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Do I turn left or right? Effects of sex, age, experience and exit route on maze test performance in sheep

Short title: Maze learning in sheep

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Brain development and function are susceptible to perturbation by environmental factors. Sheep are increasingly being used as a neurodevelopmental model due to timing similarities with humans, but effects of age, experience and sex on cognition are not well characterised in this species. We therefore studied memory and reversal learning in sheep using a modified Y-maze at two ages: naive 18 week olds (18N: 23 male, 17 female), experienced 40 week old sheep that had previously been tested at 18 weeks (40E: 22 male, 17 female), and naive 40 week olds (40N: 4 male, 10 female). Younger naive animals (18N) required more trials and time to solve the first reversal task (Task R1) than 40E ($P = 0.007$ and $P < 0.001$ respectively). Experience also improved outcomes, with 40N sheep requiring more time to solve Tasks L ($P = 0.034$) and R1 ($P = 0.002$) than 40E. Increasing age (40N cf. 18N) decreased bleat frequency in Tasks R1, M2 and R2 (each $P < 0.05$). In 40N females, outcomes also differed by exit method in Task R1, with those that exited via an indirect route taking less time to pass Tasks R1 ($P = 0.009$) and R2 ($P = 0.015$) than those that used a direct route . Age plus experience improved learning outcomes, demonstrating knowledge retention for 22 weeks in this species, whilst age alone affected mostly behavioral responses. These results provide comparison data, and can be utilised to improve experimental design, for studies of neurodevelopment in the sheep.

Keywords:

Sheep, learning, cognition, cognitive flexibility, age, experience

1. Introduction

Brain development and function are susceptible to perturbation by various exposures and environmental factors in early life. For example, in humans preterm birth [1, 2], prenatal undernutrition [3] and intrauterine growth restriction [2, 4] are associated with a lower intelligence quotient (IQ) and poorer learning, memory and executive function in children and adolescents. Techniques such as magnetic resonance imaging [5] and transcranial magnetic stimulation [6] allow for study of the morphological and some functional determinants of these cognitive capacities. Use of non-human species extends this to enable studies of the molecular basis and causal pathways that underlie associations between brain development and postnatal function, and early testing of interventions. Further, non-human species enable minimisation of confounding factors affecting neurodevelopment and postnatal function and loss due to drop out and the more rapid follow-up of long term outcomes than is possible in humans.

The sheep is an appropriate species in which to test early life environmental effects on brain development and function, in part due to similar timing of key neurodevelopmental events with humans. In humans and sheep, neurogenesis commences in the first third of gestation [7, 8], and myelination by the last third of gestation [7, 9, 10], although in humans myelination commences after birth in some of the higher brain structures including cerebrum [10]. In contrast, in the rat, neurogenesis does not commence until ~ the last third of gestation (~57% of term), and myelination occurs postnatally [8, 11, 12]. Sheep are also an intelligent species, capable of fine object, brightness, face and plant species discrimination, can extrapolate visual information to recognise individuals from different viewpoints or earlier ages [13], and can be trained to make use of these skills [13-18]. Sheep can learn to navigate mazes [19-23], and are the only large animal model aside from primates in which complex executive function has been demonstrated, with cognitive flexibility demonstrated using tests of reversal learning and intra- and extra-dimensional set-shifting [20, 22-24]. Together these characteristics make them a suitable species in which to examine effects of early life factors on neurodevelopment and subsequent behaviour.

To date there have been few studies examining cognition even in healthy sheep as they mature, limiting their usefulness for developmental studies. Memory and reversal performance in maze tasks improved with age in pre-pubertal lambs [23]. Changes in side-preference and reversal learning have also been examined in 4- and 18-month old control and periconceptionally undernourished sheep [22], however the effects of prior learning were not controlled for, and therefore effects of age and experience could not be separated. Because learning may also differ between sexes, and this effect may also differ with ageing due to effects of sex steroids on reactions to stressful situations [25, 26] and effects of stress hormones on learning [27-29], it is also important to characterise learning development in both sexes.

We therefore tested learning, memory and cognitive flexibility in 18- and 40 week old sheep, ages corresponding to pre-puberty and young adulthood [30, 31], in a cohort that were habituated to frequent human contact and handling. We also recorded numbers of entries into each arm of the maze, allowing us to assess patterns of entries prior to successful exit of the maze. Three comparisons were performed to differentiate effects of age and experience and their interactions with sex. Firstly, we compared performance in the same sheep tested twice, at 18 and 40 weeks of age, where differences reflect age and experience. Second, we compared performance in sheep tested at 18 weeks and in those tested only at 40 weeks, where both groups were naive to the task, to assess effects of age alone. Third, we compared performance of 40 week old sheep between those that had been tested previously (at 18 weeks of age) and those who were tested only at 40 weeks of age, to examine the effects of experience alone.

2. Methods

2.1 Ethics Statement

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* [32].

2.2 Animals

Merino x Border Leicester ewes were mated with Merino rams in a timed mating program [33], and delivered spontaneously at term between July 2010 and November 2012. Animal management was as described previously [34]. Briefly, ewes were housed in individual pens in an animal holding room with a 12 h light:12 h dark cycle from day110 of pregnancy until four weeks after birth, then group housed in pens until the lambs were weaned at 13 weeks of age. Ewes were fed 1 kg Rumevite pellets/day (Ridley AgriProducts, Victoria, Australia), and had *ad libitum* access to lucerne chaff and water. Weaned lambs were housed in outside paddocks in groups of the same sex and similar ages. Lambs were group-fed each day with

0.5 $kg.d^{-1}.lamb^{-1}$ Rumevite pellets, with *ad libitum* access to oaten hay, seasonal pasture and water.

Lambs were handled frequently from birth, having direct contact with the experimenters during individual measures of growth every second day from birth to 16 days of age, weekly to weaning, and then at 5 week intervals. Studies of immune function between 20 and 28 weeks of age [34] also required frequent handling of small groups of sheep in yards adjacent to paddocks. Daily feeding in small groups during pen and then paddock housing also provided frequent human contact and ensured lambs were habituated to the presence of humans. Learning was tested in these sheep either at both 18 and 40 weeks, or at only 40 weeks of age, resulting in three groups – naive 18 week old (18N: 23 male, 17 female), experienced 40 week old (40E: 22 male, 17 female) and naive 40 week old (40N: 4 male, 10 female). The 40N group was generated due to delays in maze construction at the start of the project, which meant that the maze was not available for testing of the first three lambing groups when they reached 18 weeks of age, and so animal numbers in this group were limited. One male sheep died between 18 and 40 weeks of age and is only included in 18N data. All other sheep tested at 18 weeks completed all tasks at both 18 and 40 weeks. Of the sheep tested only at 40 weeks (40N), one 40N female failed Task R2, and results from this animal were excluded only for this task.

2.3 Learning Evaluation

A diamond-shaped maze was constructed of opaque panels under a 3 m high pergola covered by shade-cloth, to remove the confounding effects of shadow or light glare on side-preference that were observed during preliminary trials (data not shown). Both maze arms were constructed to be identical mirror images, and therefore the major spatial cue was position of each maze arm relative to the starting arm containing the entry gate. Exit gates were not visible from the Y-intersection, and the sheep had to commit to picking a lane, walk 1.5 m along that lane-way from the intersection, and turn a corner before these came into sight. The gates were non-opaque, such that sheep could see and hear flock mates through the gate, be it open or closed.

We adapted the assessments described previously by Erhard *et al.* [20] and Hernandez *et al.* [22]. Briefly, the test protocol consisted of 3-5 days of testing. The first day consisted of an initial habituation task, in which sheep were habituated to the handling protocol and the maze apparatus, and trained to exit the maze through the open gates. The initial side chosen for the guided runs was randomized to prevent any introduction of side bias. Following this, a sequence of memory and reversal tasks took place on subsequent days (Table 1). Successfully completing the day's tasks resulted in graduation to the next day's testing in the sequence. Failure to complete either task on Days 2 or 3 resulted in the sheep repeating that day's tests on following days until successful on both tasks. Sheep had a maximum of five days to finish the entire three day sequence, with a maximum of six trials to learn Task L on day 1, and a maximum of ten trials to solve each task on sequence days 2 and 3. If sheep failed to solve a task, it repeated the entire day's sequence of tasks on the subsequent day, until the sheep either passed both tasks for that day or had finished five consecutive days of testing. The criterion to complete each task consisted of three consecutive exits from the maze in three minutes or less, within the allocated number of trials to learn this task (Table 1). These final three consecutive successful trials of each task (i.e. successfully exiting the maze in < 3 minutes per trial) were defined as the criterion trials in subsequent analyses. The reward for solving the maze was the capacity to exit into the reward pen, allowing for close proximity with flock-mates, and access to a lucerne chaff food reward during the ≥10 second rest period between runs. The only penalty for not solving the maze was the inability to leave the maze during that trial, consistent with previous studies [20, 22]. Once the failed trial was complete (after 3 minutes without exiting), sheep were steered out of the correct exit to the reward pen, and remained there for a ≥10 second rest period as above.

Measures recorded during the maze tasks included total trials and time taken to complete each trial, numbers of vocalizations in each trial, and number of arm entries and average time per trial in criterion trials. Sheep were also classified according to the method they used to exit the maze (complete the task) within the three minutes available. Those sheep that exited the maze via a direct route to the open gate on the majority of criterion trials were classified as using a direct exit method. Sheep which initially entered the closed maze arm before reversing direction and exiting via the open gate in the majority of their criterion trials were classified as using an indirect exit method (Figure 1).

2.4 Statistical analysis

Effects of sex and task on continuously distributed outcomes were initially analyzed within each group for data from all tasks using mixed model analysis, recognizing the multiple measures on each individual sheep, with post-hoc Bonferroni comparisons used to compare differences between each task. Effects of sex plus group (18N vs. 40E, in which differences reflect both age and experience), age (18N vs. 40N) or experience (40N vs. 40E) on continuously distributed outcomes were then analyzed for data within each task separately using mixed effects models including repeated observations on each individual sheep for the comparison of 18N and 40E groups. These data were log-transformed prior to analysis to reduce skew. Variables that were counts of events (i.e. total trials per task) were analyzed using a Poisson distribution with log link . Where effects of group, age, or experience differed between sexes (e.g. age*sex interactions), subgroup analysis was carried out. Chi-squared tests of association were used to examine proportions of animals within each group and sex with each exit method, and to determine whether exit method in Tasks L (learning task) and R1 (reversal task) predicted exit method utilized in later tasks. Effects of exit method in Task R1 on outcomes in task R1 and the subsequent tasks, M2 and R2, were analyzed by mixed effects models for effects of sex and exit method within each group (18N, 40N, and 40E). As there were no male 40N sheep that used a direct route exit method, and only 4 male sheep in the 40N group in total, effects of exit method in the 40N group were examined in females only. 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 Between task differences

Data for outcomes in each task is shown in Figure 2 – 6. The number of trials required to complete tasks differed between tasks in 18N and 40E (each $P < 0.001$) but not 40N ($P > 0.2$), whilst the total time required to complete the task differed between tasks in all groups of sheep (18N, 40E and 40N; $P < 0.01$ for all). In 18N sheep, task R1 required more trials and longer total time to complete than all other tasks ($P \leq 0.005$ for all). Also in 18N sheep, task R2 required more trials than task L ($P = 0.023$) or task M2 ($P = 0.007$), and took longer to complete than task M1 ($P = 0.014$). These naïve 18-week old sheep also bleated more in each trial for task R1 than tasks L, M1 or M2 (each $P \leq 0.005$) but not task R2 ($P > 0.1$), and female 18N sheep bleated more than male 18N sheep overall ($P = 0.005$). In 40N sheep, the number of trials required to complete tasks did not differ between tasks ($P > 0.2$), but the total time required to solve task R1 was longer than for task M2 ($P = 0.013$) with a similar trend for task M1 ($P = 0.088$). Also in 40N sheep, task differences in bleat frequency differed between sexes (task*sex interaction $P = 0.044$), but did not differ between tasks in either sex when analyzed separately (males $P > 0.4$, females $P = 0.083$). In 40E sheep, task R1 required more trials and longer total time to compete than task L ($P = 0.018$ and $P = 0.002$ respectively) and required longer total time than task M1 ($P = 0.003$). Also in 40E sheep, task R2 required more trials and longer total time than task L ($P = 0.014$ and $P = 0.012$ respectively) and required longer total time than task M1 ($P = 0.008$). These experienced 40-week old sheep bleated more in task R1 than task M1 ($P = 0.025$) but bleats per trial did not differ between other tasks.

3.2 Outcomes in learning task (Task L)

3.2.1 – Effects of age and experience (18N vs 40E)

For task L, numbers of trials, total time required to solve the task and average time per criterion trial did not differ between groups (each $P > 0.4$) or sexes (each $P > 0.5$, Figure 2). Bleat frequency in task L (Figure 2) was greater in 18N than 40E ($P = 0.023$) and did not differ between sexes ($P > 0.4$). Effects of group on the number of arm entries per trial (Figure 2) differed between sexes (group*sex interaction $P = 0.044$), such that 18N males made less arm entries per trial than 40E males ($P = 0.001$), whilst arm entries per trial did not differ between 18N and 40E females ($P > 0.9$).

3.2.2 – Effects of age in naïve learners (18N vs 40N)

Age and sex did not affect any outcomes in task L (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 18N and 40N sheep (each $P > 0.1$, Figure 2).

3.2.3 – Effects of experience in 40-week old sheep (40N vs 40E)

Greater experience reduced the total time required to solve task L (40N > 40E, $P = 0.034$) but experience did not affect number of trials, average time per criterion trial, bleat number per trial or arm entries per trial (each $P > 0.1$, Figure 2). Outcomes in task L did not differ between sexes in comparisons of 40N and 40E sheep (each $P > 0.1$).

3.3 Outcomes in first memory task (Task M1)

3.3.1 – Effects of age and experience (18N vs 40E)

Group and sex did not affect (each $P > 0.1$) number of trials, total time required to solve the task, average time per criterion trial, or arm entries per trial in task M1 in comparisons of 18N and 40E sheep (Figure 3). Effects of group on bleat frequency in task M1 differed between sexes (group*sex interaction $P = 0.008$). Bleat frequency did not differ between 18N and 40E males ($P > 0.4$), whilst 18N females bleated more than 40E females ($P = 0.001$, Figure 3).

3.3.2 – Effects of age in naïve learners (18N vs 40N)

Age and sex did not affect (each P > 0.1) number of trials, total time required to solve the task, average time per criterion trial, or arm entries per trial in task M1 in comparisons of 18N and 40N sheep (Figure 3). Naïve 18-week old sheep bleated more often in task M1 than naïve 40-week old sheep (P = 0.006, Figure 3) and females bleated more than males in comparisons of 18N and 40N sheep (P = 0.039).

3.3.3 – Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial or arm entries per trial for task M1 in comparisons of 40N and 40E sheep (each P > 0.1, Figure 3).

3.4 Outcomes in first reversal task (Task R1)

3.4.1 – Effects of age and experience (18N vs 40E)

More trials and greater total time were required for 18N than 40E sheep to solve task R1 (P = 0.007 and P < 0.001 respectively), and 18N sheep bleated more frequently than 40E sheep in this task (P < 0.001), but average time per criterion trial and arm entries per trial in task R1 did not differ between 18N and 40E sheep (each P > 0.1, Figure 4). Outcomes in task R1 did not differ between sexes in comparisons of 18N and 40E sheep (each P > 0.09).

3.4.2 – Effects of age in naïve learners (18N vs 40N)

Age did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40N sheep (each P > 0.5, Figure 4). Younger naïve sheep (18N) bleated more frequently than 40N sheep in task R1 (P < 0.001, Figure 4). In 18N and 40N groups combined, female sheep tended to require more trials (P = 0.070) and more total time (P=0.079) to solve task R1 than males (P = 0.070 and P = 0.079 respectively), and bleated more often than males (P = 0.003), whilst average time per criterion trial and arm entries per trial did not differ between sexes (each P > 0.1).

3.4.3 – Effects of experience in 40-week old sheep (40N vs 40E)

In 40 week old sheep, effects of experience on the number of trials required to solve task R1 differed between sexes (experience*sex interaction P = 0.047), with 40N and 40E males requiring similar number of trials to complete this task (P > 0.8), and 40N females requiring more trials to complete task R1 than 40E females (P = 0.036, Figure 4). Bleat number per trial also differed between 40N and 40E sheep in a sex-dependent manner (experience*sex interaction P = 0.009), with 40N males bleating less than 40E males (P = 0.048) and no difference in bleat frequency between 40N and 40E females (Figure 4). Total time required to complete task R1 was greater in 40N than 40E (P = 0.002, Figure 4) and did not differ between sexes (P > 0.3). Experience and sex did not affect average time per criterion trial and numbers of arm entries per trial in task R1 in comparisons between 40N and 40E sheep (each P > 0.1, Figure 4).

3.5 Outcomes in second memory task (Task M2)

3.5.1 – Effects of age and experience (18N vs 40E)

Compared to 40E sheep, 18N sheep required fewer trials and less total time to solve task M2 (P = 0.001 and P = 0.047 respectively), and bleated more (P = 0.004, Figure 5). Average time per criterion trial and numbers of arm entries per trial in task M2 did not differ between 18N and 40E sheep (each P > 0.2). In 18N and 40E groups overall, sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40E sheep (each P > 0.4), and female sheep bleated more per trial than males (P = 0.005).

3.5.2 – Effects of age in naïve learners (18N vs 40N)

Age and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial in comparisons of 18N and 40N sheep (each P > 0.09, Figure 5). Younger naïve sheep (18N) bleated more frequently than 40N sheep (P = 0.002, Figure 5) and overall for 18N and 40N groups combined, females bleated more than males in task M2 (P = 0.021).

3.5.3 – Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect any outcomes in task M2 (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 40N and 40E sheep (each P > 0.1, Figure 5).

3.6 Outcomes in second reversal task (Task R2)

3.6.1 – Effects of age and experience (18N vs 40E)

Group and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial for task R2 in comparisons of 18N and 40E sheep (each P > 0.1, Figure 6). Younger naïve sheep (18N) bleated more frequently than older experienced sheep (40E, P = 0.007, Figure 6) and in 18N and 40E groups overall, females bleated more than males in task R2 (P = 0.029).

3.6.2 – Effects of age in naïve learners (18N vs 40N)

Age and sex did not affect number of trials, total time required to solve the task, average time per criterion trial or arm entries per trial for task R2 in comparisons of 18N and 40N sheep (each P > 0.1, Figure 6). Younger naïve sheep (18N) bleated more frequently than older naïve sheep (40N, P = 0.028, Figure 6) and bleat frequency did not differ between sexes for task R2 (P > 0.1).

3.6.3 – Effects of experience in 40-week old sheep (40N vs 40E)

Experience and sex did not affect any outcomes in task R2 (number of trials, total time required to solve the task, average time per criterion trial, bleat number per trial and arm entries per trial) in comparisons of 40N and 40E sheep (each P > 0.1, Figure 6).

3.6 Exit method

The exit method used for the criterion trials (final three successful trials) of each task did not differ between groups, age, experience or sex. In task R1, 25 sheep (27%) sheep that exited directly via the open arm (direct exit method) to complete the task, and 67 (73%) sheep entered the closed arm first before exiting via the open arm (indirect exit method) in order to solve the task. Exit method in Task L predicted exit method in Task M1 only (χ^2 (2) = 30.0, P < 0.001). Exit method in Task R1 predicted exit method in Tasks M2 (χ^2 (1) = 16.8, P < 0.001) and R2 (χ^2 (1) = 8.78, P = 0.032). Exit method in Task R1 was therefore used as the factor for subsequent analyses of effects of exit method on maze performance.

3.7 Effects of exit method

3.7.1 - 18N

Differences between sheep using direct and indirect exit methods did not vary with sex within any group. In naïve 18 week-old sheep, sheep that took a direct exit route in criterion trials of task R1 required similar numbers of trials and total time to complete tasks R1 and M2 (each P > 0.1), but required more trials to complete task R2 (P = 0.024) with a similar trend for total time to complete task R2 (P = 0.065) compared to 18N sheep that exited indirectly in task R1 (Figure 7). Also in naïve 18-week old sheep, those that took a direct exit method in task R1 had faster average times per criterion trial in tasks R1 (P < 0.001) and M2 (P = 0.025) but not task R2 (P > 0.2), and bleated less often in task R1 (P = 0.012) but not later tasks, compared to sheep that exited indirectly in task R1 (Figure 7).

3.7.2 - 40N

Effects of exit method were only examined in 40N females owing to the small sample size of 40N males. In female naïve 40 week-old sheep, those that took a direct exit route in criterion trials of task R1 required more trials and time to complete task R1 than those who exited indirectly (P = 0.001 and P = 0.009 respectively, Figure 7). Similar effects and trends were seen for higher total trial number (P = 0.077) and total time (P = 0.015) in task R2 for sheep that used a direct exit method in task R1, whilst performance in task M2 did not differ between these groups (Figure 7). Average time per criterion trial and bleat frequency in tasks R1, M2 and R2 did not differ between exit methods for female naïve 40 week-old sheep (Figure 7).

3.7.3 40E

In experienced 40 week-old sheep, total time and number of trials required to solve tasks R1, M2 and R2 did not differ between those that took direct cf. indirect exit routes in criterion trials of task R1 (each P > 0.1, Figure 7). Sheep in the 40E group that used a direct exit method in task R1 took less time per criterion trial (P = 0.005) and bleated less (P = 0.001) in task R1, but not in subsequent tasks (each P > 0.3), than those that used an indirect exit method (Figure 7).

4. Discussion

There are two novel findings from this study. Firstly, we have shown that young sheep are capable of retaining knowledge of complex tasks when re-tested 22 weeks after initially learning these tasks, as experienced 40 week olds (40E) completed initial learning and the first reversal task more quickly than naïve 40 week old sheep (40N) not previously exposed to the tasks. This suggests sheep may be useful for examining long term memory, and indicates the necessity of controlling for effects of prior learning in this species. Secondly, whilst the combination of age and experience improved learning outcomes, with the same sheep performing better as experienced 40 week-olds than in their first exposure to tests as 18 week-olds, age alone affected mostly behavioural responses. Naïve sheep bleated more at 18 weeks of age than at 40 weeks of age but these groups differed in learning outcomes only in females, and only in the first reversal task.

Poorer performance in the reversal versus the learning and memory tasks in the present study was unsurprising as reversal learning is a comparatively demanding task compared to simple maze navigation. Reversal learning in Y-maze tasks requires initially learning a rule (e.g. which arm to enter to reach the open gate in Task L and M1 of the present study) and then reversing the use of this rule (e.g. entering the previously unrewarded maze arm in Task R1 and R2 in the present study). In rats reversal learning requires greater use of working memory and more complex attentional processes than simple spatial learning [reviewed in 35]. While reversal tasks are rapidly acquired by sheep in T-maze experiments, a higher proportion of sheep fail reversal tasks in early trials compared to more readily learned spatial learning tasks, further illustrating the greater difficulty and cognitive demand of this type of task [23, 24]. As such, reversal learning measures one aspect of executive function. In the rat, reversal learning activates the orbitofrontal cortex function in areas distinct from those associated with complex executive functions such as intra- and extra-dimensional set-shifting [36], and this task may thus provide a measure of function of this area in sheep.

Reversal learning in the present study in sheep does, however, involve differences in behaviors and stimuli than those experienced in rodents during maze learning. In rodent and primate studies, poor performance in reversal trials has been interpreted as unnecessary perseverance at an action that is no longer rewarded [35, 37], particularly as rats have a tendency to explore arms not visited in previous trials [38]. In contrast, sheep are reluctant to enter lanes that were not rewarded during training in maze tests [24], and the majority of sheep in the present study made very few arm entries per trial, generally waiting in the closed arm within sight of flock-mates during failed trials. Furthermore, sheep find social isolation stressful [39], and reversal learning in the present study required sheep to move away from visible flock-mates if they initially entered the incorrect arm of the maze, therefore involving two types of aversive stimuli. Stress probably magnifies group differences in inherent learning capacity during reversal tasks, since stress decreases the likelihood of approaching aversive stimuli in sheep [40], and 18N and 40E sheep in the present study vocalised more often in the first reversal task than in learning and memory tasks, indicating they may have found this task stressful. Assessing reversal learning in sheep therefore requires assessing their ability to successfully exit the maze to reach the reward, rather than proportions of time in correct and incorrect arms of the maze, as reported in many rodent studies where the reward is within the maze itself [e.g. 41, 42].

Learning performance also differed with age and experience in the present study. Older experienced sheep (40E) learnt the first reversal task (R1) more quickly and required fewer trials than young naïve sheep (18N). This suggests the 40E sheep were recalling executive function skills in Task R1 that they learnt at 18 weeks of age. We doubt faster learning speed in 40E sheep compared to their performance at 18 weeks is solely an effect of habituation to human handling, as sheep were handled regularly from birth, although habituation to the maze test itself may have reduced stress and improved learning. While it has been established previously that sheep can identify and recall the faces of individuals for over two years [17] retention of more complex tasks over long periods has not previously been demonstrated. Our results suggest executive function skills learned at 18 weeks are remembered for at least five months after the initial learning in sheep.

Age alone did not affect learning performance in comparisons between naïve 40 week-old (40N) and 18 week-old sheep (18N), but bleat frequency was higher in the younger group in all tasks except the initial learning task. Johnson and colleagues reported that 14 week-old lambs learned more quickly than 9 week-olds regardless of sex [23]. Since the lambs in that study were all pre-pubertal in age, these age-related improvements may have been a consequence of pre-adulthood brain maturation resulting in gain of function in this younger group [23]. Both ages in the present study were older than the animals tested by Johnson, and our findings suggest learning does not differ between these older ages in sheep.

The sex-specific effects of age and experience on learning in sheep in the present study, with fewer trials required to solve the first reversal task in 18N females than 40N females, and in 40E than 18N or 40N females, but not males, may be related to effects of sex steroids, particularly after puberty. Merino ewes enter puberty between 23-43 weeks of age, at an average age of 31 weeks [31], and therefore 18N and 40N females were exposed to different hormonal environments during their initial learning. Oestrus, but not dioestrus, female rats have impaired performance in Morris Water Maze hidden platform tasks compared to males, and this seems to be a consequence of oestrogens interfering with task acquisition rather than recall [43]. This may also explain why in females, 40E sheep that had already learned this reversal task performed better than 40N sheep that were learning the task for the first time. Age and sex differences may also be mediated by emotional reactivity, which is reduced by testosterone in sheep [25, 26]. Lower emotional reactivity due to testosterone in rams would decrease their stress responses and protect their capacity to overcome aversive stimuli [40] and hence will improve reversal performance within this maze design. Our observation of similar bleat frequencies in females in both 40-week old groups, suggests that differences in emotional reactivity do not explain sex-specific effects of experience in learning outcomes in the present study, however. These conclusions are limited by small numbers in the naïve 40 week-old group, particularly the males, however, and effects of sex and its interactions with prior learning and age need to be confirmed in subsequent larger studies. Generation of the 40N group was opportunistic with a relatively small sample size, due to the timing of maze construction after part of the flock had passed 18 weeks of age. In addition, testing at different phases of the oestrous cycle may provide clearer information about the probable effects of sex steroids on learning in sheep, as previous studies of hormonal status have examined changes in emotional reactivity [25, 26] but not learning.

In addition to effects of group, experience, age and sex on maze performance, we also observed differences according to the exit method sheep used to leave the maze during the criterion trials. Because we did not observe sex or age differences in numbers of arm entries per trial in any memory or reversal tasks throughout the protocol in these sheep, we suggest arm entries are not an indicator of general activity in sheep, unlike the rat [38]. Surprisingly, sheep that learned to turn around at dead ends in the first reversal task (indirect exit method) were quicker to learn reversal tasks (less total time and trials required in task R1 in 40-week-old naïve females and fewer trials required in task R2 in naïve 18 week-old sheep) than those learnt to directly enter the open arm of the maze. Consistent with a longer path length to exit in the indirect group, time per criterion trial was greater in 18N and 40E indirect exit method sheep than in those who used a direct exit method for the first reversal task. Behavior also differed between direct and indirect learners in the first reversal task, when sheep using an indirect exit method bleated more often than those who exited directly, in the 18N and 40E groups. Interestingly, this difference in bleat frequency was not observed in 40N sheep in task R1 or task R2 or in 18N sheep in task R2, where indirect learners completed the task faster than direct learners. This suggests that different exit methods might reflect temperament differences such as lower flocking instinct and hence a greater willingness to leave sight of flock mates at the closed gate, and/or cognitive differences such as superior executive function and hence better reversal learning, and that temperament differences might mask differences in cognitive outcomes under conditions of stress.

In summary, effects of age and experience on learning performance in maze tasks vary between sexes in healthy sheep. Our data emphasises the importance of studying both sexes, and with gonadally-intact animals, if the intention is to draw comparisons to the human condition, particularly after puberty, and to control for prior learning and handling in studies of behavioural outcomes in the sheep. These results provide comparison data for studies of neurodevelopment in the sheep, as well as longitudinal information that will allow for improved experimental design.

5. Acknowledgements

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Table 1 – Learning tasks and testing schedule.

Day	Task	Description	Maximum number of trials	Maze exits open
1	*Training*	Habituation task in which both gates were open. Sheep could exit maze out of either, side most frequently used to exit was used in later tasks as their preferred side.	5	Both
	Guided runs	Habituation task in which sheep were guided down one of the maze arms to a closed gate and trained to turn around at this dead end and exit through the other.	2	Left and right sequentially. Initial direction randomized
	Learning task (Task L)	Training sheep to exit maze via preferred side.	6	Preferred side
2+	*Memory Task 1 (Task M1)*	Learning consolidation, in which task L was repeated.	10	Preferred side
	Reversal task 1 (Task R1)	Reversal 1 training, in which the open gates were swapped and the lamb had to unlearn the previous route out of the maze and learn to exit through the other gate	10	Non-preferred side
3+	*Memory Task 2 (Task M2)*	Reversal 1 learning consolidation – repetition of R1	10	Non-preferred side
	Reversal task 2 (Task R2)	Re-reversal, with open gate swapped once more to preferred side.	10	Preferred side

Captions:

Figure 1 – Exit method. Sheep were classified as using a direct or indirect exit method for each task, according to the route they used to exit the maze in the majority of their criterion runs for Task R1.

Figure 2 – Performance and behaviour in Task L in naïve 18 week-old sheep (18N, white bars), naïve 40 week-old sheep (40N, gray bars) and experienced 40 week-old sheep (40E, black bars). Comparisons between groups (18N vs 40E), ages (18N vs 40N) and experience (40N vs 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P<0.05$, †), sex*age ($P<0.05$, ‡), sex*experience ($P<0.05$, &).

Figure 3 – Performance and behaviour in Task M1 in naïve 18 week-old sheep (18N, white bars), naïve 40 week-old sheep (40N, gray bars) and experienced 40 week-old sheep (40E, black bars). Comparisons between groups (18N vs 40E), ages (18N vs 40N) and experience (40N vs 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group ($P<0.05$, †), sex*age ($P<0.05$, ‡), sex*experience ($P<0.05$, &).

Figure 4 – Performance and behaviour in Task R1 in naïve 18 week-old sheep (18N, white bars), naïve 40 week-old sheep (40N, gray bars) and experienced 40 week-old

sheep (40E, black bars). Comparisons between groups (18N vs 40E), ages (18N vs 40N) and experience (40N vs 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group (P<0.05, †), sex*age (P<0.05, ‡), sex*experience (P<0.05, &).

Figure 5 – Performance and behaviour in Task M2 in naïve 18 week-old sheep (18N, white bars), naïve 40 week-old sheep (40N, gray bars) and experienced 40 week-old sheep (40E, black bars). Comparisons between groups (18N vs 40E), ages (18N vs 40N) and experience (40N vs 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group (P<0.05, †), sex*age (P<0.05, ‡), sex*experience (P<0.05, &).

Figure 3 – Performance and behaviour in Task R2 in naïve 18 week-old sheep (18N, white bars), naïve 40 week-old sheep (40N, gray bars) and experienced 40 week-old sheep (40E, black bars). Comparisons between groups (18N vs 40E), ages (18N vs 40N) and experience (40N vs 40E) are indicated above the combined male and female data, unless effects differed between sexes for one or more comparison, in which case differences between groups are shown separately for males and females. Bars with the same letter do not differ. Interactions are indicated as follows: sex*group (P<0.05, †), sex*age (P<0.05, ‡),

sex*experience (P<0.05, &).**Figure 7 – Performance and behaviour in Task R1, Task M2 and Task R2 in sheep that used a direct (plain bars) or indirect (striped bars) exit method in task R1.** Outcomes were compared within each group (18N, 40N and 40E) between sheep using a direct and indirect exit method in criterion trials of task R1, and are shown as the mean ± SEM for each exit method group, for males and females combined in 18N and 40E sheep, and in females only for 40N sheep. Bars with the same letter within each group do not differ.

Figure 1

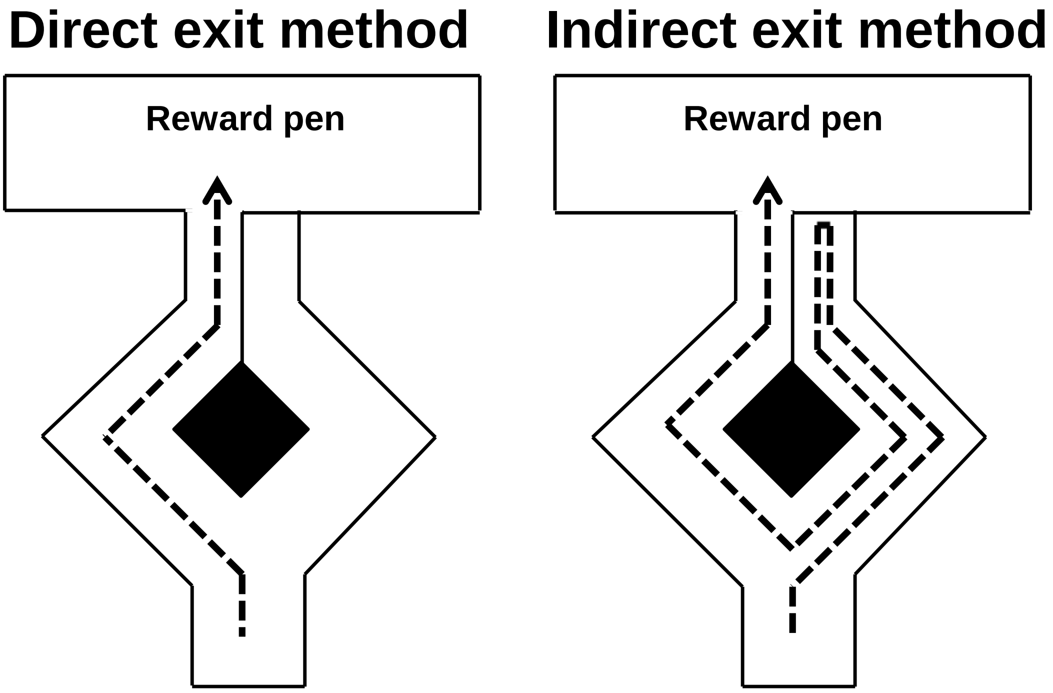


Figure 2

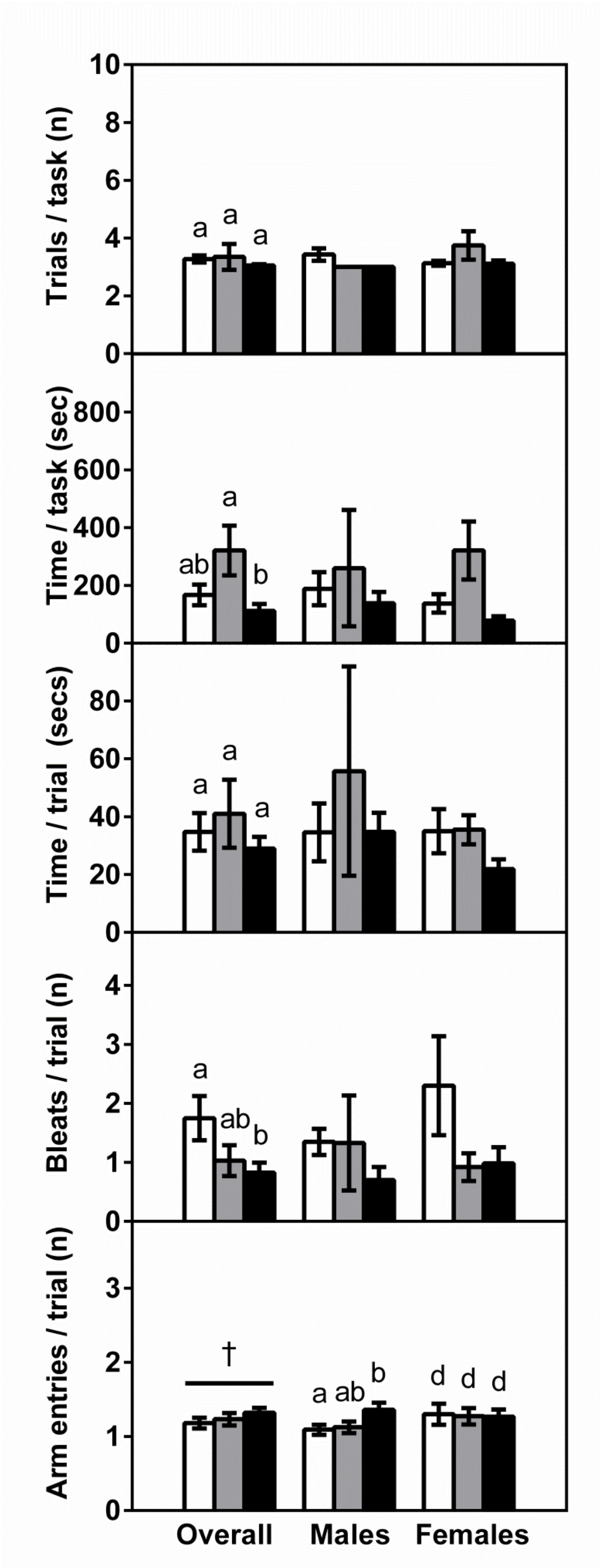
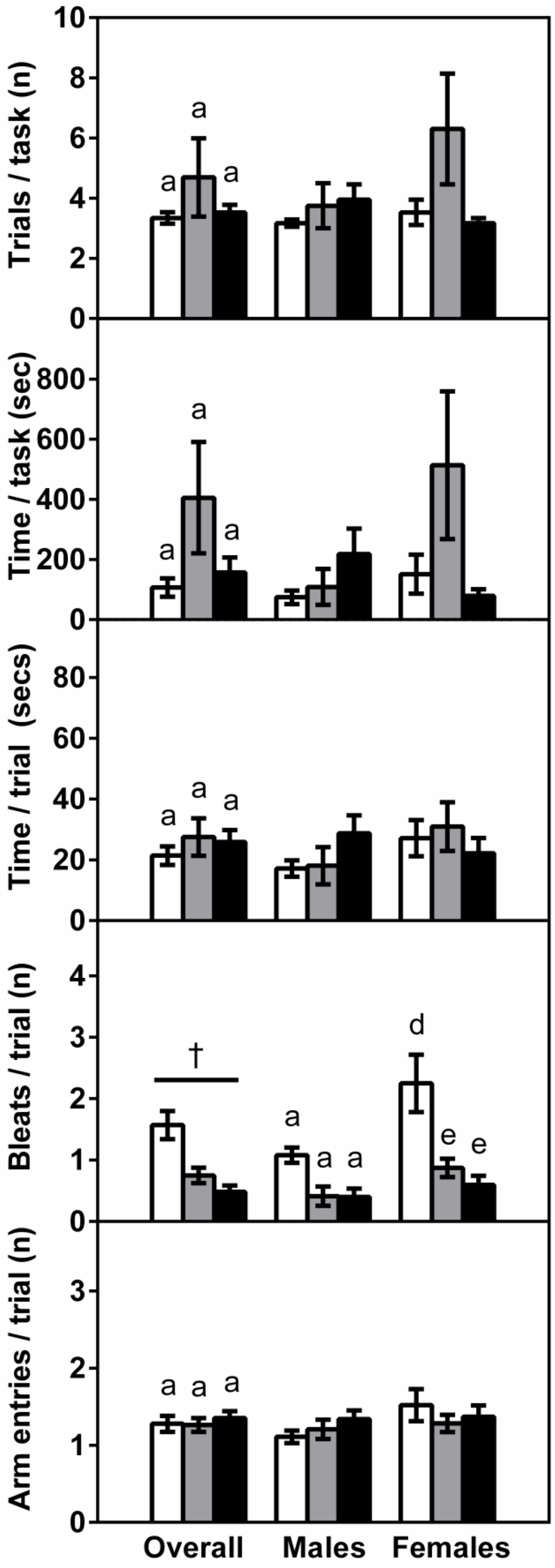

Trials / task (n)
10
8
6
4
2
0
a
a
a
Time / task (sec)
800
600
400
200
0
a
ab
b
Time / trial (secs)
80
60
40
20
0
a
a
a
Bleats / trial (n)
4
3
2
1
0
a
ab
b
Arm entries / trial (n)
3
2
1
0
†
a
ab
b
d
d
d
Overall
Males
Females

Figure 3


Trials / task (n)
10
8
6
4
2
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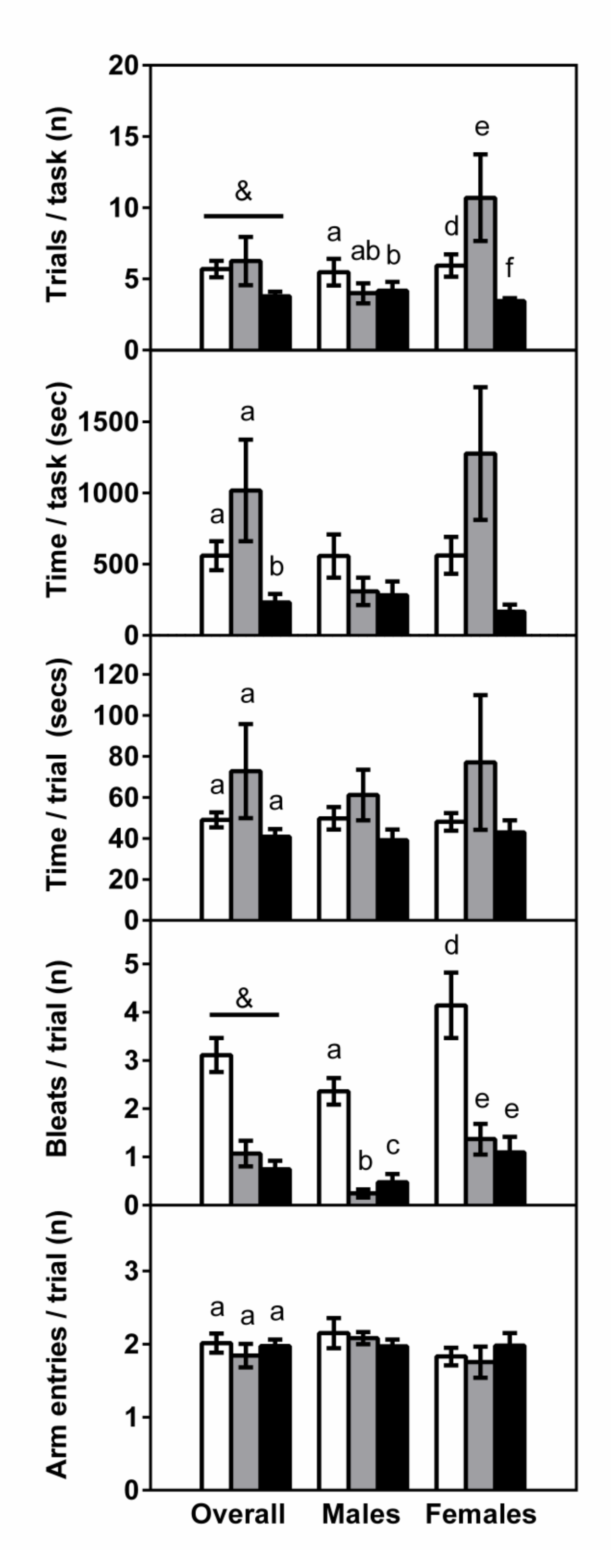
a
Time / task (sec)
800
600
400
200
0
a
a
a
Time / trial (secs)
80
60
40
20
0
a
a
a
Bleats / trial (n)
4
3
2
1
0
†
a
a
a
d
e
e
Arm entries / trial (n)
3
2
1
0
a
a
a
Overall
Males
Females

664

Figure 4


Trials / task (n)
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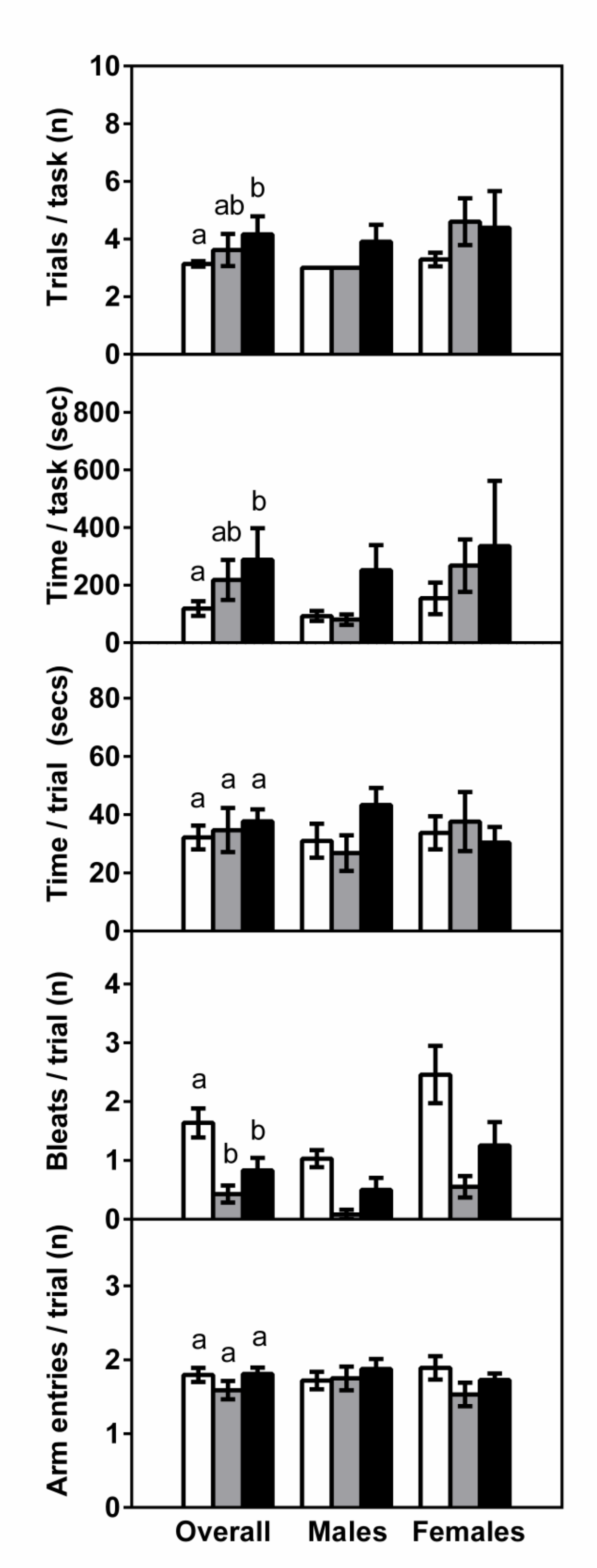
15
10
5
0
&
a
ab
b
d
e
f
Time / task (sec)
1500
1000
500
0
a
a
b
Time / trial (secs)
120
100
80
60
40
20
0
a
a
a
Bleats / trial (n)
5
4
3
2
1
0
&
a
b
c
d
e
e
Arm entries / trial (n)
3
2
1
0
a
a
a
Overall
Males
Females

Figure 5


Trials / task (n)
10
8
6
4
2
0
a
ab
b
Time / task (sec)
800
600
400
200
0
a
ab
b
Time / trial (secs)
80
60
40
20
0
a
a
a
Bleats / trial (n)
4
3
2
1
0
a
b
b
Arm entries / trial (n)
3
2
1
0
a
a
a
Overall
Males
Females

671 **Figure 6**

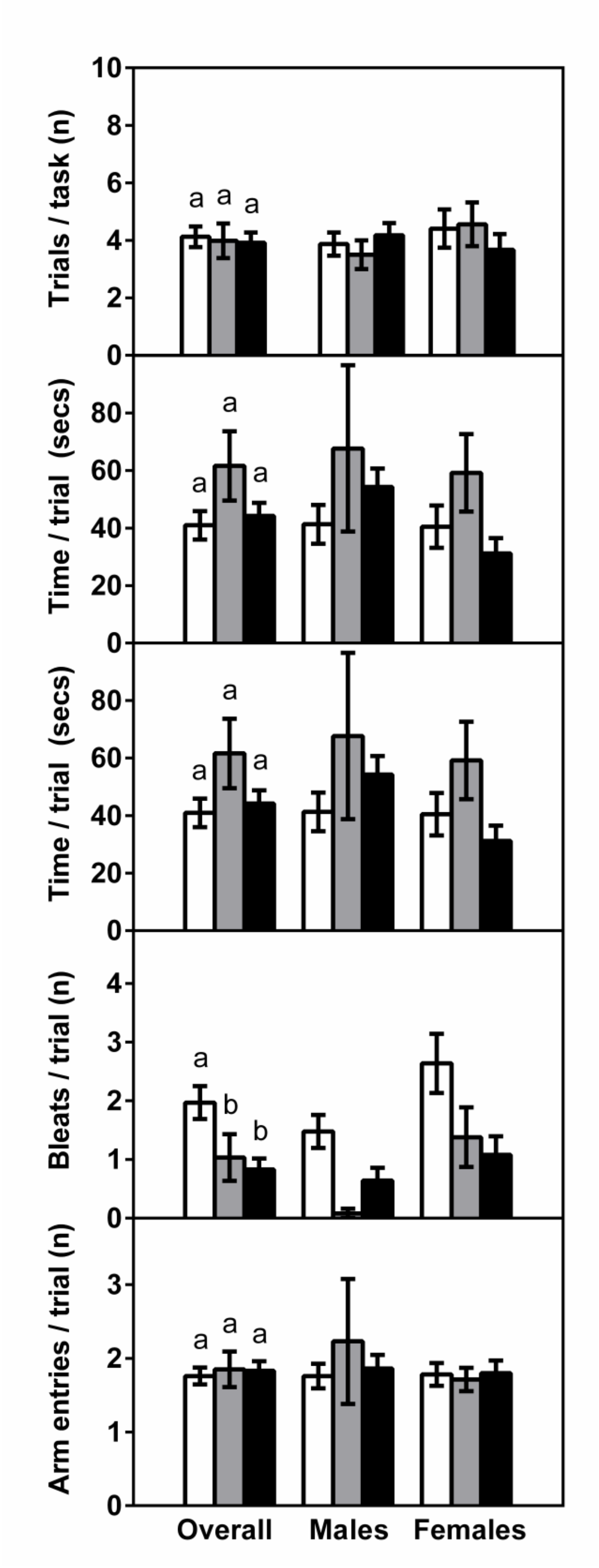


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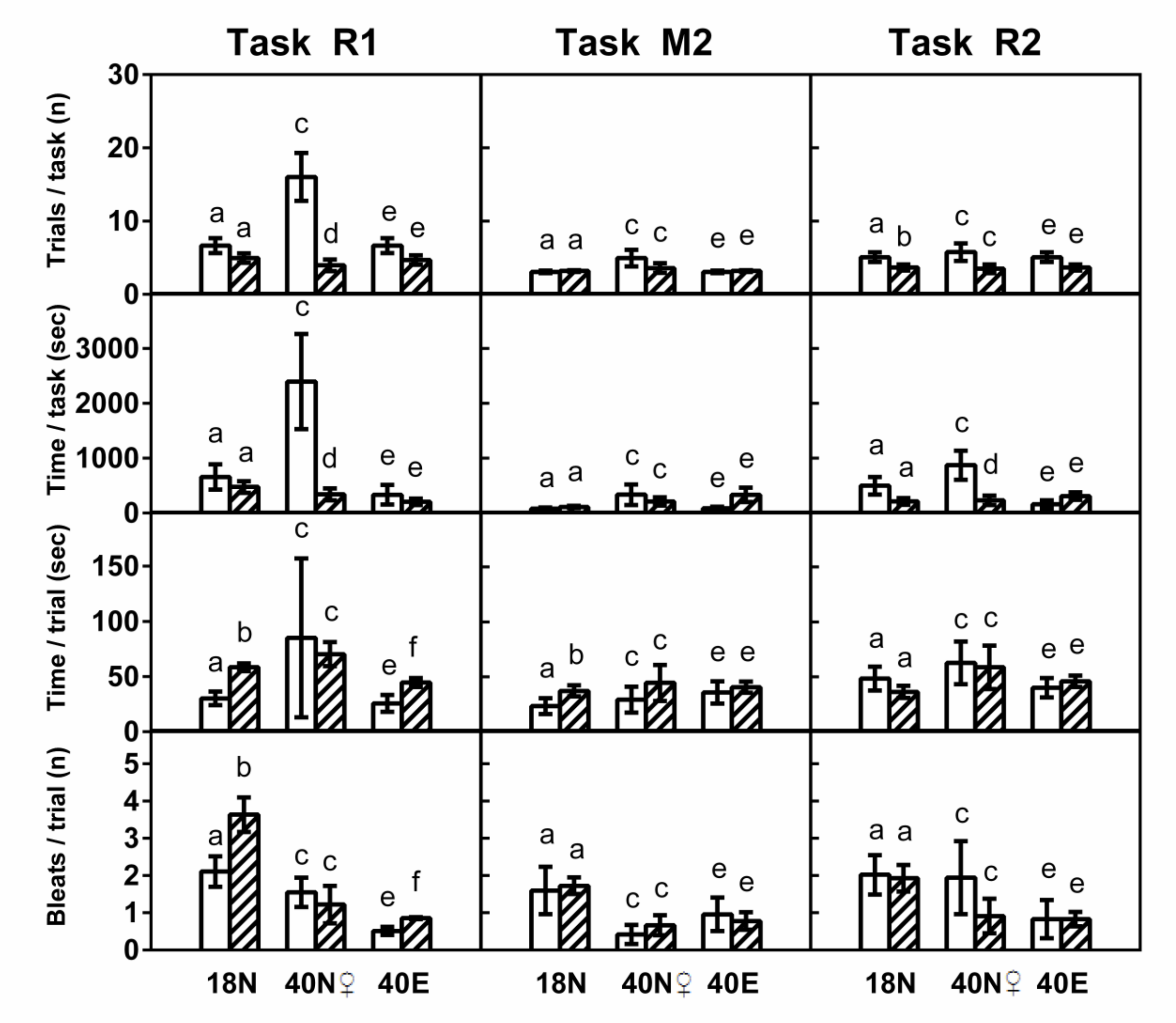
Figure 7



Task R1
Task M2
Task R2
Trials / task (n)
Time / task (sec)
Time / trial (sec)
Bleats / trial (n)
0
10
20
30
1000
2000
3000
50
100
150
1
2
3
4
5
a
b
c
d
e
f
18N
40N♀
40E