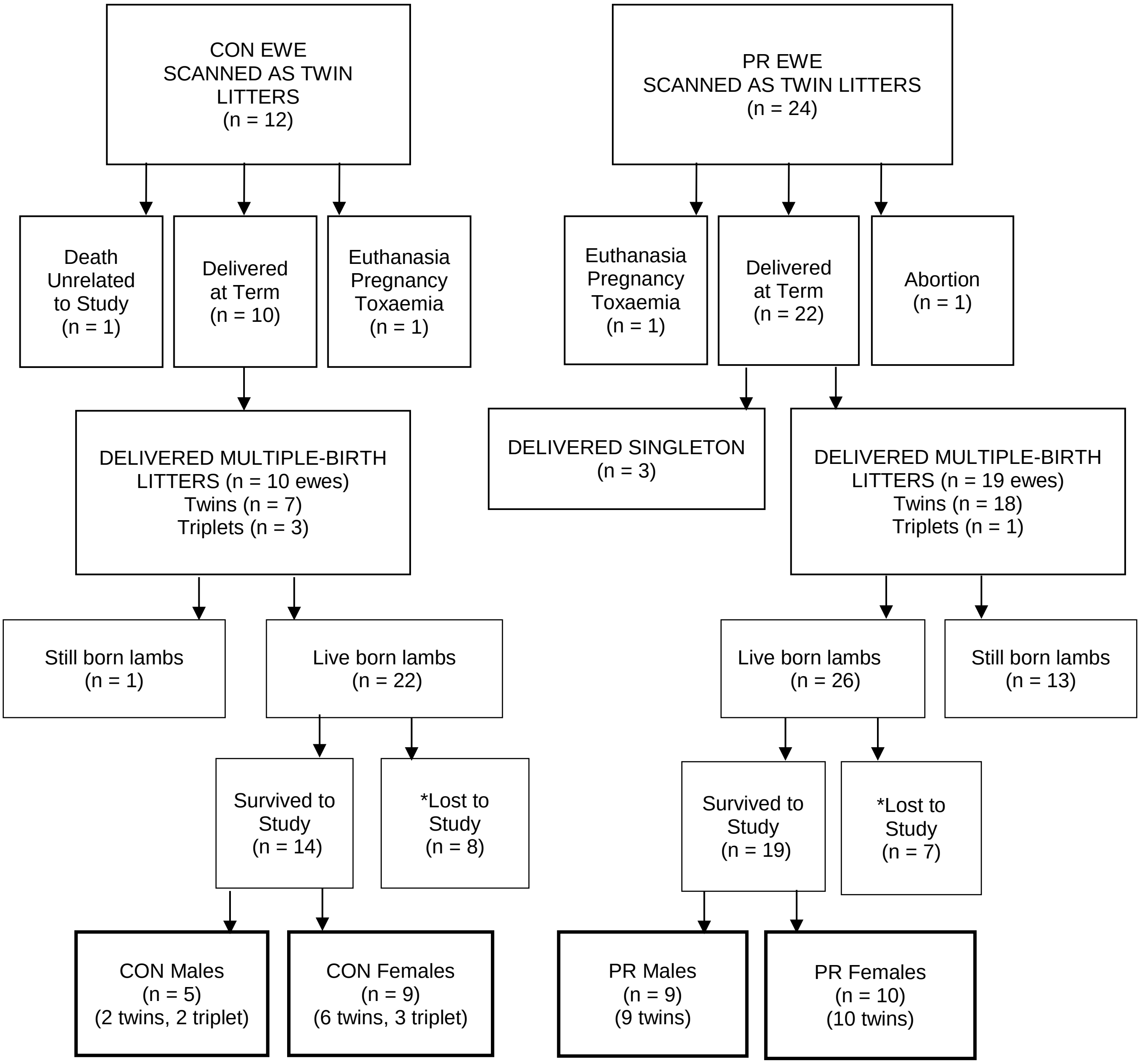
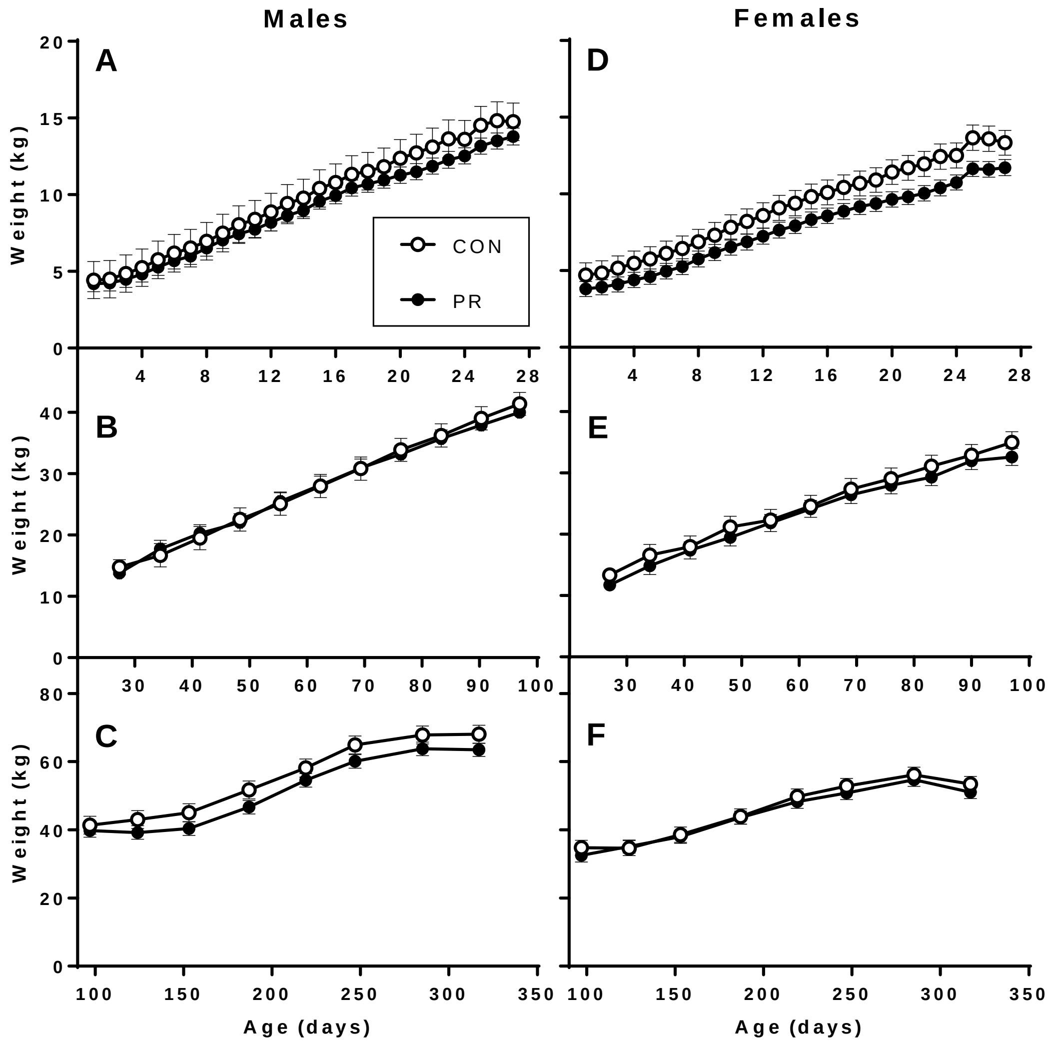

CON EWE
SCANNED AS TWIN LITTERS
(n = 12)
PR EWE
SCANNED AS TWIN LITTERS
(n = 24)
Death Unrelated to Study
(n = 1)
Delivered at Term
(n = 10)
Euthanasia Pregnancy Toxaemia
(n = 1)
Euthanasia Pregnancy Toxaemia
(n = 1)
Delivered at Term
(n = 22)
Abortion
(n = 1)
DELIVERED MULTIPLE-BIRTH LITTERS (n = 10 ewes)
Twins (n = 7)
Triplets (n = 3)
DELIVERED SINGLETON
(n = 3)
DELIVERED MULTIPLE-BIRTH LITTERS (n = 19 ewes)
Twins (n = 18)
Triplets (n = 1)
Still born lambs
(n = 1)
Live born lambs
(n = 22)
Live born lambs
(n = 26)
Still born lambs
(n = 13)
Survived to Study
(n = 14)
*Lost to Study
(n = 8)
Survived to Study
(n = 19)
*Lost to Study
(n = 7)
CON Males
(n = 5)
(2 twins, 2 triplet)
CON Females
(n = 9)
(6 twins, 3 triplet)
PR Males
(n = 9)
(9 twins)
PR Females
(n = 10)
(10 twins)

Figure 2.



Males
Females
A
B
C
D
E
F
Weight (kg)
Weight (kg)
Weight (kg)
CON
PR
Age (days)
Age (days)

Figure 3.

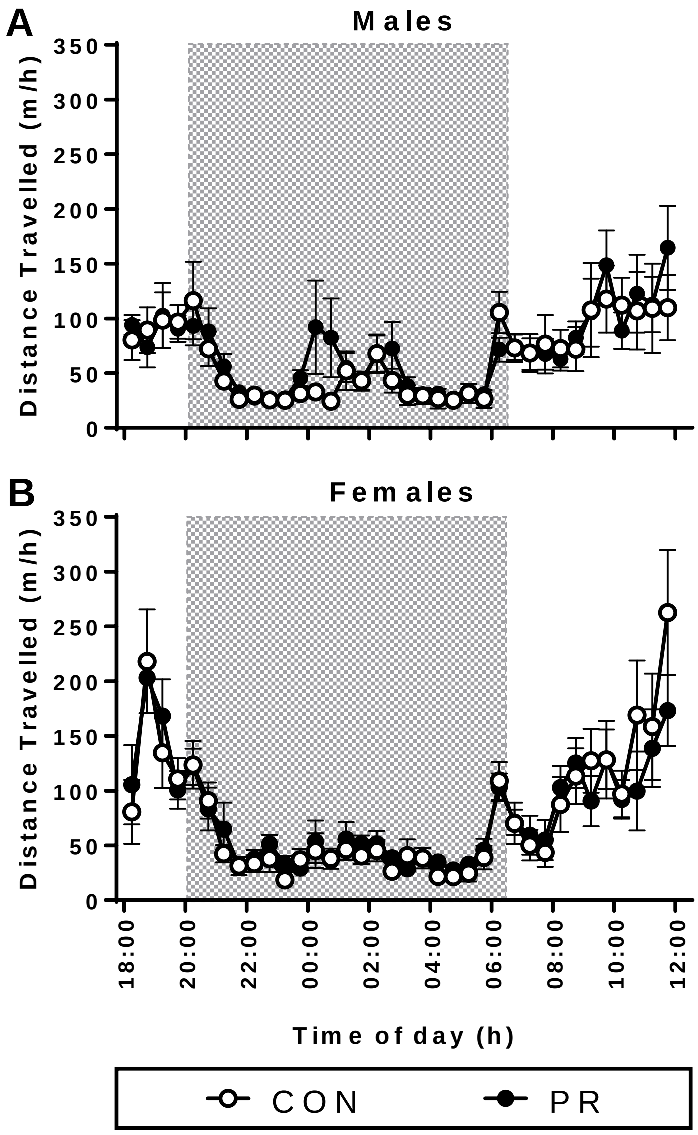
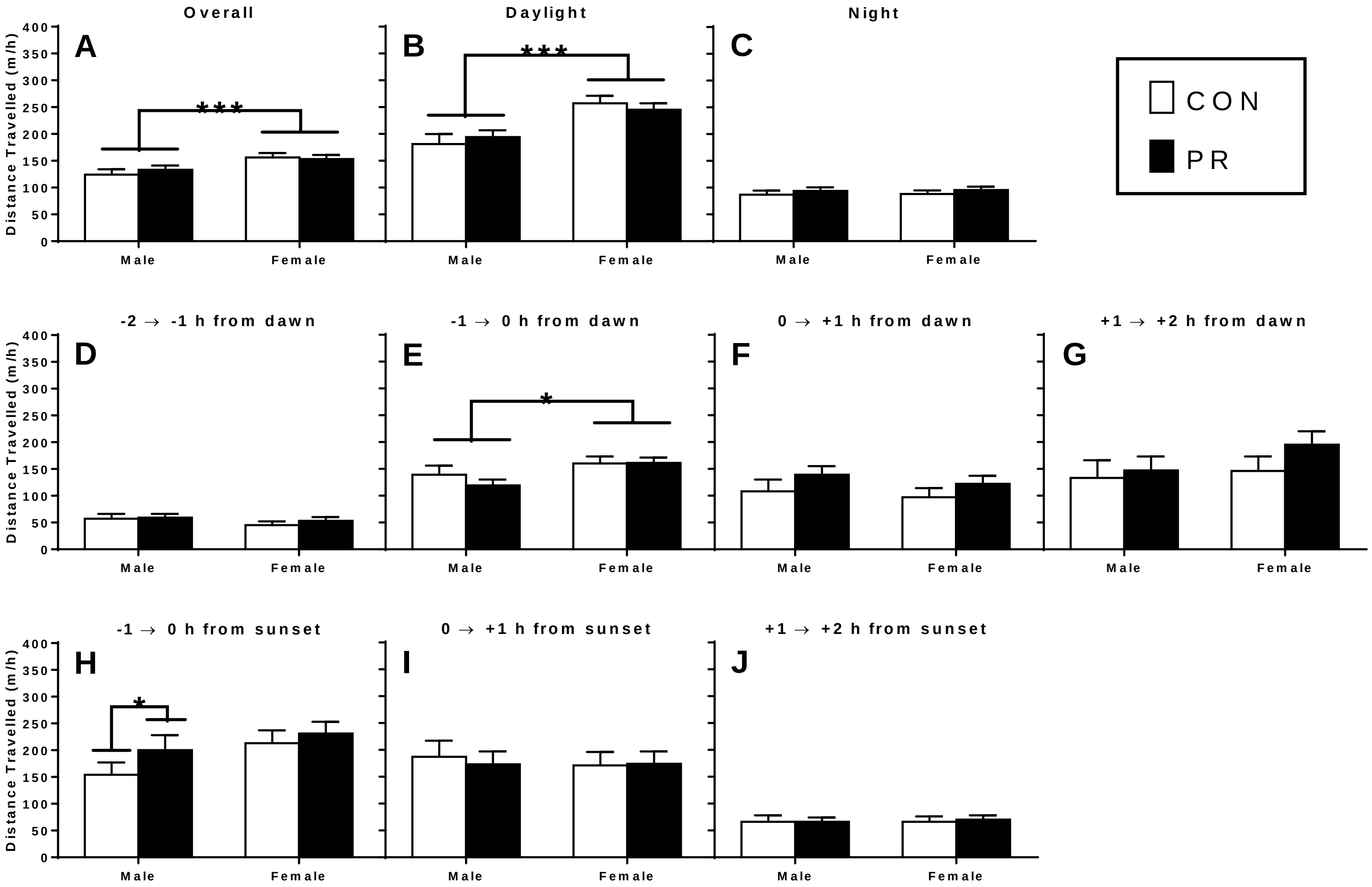
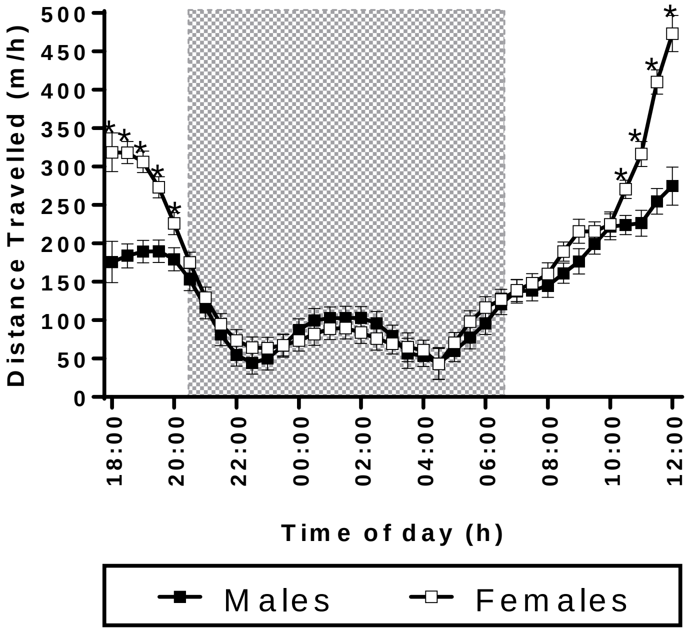

A
Males
Distance Travelled (m/h)
350
300
250
200
150
100
50
0
B
Females
Distance Travelled (m/h)
350
300
250
200
150
100
50
0
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
Time of day (h)
CON
PR

Figure 4.



Overall
Daylight
Night
A
B
C
***
***
CON
PR
-2 → -1 h from dawn
-1 → 0 h from dawn
0 → +1 h from dawn
+1 → +2 h from dawn
D
E
F
G
*
-1 → 0 h from sunset
0 → +1 h from sunset
+1 → +2 h from sunset
H
I
J
*
Distance Travelled (m/h)
0
50
100
150
200
250
300
350
400
Male
Female

Figure 5.



500
450
400
350
300
250
200
150
100
50
0
Distance Travelled (m/h)
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
Time of day (h)
Males
Females

Figure 6.

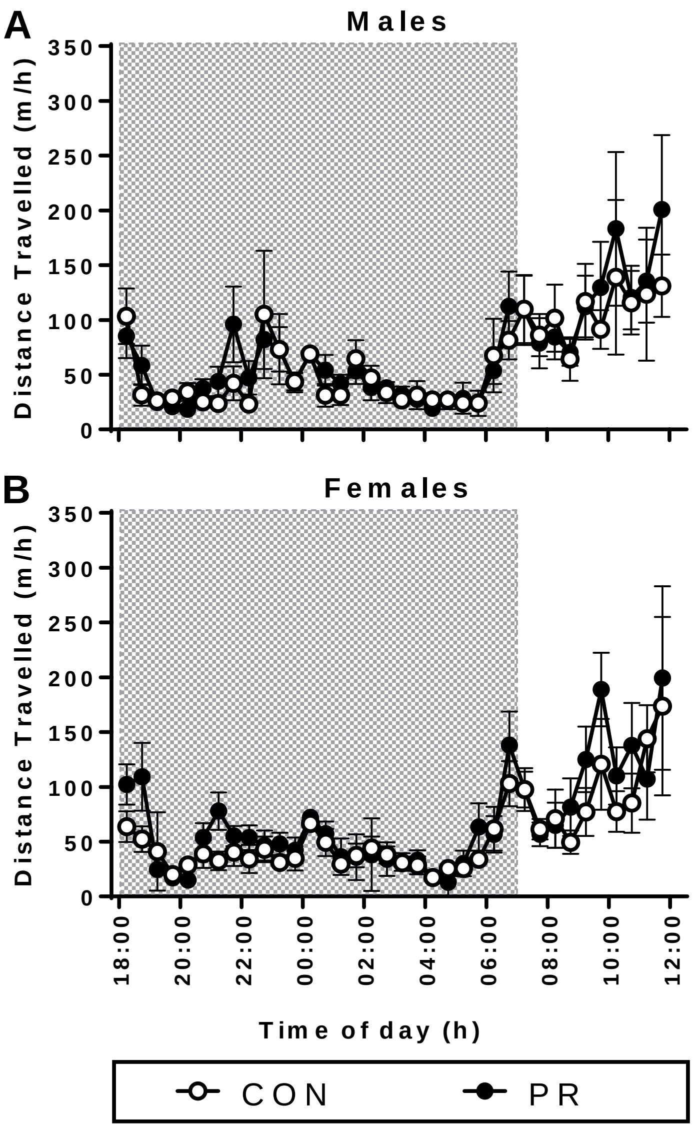
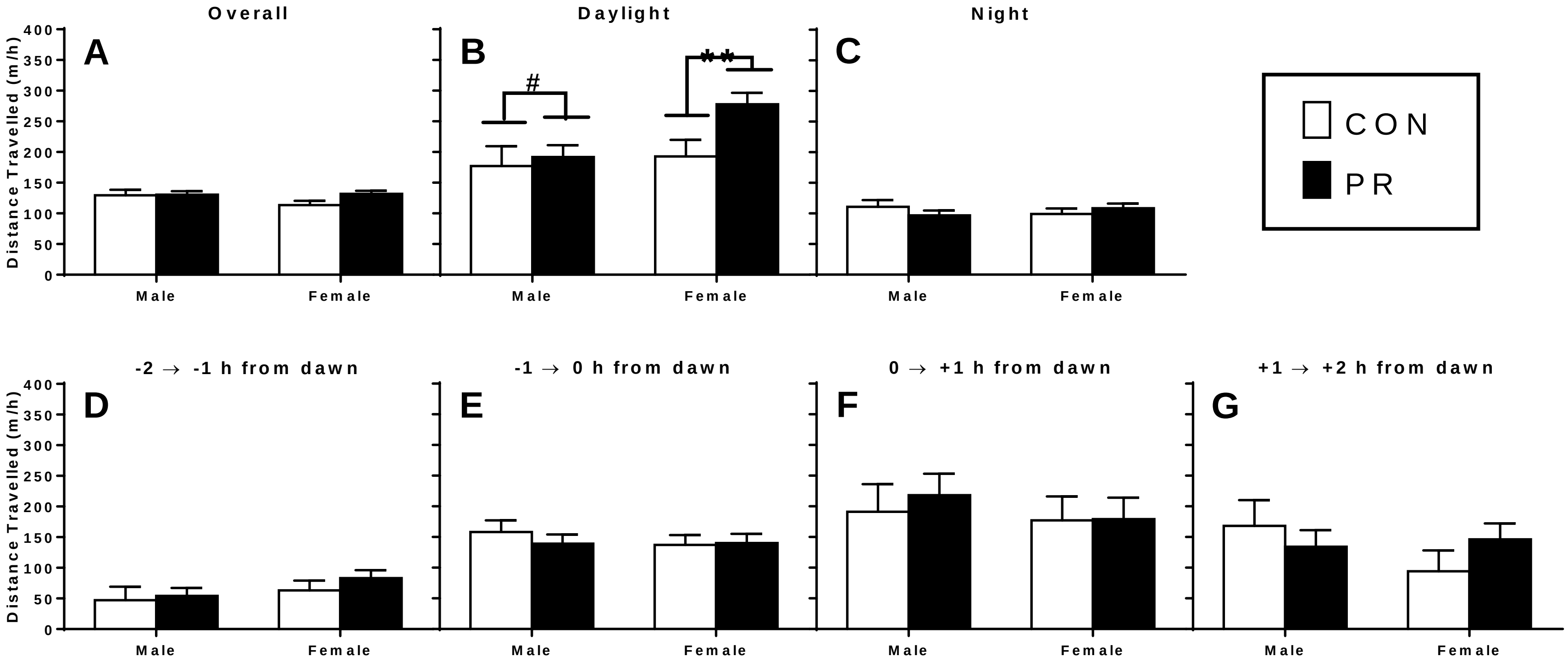
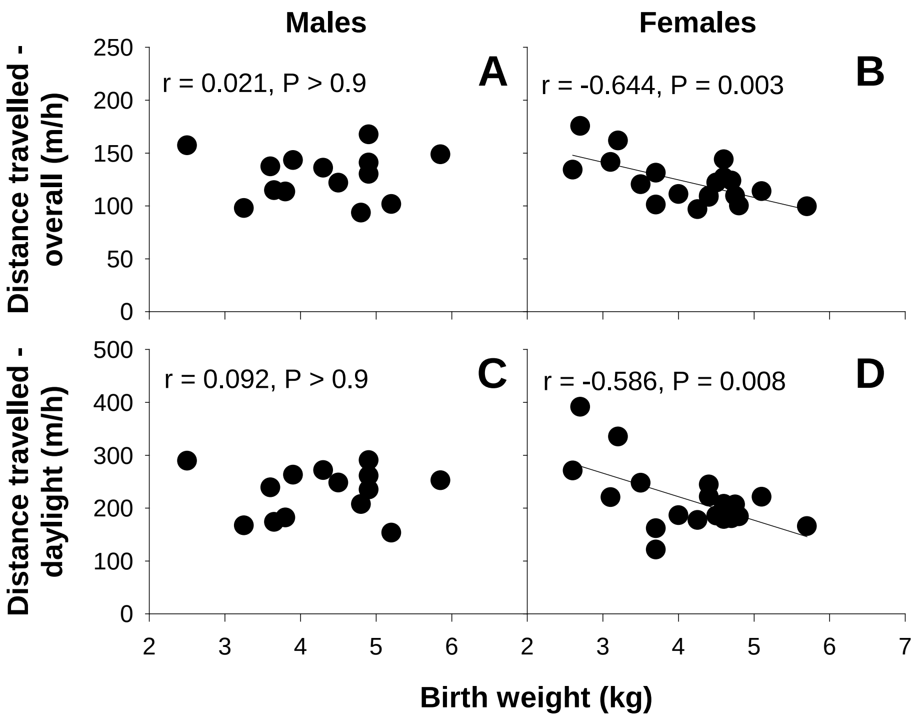

A
Males
B
Females
Distance Travelled (m/h)
350
300
250
200
150
100
50
0
18:00
20:00
22:00
00:00
02:00
04:00
06:00
08:00
10:00
12:00
Time of day (h)
CON
PR

Figure 7.



Overall
Daylight
Night
A
B
C
CON
PR
#
**
Distance Travelled (m/h)
400
350
300
250
200
150
100
50
0
Male
Female
-2 → -1 h from dawn
-1 → 0 h from dawn
0 → +1 h from dawn
+1 → +2 h from dawn
D
E
F
G

Figure 8



Males
Females
Distance travelled - overall (m/h)
Distance travelled - daylight (m/h)
r = 0.021, P > 0.9
A
r = -0.644, P = 0.003
B
r = 0.092, P > 0.9
C
r = -0.586, P = 0.008
D
250
200
150
100
50
0
500
400
300
200
100
0
2
3
4
5
6
2
3
4
5
6
7
Birth weight (kg)

Table 1. Birth weight and neonatal growth. Neonatal growth rates from birth to 30 days of age were calculated by linear regression for lambs included in spontaneous physical activity measures only.

	CON		PR		Significance		
	Male	Female	Male	Female	Treatment	Sex	T*S
N lambs	5	9	9	10			
Birth weight (kg)	4.49 ± 0.69	4.54 ± 0.67	4.21 ± 1.00	3.78 ± 0.79	0.181	0.294	0.320
Neonatal growth rate (kg/d)	0.39 ± 0.03	0.33 ± 0.03	0.38 ± 0.02	0.33 ± 0.02	0.936	0.012	0.953
Neonatal growth rate (%/d)	8.13 ± 0.75	7.17 ± 0.61	9.02 ± 0.52	8.57 ± 0.49	0.124	0.161	0.614

Treatment*sex interaction is indicated by T*S. Data are actual means ± SEM.

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