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Placental and fetal growth restriction, size at birth and neonatal growth alter cognitive function and behavior in sheep in an age- and sex-specific manner.

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Intrauterine growth restriction and slow neonatal growth in humans are each associated with poorer learning, memory and cognitive flexibility in childhood and adulthood. The relative contributions of pre- and post-natal growth to cognitive outcomes are unclear, however. We therefore compared performance in learning, memory and reversal tasks using a modified Y-maze at 18 and 40 weeks of age in offspring of placentally-restricted (PR: 10 M, 13 F) and control (23 M, 17 F) ovine pregnancies. We also investigated relationships between size at birth, neonatal growth rates and cognitive outcomes. PR males required more trials to solve the initial learning task than controls ($P = 0.037$). PR sheep of both sexes completed reversal tasks more quickly than controls at 18 weeks of age (each $P < 0.05$). In males, neonatal growth rate correlated negatively with numbers of trials and total time required to solve memory tasks at 40 weeks of age (each $P < 0.05$). In females, bleat frequency in the first reversal task at 18 weeks of age correlated positively with birth weight ($r = 0.734$, $P < 0.05$) and neonatal growth rate ($r = 0.563$, $P < 0.05$). We conclude that PR induces age- and sex-specific effects on cognitive outcomes in sheep, with some evidence of impaired learning in males, but little effect on memory or cognitive flexibility in either sex. Rapid neonatal growth predicted improved memory task performance in males, suggesting that strategies to optimize neonatal growth may have long-term cognitive benefits but that these may be sex-specific.

Keywords: Sheep; IUGR; birth weight; neonatal growth; cognition; maze

Abbreviations:

Appropriate birth size for gestational age; AGA

Birth weight; BW

Control; CON

Fractional growth rate; FGR

Gestational age; GA

Intrauterine growth-restriction; IUGR

Placentally-restricted; PR

Small birth size for gestational age; SGA

1. Introduction

Intrauterine growth-restriction (IUGR) is associated with impaired neurodevelopment, with life-long consequences for cognitive function [1]. Small size at birth corrected for gestational age (SGA, size at birth below the 10th centile for gestational age) is often used as a surrogate marker of IUGR in humans when repeated measures of fetal growth are not available. Children born small for gestational age (SGA) have, on average, IQs 6-11 points lower than their peers, poorer language skills, impaired spatial learning and memory, and higher incidences of behavioral and attentional problems [2-5]. These deficits have functional consequences, as SGA is also associated with poorer academic outcomes in children [6] and adults [7, 8].

The effects of IUGR on neurodevelopmental outcomes may be ameliorated by catch-up growth in early life, suggesting an important role for post-natal growth. Catch-up growth following IUGR is common across species, including humans, where it occurs mostly during the first two months after birth [9, 10]. Catch-up growth is associated with better visuomotor and problem solving skills, intelligence quotients, IQ and academic performance in SGA children, starting from 18 months and continuing into adulthood, compared to those with failure of catch up growth [3, 4, 11, 12]. SGA children do not always catch up in head circumference compared to peers born at an appropriate weight for their gestational age (AGA) [3, 13-15], even if they are among the 86% of SGA children that catch up in height and weight [16, 17]. Head circumference is an important surrogate marker for neurodevelopment, because it is strongly correlated with IQ, language, visuomotor and neurodevelopmental scores in SGA children [12, 14], a relationship that strengthens with age [14].

Disentangling the influences of fetal and postnatal growth on neurodevelopmental outcomes is complicated by the common comorbidity between IUGR and preterm birth (birth before 37 completed weeks of gestation) in humans, both of which separately impair neurodevelopment and learning outcomes [4, 18], with compounding effects in combination [19, 20]. Human studies can

also be confounded by shared prenatal and postnatal environments, and complicated by variation due to genetics and environmental factors. For example, lower socioeconomic status is associated with increased risk of SGA, a reduction in postnatal catch-up growth [21-23], and poorer cognition and executive function in both healthy [24], and SGA children [3, 4, 6, 20]. Therefore, an animal model of fetal growth retardation, with IUGR offspring born at term, is required to further investigate the influence of fetal and neonatal growth on neurodevelopmental outcomes.

Sheep have a similar ontogeny of neurodevelopment to humans with neurogenesis, oligodendrocyte development and myelination commencing prenatally in both species [25, 26]. Importantly, sheep demonstrate higher cognitive processing, including executive functions and problem solving [27, 28], and learning, memory and cognitive flexibility can be tested in this species using maze tasks [28-32]. Impaired placentation, which reduces the supply of nutrients and oxygen reaching the fetus, is a major cause of IUGR in developed countries [33]. Restriction of placental growth (PR) in sheep, by surgical removal of placental attachment sites prior to pregnancy, reduces nutrient and oxygen supply and is associated with similar fetal outcomes as occurs in human IUGR, including endocrine adaptations [34-37]. PR results in delivery of full-term lambs with reductions in average birth weight of 20-31% [38, 39]. PR lambs undergo neonatal catch-up growth, with incomplete catch-up of skull width [40, 41], consistent with growth patterns in IUGR infants [39-42]. This model allows effects of IUGR to be tested independent of confounders such as preterm birth and environmental differences, since all individuals share a common postnatal environment. We therefore tested the hypothesis that in adolescent and adult sheep, PR, low birth weight and slow neonatal growth each impair learning, memory and cognitive flexibility.

2. Methods

All procedures were jointly approved by the University of Adelaide Animal Ethics Committee (M-2009-145 and M-2011-055) and the SA Pathology Animal Ethics Committee (135a/09) and complied with the *Australian Code of Practice for the Care and Use of Animals for Scientific Purposes* [43].

2.1. Animals

Generation and management of this cohort has been described previously [39]. Briefly, placental growth and function of primiparous Merino x Border Leicester ewes was restricted by surgical removal of all but four visible endometrial placental attachment sites (caruncles) from each uterine horn [44, 45] at least 10 weeks prior to timed mating to Merino rams. Control ewes were un-operated and were also included in the timed mating program. Pregnant control (CON) and PR ewes were housed indoors from day 110 of gestation until their spontaneously-born lambs were weaned at 13 weeks of age. Groups of lambs were born at five-week intervals between July 2010 and December 2012. Ewes were fed 1 kg Rumevite pellets daily (Ridley AgriProducts, Melbourne, Australia), with *ad libitum* access to lucerne chaff and water. Gestational ages in days (GA), birth weight (BW) and litter sizes were recorded. After weaning, progeny were housed in outside paddocks in same sex groups of similar ages and fed 0.5 kg Rumevite pellets/sheep daily, with *ad libitum* access to oaten hay, pasture and water. Progeny were handled frequently from birth, with measures of weight recorded every second day from birth to 16 days of age to calculate fractional growth rate for weight [FGR, 46], followed by weekly weighing until weaning. All animals were fed daily by an animal technician, providing frequent human contact and ensuring lambs were habituated to humans.

2.2. Learning evaluation

Maze tests were performed at 18 and 40 weeks of age as described previously for control animals [32] using a protocol modified from Erhard *et al.*[30] and Hernandez *et al.* [31]. Here we report outcomes from animals tested at both ages; consisting of 40 control progeny (1 male and 1 female from singleton births, 22 male and 16 female from multiple births) and 23 PR progeny (5 male and 10 female from singleton births, 5 male and 3 female from multiple births).

Briefly, the test protocol consisted of 3-5 days of testing [32]. The first day commenced with a habituation task, in which sheep had five trials to exit the maze through either of the open gates, allowing for habituation to human handling, the maze and maze protocols. The gate most frequently exited in this task was recorded as their preferred side. Sheep then completed guided runs followed by a learning task in which they were required to exit the maze only through their preferred side (Task L). On day 2, sheep first performed a memory task (Task M1) which involved repetition of task L from the previous day. This was followed by a reversal task, requiring completion of the maze with the open gate switched to the non-preferred side (Task R1). On day 3, the sheep performed a memory task (Task M2); repeating task R1 with the gate on the non-preferred side, and then the open gate was switched back to the preferred side for the final reversal task (Task R2). The criterion that had to be met to complete each task was three consecutive correct exits from the maze within either 6 trials (Task L) or 10 trials (Tasks M1, R1, M2 and R2), with each trial completed within three minutes. The reward for solving the maze was access to the reward pen for 10 seconds, allowing access to flock-mates in the neighboring pen and a food reward. The only penalty for not solving the maze was the inability to leave the maze during that trial. Sheep that failed a trial (>3 minutes in maze), were then steered through the correct exit to the reward pen, where they stayed for 10 seconds before the next run. Successfully completing the tasks for each day resulted in graduation to the next day of testing in the sequence, whereas failure to complete tasks M1, R1, M2

or R2 resulted in the sequence being repeated, with a maximum of five days permitted to complete the sequence. Cognitive measures included total time and number of trials taken to solve each task and average time per criterion trial (i.e. the final three successful trials of each task). Behavioral measures included number of bleats [as a measure of stress, eg. 47] and maze arm entries per trial, indicating the number of times the sheep entered each maze arm.

2.3. Statistical analysis

Effects of treatment (control or PR), sex and litter size (singleton or multiple birth), and interactions between these variables on gestational age, size at birth and neonatal growth were analyzed using generalized linear mixed models, including the mother as a random factor. Effects of treatment, sex, litter size and age on maze task outcomes were analyzed for data within each task separately using generalized linear mixed models, including the mother as a random factor, with only main effects for litter size, and recognizing the multiple measures on each individual sheep, with post-hoc Bonferroni comparisons used to compare differences between each treatment, sex or age. Continuously distributed variables (i.e. time and growth measures) were log-transformed prior to analysis to reduce skew and were analyzed assuming a normal distribution and identity link, while variables that were counts of events (i.e. total trials per task) were analyzed using a Poisson distribution with log link. Subgroup analyses were run when interactions were significant. Correlations between BW, GA and FGR were tested by multiple linear regression for continuously distributed variables, and Poisson regression for count variables. Ewe identity did not influence these correlations with continuously distributed variables and was therefore excluded from correlation analysis. Effect of treatment on litter size was analyzed by χ^2-test. All analyses were carried out using SPSS 20.0 (IBM, Armonk, USA). Data are presented as mean ± SEM unless otherwise stated and statistical significance was accepted at $P < 0.05$.

3. Results

3.1. Effects of PR on size at birth and neonatal growth

Overall, PR did not alter BW, FGR or GA, which overlapped between treatments (Figure 1). The greater proportion of twins in CON than PR adult offspring ($P < 0.001$) may have contributed to this, however in singletons alone, PR did not alter FGR (CON: 0.070 ± 0.013 $1.d^{-1}$, PR: 0.083, ± 0.006 $1.d^{-1}$, $P > 0.3$, GA (CON: 146.5 ± 0.83 d, PR: 145.66 ± 0.37 d, $P > 0.3$), or BW (CON: 5.45 ± 0.525 kg, PR: 4.30 ± 0.233 kg, $P = 0.077$), and in twins there were also no differences in BW, FGR or GA. BW correlated positively with GA ($r = 0.440$, $P < 0.001$), and FGR correlated negatively with BW ($r = -0.515$, $P < 0.001$) but not with GA.

3.2. Effects of PR on cognitive and behavioral outcomes

3.2.1 Learning task (task L)

Effects of treatment and age on the number of trials required to solve task L differed between sexes (interactions: treatment*sex $P = 0.023$, age*sex $P = 0.023$, Figure 2A) and did not differ between singleton- and multiple-birth sheep ($P > 0.6$). PR males required more trials than CON males ($P = 0.037$) and 18 week-old males required more trials than 40 week-old males ($P = 0.001$) to complete task L. In females, treatment did not affect the number of trials required to complete task L, and similar to the pattern in males, 18 week-old females required more trials than 40 week-old females in the two treatment groups combined ($P = 0.040$). The total time required to solve task L did not differ between males and females ($P = 0.072$) or between treatments, litter sizes or ages (each $P > 0.15$, Figure 2B). The average time in criterion trials did not differ between treatments, litter sizes, sexes or ages (each $P > 0.17$, Figure 2C). Younger sheep (18 week olds) bleated more ($P = 0.004$) and made fewer arm entries per trial ($P = 0.002$) than older sheep (40 week olds), and these outcomes did not differ between treatments, litter sizes or sexes (each $P > 0.2$, Figure 2D,E).

3.2.2. First memory task (task M1)

The number of trials required to solve task M1 differed between treatments and ages in a sex-specific manner (interaction: treatment*sex*age $P = 0.026$, Figure 3A) and did not differ between litter size groups ($P > 0.5$, data not shown). In males, there was an interaction between treatment and age ($P = 0.039$), but treatment did not affect the number of trials required to solve task M1 in either 18 week-old males ($P > 0.1$) or 40 week-old males ($P > 0.7$). The number of trials required to solve task M1 did not differ between ages in either CON or PR males (each $P > 0.1$). In females, the number of trials required to solve task M1 did not differ with age or treatment. The total time required to solve task M1 and average time in criterion trials differed between treatments and ages in a sex-specific manner (interaction for total time: treatment*sex*age $P = 0.028$; interaction for time per criterion trial: treatment*sex*age $P = 0.030$, Figure 3B,C), and did not differ between litter sizes (each $P > 0.5$, data not shown). Despite the overall interaction, when each sex was analyzed separately, effects of treatment and age on these outcomes were not different in either sex (all $P > 0.6$). Bleat frequency also differed between treatments and ages in a sex-specific manner (interaction: treatment*sex*age $P = 0.005$, Figure 3D), and did not differ between litter sizes ($P > 0.1$, data not shown). In males, bleat frequency did not differ between treatments ($P > 0.8$) and was greater at 18 weeks of age than at 40 weeks of age ($P = 0.023$). In females, effect of treatment changed with age (interaction: treatment*age $P = 0.008$). Bleat frequency was not different in CON and PR females within either age group (each $P > 0.3$). Bleat frequency decreased from 18 to 40 weeks of age in CON females ($P < 0.001$), but not in PR females ($P > 0.6$). Arm entries per trial in task M1 did not differ between treatments, litter sizes, sexes or ages (each $P > 0.2$, Figure 3E).

3.2.3. First reversal task (task R1)

The number of trials required to solve task R1 did not differ between treatments, sexes or litter sizes (all $P > 0.2$), and was greater at 18 than 40 weeks of age ($P = 0.003$, Figure 4A). The total time required to solve task R1 differed between treatments and sexes in an age-specific manner

(interactions: treatment*age P = 0.009; sex*age P = 0.003, Figure 4B), and did not differ between singleton-born and multiple-born sheep overall (P = 0.073). In males, effects of treatment differed between ages (interaction: treatment*age P = 0.009), such that in 18 week-old males, control sheep required more time to solve task R1 than PR sheep (P = 0.023), but in 40 week-old males, treatment did not affect this outcome (P > 0.9). In females, both treatment and age affected the total time required to solve task R1. Overall, control females required more time to solve task R1 than PR females (P = 0.026), and 18 week-old females required more time to solve task R1 than 40 week-old females (P < 0.001). Time per criterion trial in task R1 differed between treatments in an age- and sex-specific manner (interaction: treatment*sex*age P = 0.012, Figure 4C). In males, effects of treatment on average time in criterion trials changed with age (interaction: treatment*age P = 0.009). At 18 weeks of age, control males were slower in criterion trials than PR males (P = 0.023), and at 40 weeks of age, control and PR males completed criterion trials in similar times (P > 0.5, Figure 4c). In females, time in criterion trials was unaffected by treatment (P > 0.1), age (P = 0.054), or litter size (P = 0.089, data not shown). Bleats and arm entries per trial in task R1 did not differ between treatments, litter sizes or sex (each P > 0.1, Figure 4D,E). Bleat frequency was greater at 18 than 40 weeks of age (P < 0.001), but there was no age difference in arm entries per trial (P>0.1).

3.2.4. Second memory task (task M2)

The number of trials required to solve task M2 did not differ between sexes or litter sizes (each P > 0.7), and effects of treatment varied with age (interaction: treatment*age, P = 0.041, Figure 5A). At 18 weeks, the number of trials required to solve task M2 did not differ between treatments, sexes or litter size groups (each P > 0.9). At 40 weeks of age, the number of trials required to solve task M2 did not differ between treatments (P = 0.06), nor between sexes or litter size groups (each P > 0.2). CON sheep required more trials to solve task M2 at 40 than 18 weeks of age (P = 0.013, Figure 5A), but the number of trials to solve task M2 did not change with age in PR sheep (P = 0.082). The total

time required to solve task M2 did not differ between treatments or litter size groups (each $P > 0.2$), and effects of age differed between sexes (interaction: age*sex $P = 0.020$). In males, 40 week-olds took more time to solve task M2 than 18 week-olds overall ($P = 0.039$), and in females, this outcome did not change with age. Average time in criterion trials similarly did not differ between treatments or litter size groups (each $P > 0.6$), and effects of age differed between sexes (interaction: age*sex $P = 0.040$). In males, 40 week-olds had longer average time in criterion trials than 18 week-olds ($P = 0.023$), but time in criterion trials did not change with age in females ($P = 0.080$). Bleat frequency did not differ between treatments and litter size groups (each $P > 0.7$), was greater in females than males ($P = 0.008$) and greater at 18 than at 40 weeks of age ($P < 0.001$). Arm entries per trial did not differ between treatments and litter size groups (each $P > 0.2$), and differed between ages in a sex-specific manner (interaction: age*sex $P = 0.026$). Numbers of arm entries did not change with age in males ($P > 0.1$) or females ($P = 0.055$).

3.2.5. Second reversal task (task R2)

The number of trials required to complete task R2 and arm entries per trial in task R2 did not differ between treatments, sexes, litter size groups or ages (Figure 6, all $P > 0.1$). The total time required to complete task R2 was greater in CON than PR sheep ($P = 0.047$, Fig 6B) and did not differ between sexes, ages or litter size groups. Average time per criterion trial did not differ between treatments, litter size groups or ages (all $P > 0.1$), and was greater in males than females ($P = 0.047$, Fig 6C). Bleat frequency was greater at 18 than 40 weeks of age overall ($P = 0.003$, Fig 6D) and did not differ between treatments, sexes and litter size groups (all $P > 0.1$).

3.3. Relationships of cognitive outcomes with birth weight, neonatal growth rate and gestational age

Associations of cognitive outcomes with BW, neonatal FGR and GA in multiple linear regression analyses changed with age and differed between sexes. At 18 weeks, BW, FGR and GA rarely

predicted cognitive outcomes (total trials, total time, time/criterion trial; Table 1). In females, time per criterion trial in task R1 correlated negatively with GA and positively with BW (Table 1). At 40 weeks, associations between cognitive outcomes, BW, FGR and GA differed between sexes (Table 2). In females, the total number of trials to solve Task R2 correlated positively with BW, whereas time per criterion trial in the same task correlated negatively with BW (Table 2). In contrast, female performance in the learning, memory and the first reversal task was not associated with BW, FGR and GA. In 40 week old males, time per criterion trial in Task L correlated positively with FGR, and the number of trials, and total time required to solve the first memory task (M1) correlated negatively with FGR (Table 2). Outcomes in memory or reversal tasks did not correlate with BW or GA in these older males, however.

3.4. Relationships of behavior during maze tests with birth weight, neonatal growth and gestational age

Correlations between behavior, BW, neonatal FGR and GA in multiple linear regression analyses changed with age and differed between sexes. In 18 week old males (Table 1), bleat frequency did not correlate with BW, FGR or GA. In 18 week old females, bleat frequency during task M1 correlated positively with GA ($P < 0.05$), and bleat frequency during task R1 correlated positively with BW and FGR (Table 1). In 18 week old males, numbers of arm entries in task R1 correlated negatively with BW and positively with GA (Table 1). In these young males, associations between numbers of arm entries and FGR differed between tasks, such that number of arm entries in task L correlated positively with FGR, whereas arm entries in task M2 correlated negatively with FGR. In 18 week old females, numbers of arm entries did not correlate with BW and FGR, whilst numbers of arm entries in tasks L and M1 correlated positively with GA (Table 1). Few associations were observed between behavior during maze tests at 40 weeks and BW, FGR and GA. In 40 week old males, bleat frequency in task L, but not other tasks, correlated negatively with BW and FGR. In 40 week old females, bleat frequencies in maze tasks did not correlate with BW, FGR or GA (Table 2).

Numbers of arm entries were not correlated with BW, FGR or GA in either sex or in any task in these older animals.

4. Discussion

In the present study, PR impaired initial learning performance, but did not impair measures of memory or reversal learning, and in fact we saw evidence of improved performance in reversal learning tasks in PR compared to control sheep. Although impaired initial learning in PR sheep is consistent with results from human studies, effects of PR on reversal learning differ from those previously reported in IUGR humans. This is possibly due to differences in the measures of executive function used, or because PR did not significantly reduce birth weight in the present cohort. In 40 week-old males only, early postnatal growth rate positively predicted performance in the memory task conducted the day after the initial learning task, suggesting that early postnatal growth benefits learning retention in adult male sheep. This adds to studies showing higher IQ in SGA infants who caught up in head circumference in the first 12-36 months of life compared to those with failure of catch-up growth [3, 19]. Furthermore, because the present study was conducted in term-born animals raised in a common postnatal environment, the results of the present study are independent of confounders common in human studies. The reversal tasks are the most challenging and stressful in the maze test series [27, 32], and in females size at birth and early postnatal growth correlated positively and much more strongly with behavioral outcomes than cognitive outcomes in these tasks. We hypothesise that altered emotional reactivity, including sex-specific changes to stress responses, might contribute to adverse effects of IUGR seen in humans undertaking more complex learning tasks requiring higher-order executive function than used here in sheep [4, 12, 48].

In males, PR sheep required more trials than CON to solve task L, the initial learning task in the

maze series. Impaired initial learning after PR is consistent with results of human studies, where LBW (<2500 g) and SGA children (lowest 10^{th} percentile of population birth weight) had poorer visuomotor skills compared to AGA [49], including poorer maze learning, evidenced by a greater proportion of incorrect arm entries in a radial maze and poorer spatial orientation, based on Kaufman-ABC results [50]. The fact that SGA children also have a greater incidence of learning deficits compared to AGA [51] suggests they also have learning difficulties in areas additional to the spatial learning we examined in task L of the present study. This impaired learning may also reflect the greater incidence and severity of attention deficits in preterm and term IUGR infants and term-born IUGR children compared to term-born AGA, which in turn has been suggested to impair learning [5, 52]. IUGR children do not differ from AGA in terms of hippocampal memory decay [51], however, suggesting that both groups are equally able to recall learnt information. Our observation of learning deficits in males only in the PR sheep contrasts with results of human studies, where SGA is associated with learning impairments in both sexes, although there is some evidence of more severe effects of SGA on different cognitive outcomes in each sex from those studies in which sex-specific outcomes have been reported. SGA boys have a higher incidence of learning difficulties than SGA girls, although in both sexes the incidence is higher in SGA than AGA [53]. Conversely, the lower Wide Range Achievement Test reading scores in SGA than AGA [53], and lower Rey Osterrieth Complex Figure Test scores in IUGR than non-IUGR [4], were observed only in girls and not in boys. Other studies observed no sex differences in effects of IUGR status on outcomes of the Visual-Aural Digit Span Test [5], or on relationships between birth weight and scores gathered from a neuropsychological battery of tests [6].

Somewhat surprisingly, we saw some evidence of better performance in reversal tasks in PR than CON. In both R1 and R2, PR took less total time per task than CON; seen in task R1 overall at 18 weeks and also at 40 weeks but only in females; and overall (across ages and treatments) in task R2. We have reported previously that the reversal tasks, particularly task R1, are the most challenging

for sheep within the series of maze tests performed in the present study [32]. It was therefore surprising that PR decreased the time required to solve this reversal learning task, because in humans SGA children and adults have lower test performance on measures of executive function than AGA individuals [4, 48]. Memory decay does not appear to contribute to executive function deficits in IUGR, however, with normal hippocampal memory decay in IUGR humans [51]. SGA children also show greater perseverative errors than AGA controls in the Wisconsin Card Counting test, a measure of problem solving and executive function [12]. Perseverative errors are characteristic of failure of reversal learning, particularly following damage to the prefrontal cortex and hippocampus [54] and these outcomes in SGA children suggest that their reversal learning is also likely to be similarly impaired, although this aspect of performance was not reported separately in that study [12]. The lower total time in PR than CON sheep in the reversal task did not reflect fewer trials to solve the task. Greater general speed of PR sheep also does not appear to explain the faster overall completion of the reversal tasks, because average trial time for criterion trials was greater in PR than CON only in 18-week old males, and not in 18-week old females or in 40-week-old sheep of either sex. PR and CON animals also did not differ in bleat frequency, a measure of behavioral stress response [55], in either reversal task in the present study. This suggests that differences in perceived stress also do not explain the better performance of PR than CON sheep in reversal learning tasks. We hypothesise that the faster completion of reversal learning tasks in PR than CON sheep actually reflects weaker initial learning during the first learning task at 18 weeks of age, reducing proactive interference during learning of the reversed route in the subsequent reversal task.

Measures of early postnatal growth positively predicted performance in memory tasks, conducted the day after initial learning tasks, suggesting that faster early postnatal growth benefits learning retention in sheep, albeit in a sex-specific manner. Slow neonatal growth predicted poorer cognitive outcomes in memory tasks (total time and trials required) at 40 weeks of age in males, with a

similar trend for effects of low birth weight. Birth weight and neonatal growth did not predict memory task performance in females. Our data suggests that neonatal growth as well as prenatal growth affects adult memory, in males but not females. Impaired memory may therefore be one mechanism explaining the adverse effects of poor neonatal growth on IQ and intellectual performance, consistent with the observation that SGA children that do not undergo catch-up growth have lower IQ and intellectual performance at 2-4 [3] or 8 years of age [19] compared to SGA with catch-up growth or AGA, and these effects persisted until adulthood [3]. Although working memory at 7-9 years of age does not differ between SGA children who did or did not catch-up in head circumference within the first 9 months of life [12], our data suggest that learning retention to the next day (long-term memory), may be positively affected by neonatal growth. Geva and co-authors [4] have suggested that poorer memory in IUGR compared to AGA children might be explained by their lower grey matter volume [56], including in areas important for memory, such as the hippocampus, as observed in preterm humans and in animal models [57, 58]. In neonatal IUGR guinea pigs, loss of hippocampal grey matter is characterised by decreased axonal and dendritic sprouting as well as neuronal and glial cell loss [57]. Because neurogenesis is completed before birth in sheep and humans [25, 59, 60], improvements in cognitive function associated with neonatal growth in these species might therefore be via postnatal synaptogenesis or glial cell division. Myelination has commenced or is complete in the majority of regions in the ovine brain prior to birth [25], and abundance of myelin basic protein in the cerebral cortex is decreased ~70% in IUGR compared to control sheep fetuses [61]. There is some evidence that white matter can recover during neonatal life following prenatal insults in the sheep, as seen after prenatal and maternal viral infection with Border disease, where axonal myelination of progeny, while not normalised, was improved at six months postnatal age compared to birth [62]. Whether accelerated neonatal growth improves white matter remodelling and this underlies the beneficial relationships observed between neonatal growth and memory in the present study remains to be investigated. It is not clear why we only observed relationships between neonatal growth and memory task

410 performance in males, as in SGA children effects of catch-up growth on IQ and intellectual
411 performance were apparent in both sexes [3, 19].
412
413 In contrast to the positive relationships between size at birth, neonatal growth and memory task
414 performance in males, low birth weight and slow neonatal growth weakly predicted better outcomes
415 in task R2 in females. Reversal task outcomes were not correlated with size at birth or neonatal
416 growth in males. These negative relationships of birth weight and neonatal growth with reversal
417 learning in females were seen only in task R2, where animals reverse to exit the maze on their
418 preferred side, and not in task R1, where animals need to exit on the non-preferred side. We
419 therefore hypothesise that these negative correlations may reflect stronger lateralization in female
420 sheep of low birth weight and slow neonatal growth. Consistent with this, SGA individuals have
421 stronger visuomotor lateralization than AGA as adolescents, whilst decreasing birth weight centile
422 correlated with stronger cortical lateralization in young adults [8, 63]. To date, effects of neonatal
423 growth on lateralization have not been directly explored in human cohorts.
424
425 Pre- and postnatal growth was correlated more strongly with behavioral than cognitive outcomes,
426 and these relationships were sex-, age- and task-specific. Low birth weight and slow neonatal
427 growth predicted lower behavioral stress, measured as bleat frequency in the first reversal task in
428 females at both ages and not in males. While bleats are an indirect behavioral measure of stress
429 response, bleating is observed as a behavioral response to exposure to frightening situations or
430 exposure aversive stimuli [64, 65] and has been used in arena tests as a behavioral indicator of
431 greater emotional reactivity to stress [55]. These proxy measures are important because sheep find
432 close contact with humans aversive and seek to maintain a minimum distance from handlers [65],
433 and therefore behavioral measures of stress response are necessary to remove the confounding
434 effect of stress associated with the handling required to take blood or saliva samples to measure
435 cortisol response. Reversal learning, particularly the first reversal task, is the hardest task in the test

sequence used in the present study [32], and therefore the most likely to expose effects of pre- and post-natal growth on stress responses. Conversely, these indicators of restricted pre- and neonatal growth correlated with greater bleat frequency in the initial learning task in males, and only as adults, and not in females. There is also evidence that prenatal growth alters postnatal stress axis function in a sex- and age-specific manner in humans. Low birth-weight is associated with reprogramming of the stress axis, including increased circulating cortisol in cord blood, increased morning peak (unstressed) cortisol levels in girls, larger stress-induced increases in cortisol in boys and greater and more sustained increases in cortisol following ACTH-stimulation in aged men [66-68]. In humans, both high and low levels of cortisol impair recall of memorised traits [69]. In the present study, greater behavioral stress responses in adult males of low birth weight and with slow neonatal growth may have impaired learning during task L and may therefore have contributed to their poorer maze performance in task M1 the following day. Reprogramming of the stress axis may particularly inhibit learning in more complex executive function tasks (e.g. set-shifting), which are more sensitive to disruption by acute stress than reversal learning [70].

The strong negative correlation between birth weight and arm entries in the first reversal task in 18 week-old males provides further evidence that restricted prenatal growth affects behavior. Arm entries in this maze task in sheep are unlikely to reflect general activity, as sheep make very few arm entries within each individual trial [32]. More frequent arm entries in low birth weight adolescent males than in those of higher birth weight may therefore indicate changes to exploratory drive or flocking instinct, since reversal from one arm to the other requires sheep to move away from flock mates. Unlike bleat frequency, neonatal growth was not correlated with arm entries for this task and was in fact positively correlated with arm entries for task L and M1 in 18 week-old males, suggesting that pre- and post-natal growth do not have consistent effects on this behavioral outcome. Consistent with adverse effects of restricted prenatal growth on behavior, low birth weight and SGA children have higher incidences of behavioral disruption, ADHD and conduct disorders

than AGA children [2, 71], particularly in girls [53]. It appears likely, therefore, that while memory may be directly impaired by poor pre- and postnatal growth, behavioral disruption – including that linked to poor attention and altered stress responses – may also contribute to learning problems after IUGR.

In conclusion, surgical restriction of placental growth impaired cognitive outcomes in a learning task but not in memory or reversal tasks, in a cohort of sheep born at term and raised in a common postnatal environment, and despite PR not reducing birth weight in this cohort. Neonatal growth correlated positively with memory task performance in adult males only, suggesting that accelerated neonatal growth may benefit cognitive function, even after completion of neurogenesis. This is consistent with the observation that neurodevelopmental outcomes from childhood to adulthood are better in SGA individuals with catch-up growth compared to SGA without catch-up [3, 19]. Low birth weight and slow neonatal growth were associated with lower behavioral stress in females during reversal tasks, measured as bleat frequency, but conversely with increased behavioral stress in males during the initial learning task in the present study. IUGR in humans alters function of the stress axis and increases incidence of attention problems and behavioral disruption [53, 71, 72]. Given the evidence for impaired memory recall with either low or elevated circulatory cortisol levels [69], we hypothesise that adverse effects of impaired prenatal and neonatal growth on complex learning are at least in part due to altered stress axis function, and suggest that additional studies of stress responses are warranted in ovine models of IUGR.

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Figure legends

Figure 1. Distribution of birth weight, fractional growth rate and gestational age in control (CON, n = 40, white bars) and placentally restricted (PR, n = 16, grey bars) sheep. A: Birth weight (kg), B: Fractional growth rate for weight ($1.d^{-1}$), C: Gestational age (days).

Figure 2 – Performance and behavior in Task L in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age. Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment (P<0.05, †), sex*age (P<0.05, ‡), sex* treatment*age (P<0.05, Φ).

Figure 3 – Performance and behavior in Task M1 in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age. Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment (P<0.05, †), sex*age (P<0.05, ‡), sex* treatment*age (P<0.05, Φ).

Figure 4 – Performance and behavior in Task R1 in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age.

Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment ($P<0.05$, †), sex*age ($P<0.05$, ‡), sex* treatment*age ($P<0.05$, Φ).

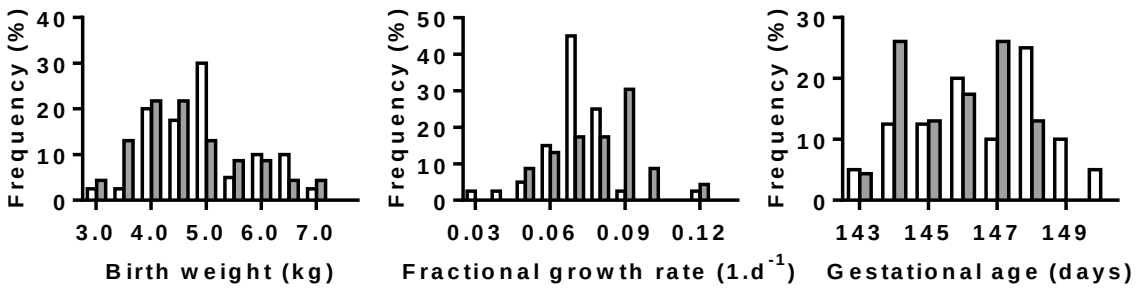
Figure 5 – Performance and behavior in Task M2 in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age.

Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment ($P<0.05$, †), sex*age ($P<0.05$, ‡), sex* treatment*age ($P<0.05$, Φ).

Figure 6 – Performance and behavior in Task R2 in control (white bars) and placentally-restricted (grey bars) sheep at 18 (unhashed bars) and 40 (hashed bars) weeks of age.

Comparisons between treatments and ages are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences are shown separately for males and females. Treatment effects are shown in text above the overall data or sex-specific data as appropriate. Different letters above bars indicate groups that differ overall (a, b), within males only (c, d) or within females only (e, f). Interactions are indicated as follows: sex*treatment ($P<0.05$, †), sex*age ($P<0.05$, ‡), sex* treatment*age ($P<0.05$, Φ).

731 **Figure 1.**



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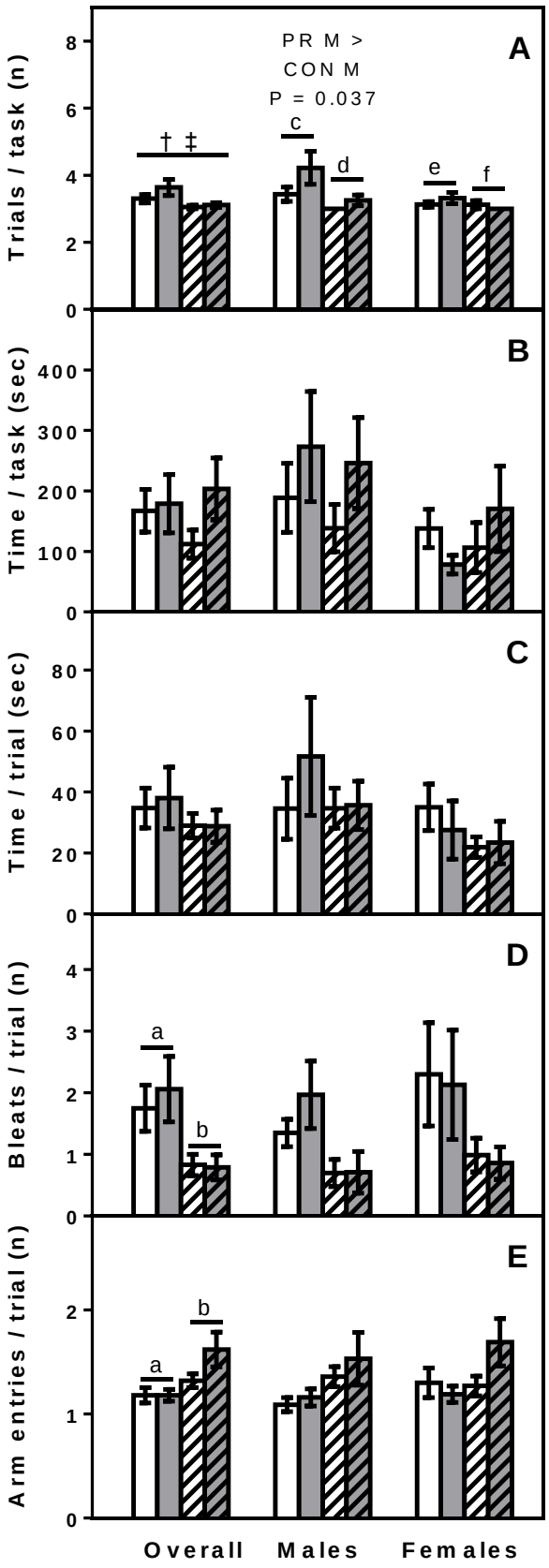
734 **Figure 2**



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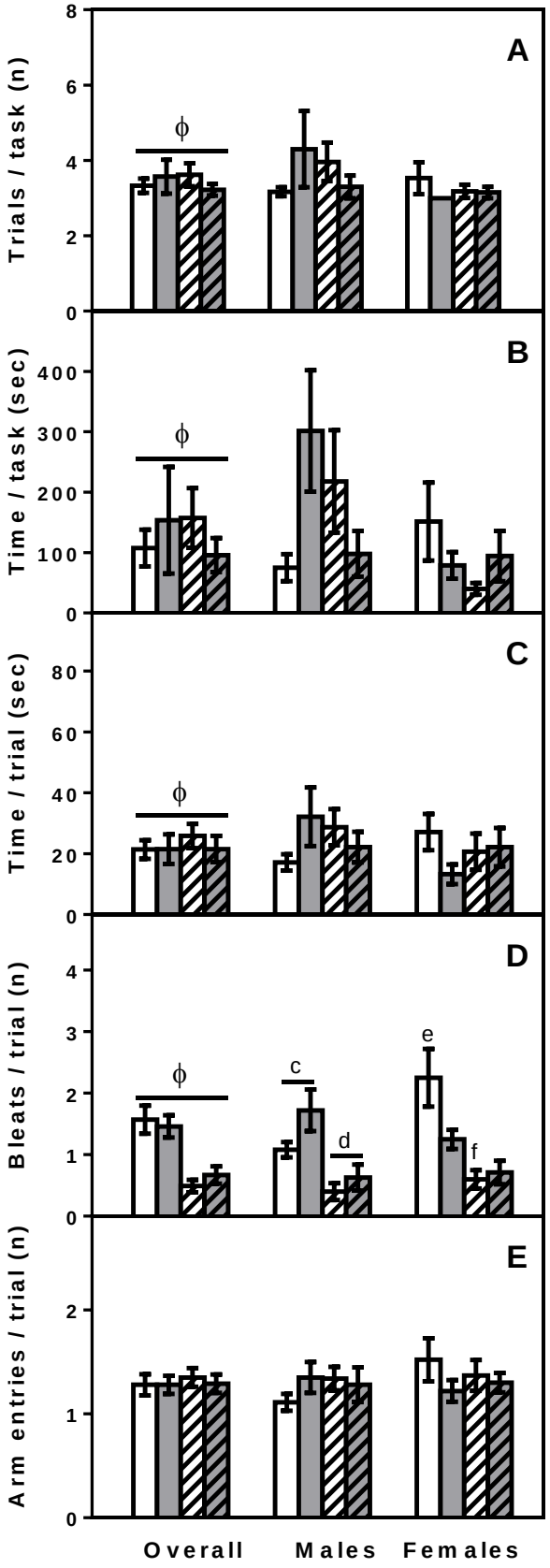
736 **Figure 3**



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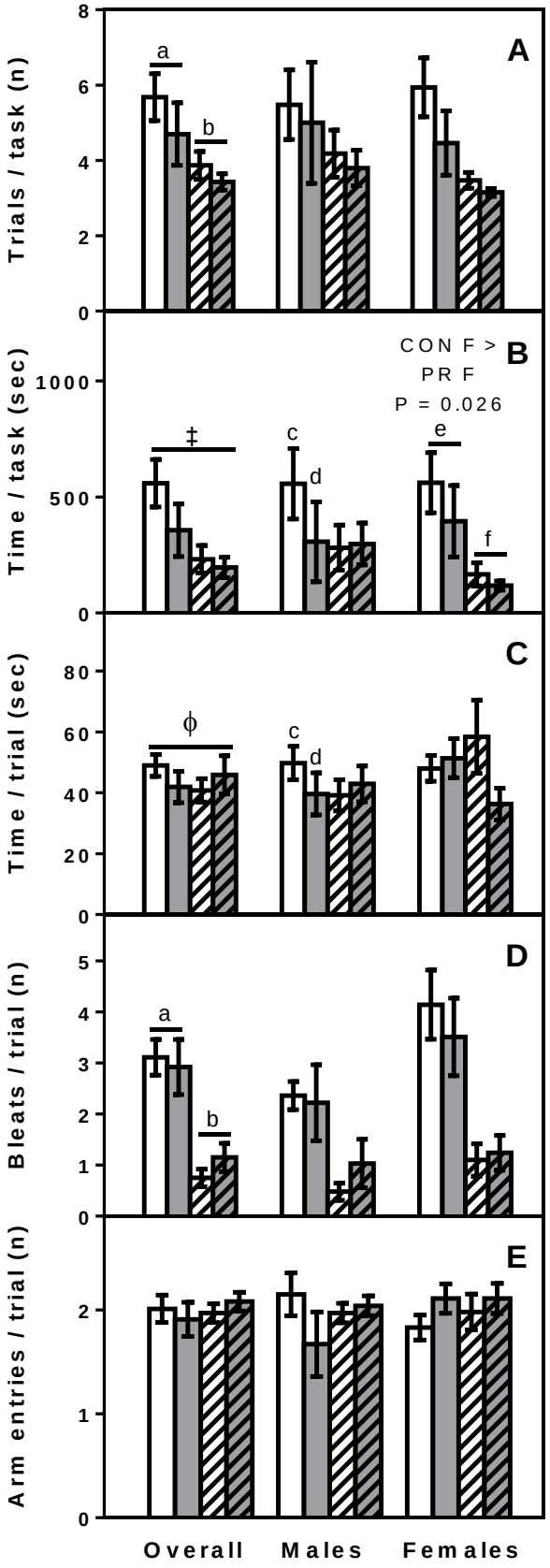
738 **Figure 4**



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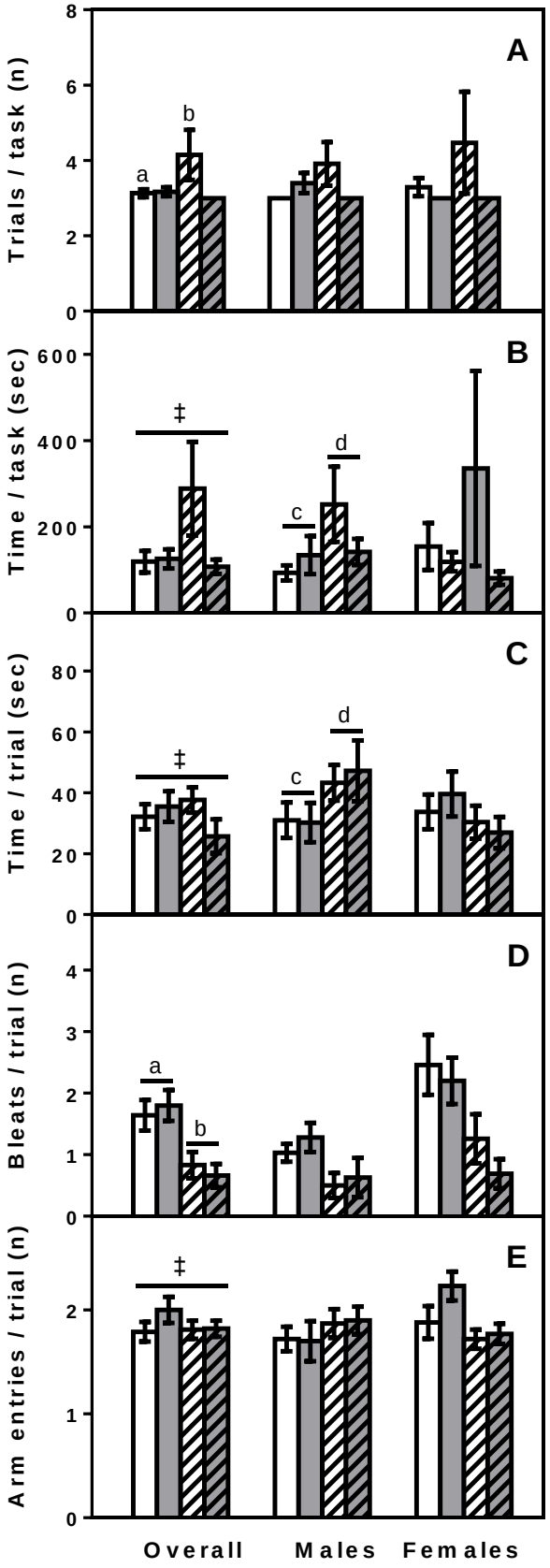
741 **Figure 5**



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744 **Figure 6**

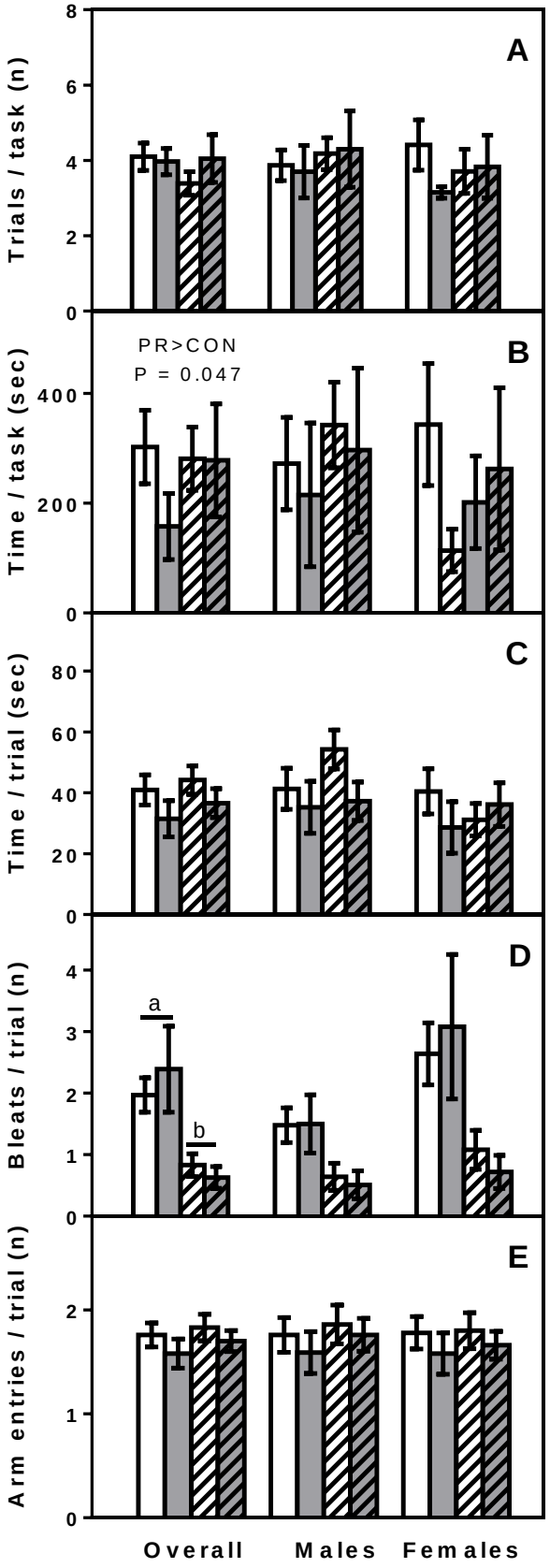

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Table 1 – Associations of maze test outcomes at 18 weeks of age with gestational age, birth weight and neonatal growth. Associations of count data (total trials) with each factor are presented as standardized beta, and associations of continuous data (total time, time / criterion trial, bleats/trial and arm entries/trial) with each factor are presented as partial *R*. Model *r* was obtained from models for continuous data but was not generated in models of count data. Significance of associations between outcomes and each factor are indicated by symbols: # P < 0.1, * P < 0.05, ** P < 0.01.

	Males				Females			
Measure	**Model *r***	**BW**	**FGR**	**GA**	**Model *r***	**BW**	**FGR**	**GA**
Task L								
Total trials		-0.004	0.082	-0.086,		0.019	0.051	-0.014
Total time	0.292	0.042	0.271	-0.058	0.249	-0.025	0.119	0.213
Time / criterion trial	0.308	-0.020	0.255	0.043	0.362	0.036	0.190	0.323
Bleats / trial	0.391	0.087	0.221	-0.340	0.232	0.089	0.199	0.124
Arm entries / trial	0.508*	-0.101	0.378*	0.217	0.463	-0.241	-0.024	0.448*
Task M1								
Total trials		-0.033	0.157	-0.143		-0.237	-0.142	0.068
Total time	0.380	0.062	0.312	-0.225	0.312	-0.280	-0.194	0.212
Time / criterion trial	0.408	-0.023	0.332	-0.134	0.317	-0.123	-0.090	0.303
Bleats / trial	0.306	0.133	0.200	-0.278	0.499#	0.246	0.245	0.395*
Arm entries / trial	0.570*	-0.325#	0.324#	0.036	0.503#	-0.121	-0.100	0.484*
Task R1								
Total trials		0.293	0.202	-0.197		-0.107	-0.130	0.007
Total time	0.204	0.183	0.112	-0.181	0.148	-0.048	-0.126	-0.077
Time / criterion trial	0.314	-0.310	-0.203	0.185	0.551*	0.447*	0.080	-0.399*
Bleats / trial	0.111	-0.053	-0.111	0.045	0.751**	0.734*	0.563*	0.011
Arm entries / trial	0.548*	-0.517*	-0.156	0.439*	0.205	0.156	0.019	-0.122
Task M2								
Total trials		0.211	0.167	-0.149		-0.103	-0.080	-0.010
Total time	0.180	-0.139	-0.001	0.005	0.220	-0.210	-0.109	0.024
Time / criterion trial	0.239	-0.212	-0.172	0.196	0.249	-0.119	0.082	0.118
Bleats / trial	0.232	0.180	0.140	0.007	0.387	0.336#	0.194	0.093
Arm entries / trial	0.404	0.344#	-0.377*	-0.252	0.374	0.358#	0.328#	-0.017
Task R2								
Total trials		0.017	0.064	0.123		-0.064	-0.107	-0.072
Total time	0.223	0.063	0.153	-0.189	0.129	-0.053	-0.099	-0.085
Time / criterion trial	0.327	0.217	0.305	-0.227	0.108	-0.062	-0.079	0.070
Bleats / trial	0.085	0.075	0.039	-0.011	0.326	0.310	0.188	0.007
Arm entries / trial	0.226	-0.019	0.173	0.068	0.236	0.046	-0.089	0.105

Table 2 – Associations of maze test outcomes at 40 weeks of age with gestational age, birth weight and neonatal growth. Associations of count data (total trials) with each factor are presented as standardized beta, and associations of continuous data (total time, time / criterion trial, bleats/trial and arm entries/trial) with each factor are presented as partial *R*. Model *r* was obtained from models for continuous data but was not generated in models of count data. Significance of associations between outcomes and each factor are indicated by symbols: # P < 0.1, * P < 0.05, ** P < 0.01.

Measure	Males				Females			
	Model *r*	**BW**	**FGR**	**GA**	**Model *r***	**BW**	**FGR**	**GA**
Task L								
Total trials		0.037	0.031	-0.025		-0.024	-0.029	-0.007
Total time	0.206	-0.150	0.039	0.127	0.317	-0.191	-0.012	-0.108
Time / criterion trial	0.492#	0.357#	0.428*	-0.058	0.252	-0.152	-0.110	-0.163
Bleats / trial	0.443	-0.372*	-0.400*	0.307#	0.288	0.088	0.093	0.247
Arm entries / trial	0.273	-0.002	0.188	0.130	0.292	-0.131	-0.098	-0.222
Task M1								
Total trials		-0.238#	-0.233*	0.197#		0.050	0.011	-0.072
Total time	0.444	-0.326#	-0.409*	0.329#	-0.096	-0.013	0.004	-0.080
Time / criterion trial	0.180	0.077	-0.013	0.087	0.146	-0.129	-0.041	0.073
Bleats / trial	0.230	-0.080	0.088	0.169	0.286	0.206	0.244	0.130
Arm entries / trial	0.289	0.112	0.277	0.080	0.228	-0.227	-0.149	0.044
Task R1								
Total trials		-0.114	-0.182	0.198		-0.008	-0.021	0.061
Total time	0.373	-0.181	-0.306	0.305	0.143	-0.087	-0.004	0.119
Time / criterion trial	0.311	0.233	0.298	-0.155	0.361	0.123	0.252	0.280
Bleats / trial	0.148	-0.079	0.020	0.127	0.439	0.365#	0.323#	0.184
Arm entries / trial	0.288	0.051	0.118	0.128	0.232	0.064	0.193	-0.045
Task M2								
Total trials		-0.091	-0.199#	0.177		-0.238	-0.206	0.020
Total time	0.351	-0.038	-0.281	0.202	0.206	-0.197	-0.180	0.03
Time / criterion trial	0.260	-0.071	-0.252	0.052	0.203	-0.162	-0.048	0.146
Bleats / trial	0.117	-0.018	0.060	0.075	0.330	0.075	0.123	0.299
Arm entries / trial	0.421	-0.202	-0.279	-0.157	0.359	-0.310	-0.142	-0.054
Task R2								
Total trials		-0.014	-0.024	0.055		0.498*	0.416#	0.108
Total time	0.137	-0.067	0.002	0.128	0.409	0.324#	0.337#	0.173
Time / criterion trial	0.069	-0.045	-0.048	-0.008	0.439	-0.395*	-0.184	0.291
Bleats / trial	0.199	-0.141	-0.154	0.001	0.358	0.219	0.095	0.192
Arm entries / trial	0.337	-0.161	0.066	-0.138	0.422	-0.358#	-0.233	0.322#