Sex impacts pain behaviour but not emotional reactivity of lambs following ring tail-docking

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Abstract

Studies in humans have shown sex differences in response to painful events, however, little is
known in relation to sex differences in sheep. Understanding sex differences would enable
improved experimental design and interpretation of studies of painful procedures in sheep. To
examine sex differences in response to pain, 80 lambs were tested across 5 cohorts of 16. The
lambs were penned in groups containing 2 male and 2 female lambs with their respective
mothers. Lambs were randomly allocated from within each block to one of four treatment
groups; FRing - Female lamb, ring tail docked without analgesia, MRing - Male lamb, ring tail
docked without analgesia, FSham – Female lamb, tail manipulated and MSham - Male lamb, tail
manipulated. Following treatment, lambs were returned to their pen and were video recorded for
45 mins for behavioural observations of acute pain and posture. An hour after treatment, lambs
then underwent an emotional reactivity test that consisted of three phases: Isolation, Novelty and
Startle. Following treatment, Ring lambs displayed more abnormal postures (mean = 2.5 ± 0.5)
compared to Sham lambs (mean = 0.05 ± 0.4, P = 0.0001). There was an effect of sex on the
display of acute pain-related behaviours in lambs that were tail docked (P < 0.001), with female
lambs displaying more acute behaviours (mean count = +2.2). This difference in behaviour
between sexes was not observed in Sham lambs. There was no effect of sex on display of
postures related to pain (P = 0.99). During the Novelty and Startle phase of the emotional
reactivity test, Ring lambs tended to (P = 0.084) or did (P = 0.018) show more fear related
behaviours, respectively. However, no effect of sex was observed. The results of this study
indicate that a pain state may alter the emotional response of lambs to novel objects and potential
fearful situations. It was also demonstrated that female lambs display increased sensitivity to the
acute pain caused by tail docking compared to males.

Introduction

Every year millions of lambs undergo a variety of painful husbandry procedures, such as
castration and tail docking, for ease of management and to reduce the risk of fly strike (French et
al. 1994). Currently, producers in Australia have access to pain-relief that can be applied at the
time of tail-docking that ameliorates the acute pain phase of the procedure (Small et al. 2021),
although pain-relief use is not mandated in Australia. There is growing evidence that the acute
pain of these procedures may manifest into chronic pain, especially when pain relief is not given.
(Johnston et al. 2022; Viñuela-Fernández et al. 2007). This has implications for animals kept in
the production system long term (such as ewes and breeding rams). Australia's sheep industry
contains a larger proportion of ewes, compared to wethers and rams, as part of self-replacement
flocks for meat and or wool production (East & Foreman 2011). There are extensive studies that
look at pain impact and pain-relief research related to the castration of male lambs (Bonelli et al.
2008; Clark et al. 2011; Colditz et al. 2012; Jongman et al. 2000; Kells et al. 2020) as well as
work looking at tail docking in male and female lambs (Small et al. 2020). There is little
comparison on the effect of these procedures between male and female lambs.

79   Previous pain research in young lambs (under 12 days of age) observed age differences in the
80   response to pain that was dependant on the sex of the lamb. In the study by Guesgen et al. (2011)
81   there was no observed difference between males and females in the response to thermal
82   nociception between the ages of 1 to 12 days. However, there was a decrease in sensitivity to
83   thermal nociception in male lambs from 1 to 12 days of age (Guesgen et al. 2011). Sensitivity to
84   thermal nociception increased in the female lambs over time, however this observation between
85   age 1 and 12 days was not significant. It is often reported that females have a higher sensitivity
86   to pain, however males have been shown to be more responsive to analgesic treatments (Mogil
87   2020). In some studies, differences were only observed in one sex when comparing the effect of
88   analgesia against a before-analgesia or no-analgesia control group (Mogil 2020).
89
90   As a prey species, sheep have evolved to protect themselves from predators and increase their
91   chance of survival by showing minimal responses to pain (Gougoulis et al. 2010). This makes it
92   challenging to assess the level of pain lambs may be experiencing following tail docking,
93   particularly after the first hour as the display of acute behavioural indicators of pain reduces
94   significantly (Molony et al. 2002). Specific behaviours have been validated as indicators of acute
95   pain in lambs, including active pain avoidance behaviours, abnormal body postures and grimace
96   scales (Grant 2004; Guesgen et al. 2016; Kent et al. 1998; Marini et al. 2021; Molony et al.
97   2002). However, these behavioural assessments still do not provide a direct measure of pain, nor
98   the emotional state of the animal. Pain is an individual emotional experience and measures of
99   affective state may complement the observation of pain-specific behaviours, to determine how
100   that animal perceives a painful experience. Emotional reactivity tests are often used to determine
101   an animal's affective state, particularly after experiencing a stressor (Doyle et al. 2011; Grillon
102   2008; Marchewka et al. 2016). Measures of attention bias can also indicate negative affect
103   (Monk et al. 2020) and it has been reported that castrated lambs are more likely to show attention
104   to the surrounding environment than non-castrated lambs (Cho et al. 2020). The use of an
105   attention bias or reactivity test in lambs following tail docking may give some insight into the
106   emotional state of lambs in pain. Such insight could provide knowledge on their experience of
107   pain in relation to duration and intensity resulting in improved assessment of pain and pain relief
108   treatments. Emotional reactivity and response to stress has also been reported to differ in sheep
109   depending on their sex and breed (Vandenheede & Bouissou 1993) and therefore we might
110   expect to see variation in response between male and female lambs.
111
112   The aim of the current study was to assess sex differences in pain using behavioural responses
113   and an emotional reactivity test as a measure of emotional state. Two hypotheses were tested.
114   The first: Female lambs undergoing tail docking would be more sensitive to the pain than male
115   lambs. Increased sensitivity to pain would constitute displaying an increase in known
116   behavioural indicators of pain following ring tail-docking. The second hypothesis: Female lambs
117   in pain would show a higher state of emotional reactivity in the test compared to male lambs in

118 pain and to lambs not in pain. A higher state of emotional reactivity would constitute an
119 increased display in fearful behaviours and a decrease in exploratory behaviours.
120

Materials & Methods

122 All animal experimentation and modifications to protocol were approved by the CSIRO
123 Armidale animal ethics committee under the NSW Animal Research Act, 1985 (ref ARA 20/18).
124 The study used a blinded controlled randomized block design, incorporating detailed individual
125 behavioural pain indicators and emotional reactivity testing. The experimental unit was the
126 individual animal. A pilot study was conducted to establish a protocol for attention bias testing in
127 young lambs as a measure of emotional state (Lee et al. 2016; Monk et al. 2018), which was
128 ultimately replaced by an emotional reactivity test in the main study (Erhard et al. 2004).
129

Animals

131 In total, 96 single-born Merino lambs between 6 - 10 weeks of age were used for this study (pilot
132 and main study), with an average body weight of 15.87 ± 0.70 kg (average $\pm$ SEM) at the time of
133 testing. All animals were obtained from the CSIRO Chiswick farm, Armidale, New South Wales,
134 Australia. When not part of the experimental protocol ewe-lamb pairs were kept as a flock in a
135 paddock assigned by the farm manager and managed under standard husbandry practice. When
136 the lambs were required, they were transferred with their mothers into an animal housing facility
137 in 6 cohorts of 16 ewe-lamb pairs/cohort. The first cohort was used in the pilot study, while the
138 other 5 cohorts were used in the main study. Lambs were penned in groups containing 2 male
139 and 2 female lambs with their respective mothers. Grouping was based on bodyweight rankings,
140 with pen allocation assigned sequentially by bodyweight within lamb sex. Lambs were marked
141 with symbols applied to the wool using coloured spray (Colourflow, Heiniger Australia, Western
142 Australia) for identification for observation of behaviour through video recording Identification
143 marks were randomised across treatments within a pen. Pen-side health inspections were carried
144 out, assessing movement and demeanour, immediately prior to entry into the animal housing
145 facility, to confirm acceptance into the trial. Each ewe-lamb pair was fed 800 g sheep pellets and
146 200 g chaff daily, water was provided ad libitum. Animals were kept in the animal housing
147 facility for a maximum of 7 days. After all lambs had completed the reactivity test the research
148 trial was concluded. Male lambs were then castrated and Sham lambs were tail docked with the
149 Numnuts® device (Senesino, Australia) with NumOcaine™ (1.5ml Lignocaine, Senesino,
150 Australia) and all lambs received Buccalgesic OTM (10mg/ml Meloxicam, Troy Laboratories,
151 Australia) as per standard husbandry and were returned to the CSIRO Chiswick farm.
152

Emotional reactivity test arena and equipment

154 Attention bias and emotional reactivity testing for the pilot trial and the main experiment
155 occurred in a 2.5 x 2.5 m arena with 2 m high opaque walls, located within the animal house.
156 The arena contained a food bowl positioned between removable clear PVC barriers, to block
157 physical access to the bowl from the side without blocking vision (Figure 1). On the opposite

158   wall to the food bowl was the location of the negative stimulus. Lambs entered the arena from a
159   guillotine door on the left-hand side of the arena.

160

161   Lambs were video recorded during habituation and behavioural testing using GoPro cameras
162   (Hero5, GoPro, USA) mounted on the rafters above the test arena as well as a camera mounted
163   inside a shelf located at lamb height in the left-hand corner between the guillotine door and
164   negative stimulus location. Identical shelves were located at the same height in the remaining
165   corners of the arena.

166

167   **Pilot study**
168   A total of 16 ewe-lamb pairs were used in the pilot study. In the first week of housing, lambs
169   were habituated to the testing arena with no negative stimulus present, to become familiar with
170   and consume feed within the arena. Habituation was conducted daily and involved removing
171   lambs from their pens and moving them to the test arena (2.5 x 2.5 m), for up to 30 min before
172   being returned to their ewes. Inside the arena, lambs were presented with four brightly coloured
173   bowls that contained feed pellets. The bowls were positioned between removable clear PVC
174   barriers. Across the week, lambs underwent three habituation sessions of up to 30 mins in groups
175   of four. Lambs then underwent two sessions of up to 30 mins in randomly assigned pairs
176   (whichever lambs were caught first). Finally, the lambs underwent two, five-minute habituation
177   sessions individually totalling seven habituation sessions/lamb.

178

179   After the habituation stage, a negative stimulus was trialled. All lambs underwent a baseline
180   attention bias assessment using the selected test configuration displayed in Fig. 1, with no threat
181   present. Each individual lamb was placed in the arena for 5 mins while behaviours were recorded
182   live via feed from GoPro cameras. The following day, the arena was arranged so that a
183   photograph of a dog was positioned on the wall opposite the positive food stimulus at the
184   beginning of the test. An individual lamb entered the arena, and the following behaviours were
185   recorded to indicate a fearful response to the threatening stimulus compared to baseline
186   behaviour: duration looking at the dog (s), freezing responses (yes/no), frequency of entering the
187   zones near the dog's location, number of high and low vocalisations, latency to eat and quantity
188   of food eaten (g). It was found that the photograph was inadequate as a negative stimulus for the
189   first 5 lambs. Subsequent lambs were therefore tested with a live dog used as a threatening
190   stimulus. The dog's location was that of the photograph and the same behaviours indicating a
191   fearful response were recorded.

192

193   During the test with a threat present, lambs did not consistently direct their attention towards the
194   photograph of a dog or the live dog. Lambs also did not consistently display a fear response
195   towards the photograph of a dog or to the live dog. It therefore appeared that the dog was not an
196   innately threatening stimulus for the young lambs used in the pilot study. The proposed method
197   used in the pilot trial for assessing attention bias was determined not to be suitable for use with

198 young lambs and an alternative test of emotional reactivity including an alternative assessment of
199 attention bias (see *Emotional reactivity test*) was used for the main study (Erhard et al. 2004).
200

Main study

202 The study was conducted across 5 cohorts of 16 lambs per cohort. Within each cohort, the lambs
203 were ranked according to weight, sequentially blocked into blocks of four. Weighted blocks were
204 rotated between pens each week. Lambs were randomly allocated, by picking coloured marbles
205 from an opaque bag, from within each block to each treatment;
206

207 1) FRing - Female lamb, tail docked using standard elastrator pliers without analgesia
208 2) MRing - Male lamb, tail docked using standard elastrator pliers without analgesia
209 3) FSham – Female lamb, tail manipulated to mimic ring tail docking
210 4) MSham - Male lamb, tail manipulated to mimic ring tail docking
211

212 This provided 20 replicates per treatment, which would allow us to define the probability
213 distribution around the mean counts with reasonable confidence to compare those 95% CI
214 brackets. The number of animals required per group was based on a sample size determination
215 calculation, using anticipated mean and standard deviation values obtained from previous tail
216 docking and attention bias studies.
217

218 During treatment application, lambs were picked at random (whichever lamb was caught first).
219 All lambs were placed on their back in a lamb marking cradle for treatment application. Ring
220 lambs had an elastrator ring applied to the tail at the level of the third palpable joint according to
221 standard industry procedures (Lloyd & Playford 2013). After treatment, lambs were placed back
222 in their pen for behavioural observation. During the main study lambs were not given analgesia
223 as the aim was to compare response to pain between male and female lambs and to measure the
224 emotional reactivity of lambs in pain. These methods comply with The Australian Animal
225 Welfare Standards and Guidelines for Sheep (AHA 2014). Sham lambs were placed in the lamb
226 marking cradle and had their tail manipulated as though a ring were to be applied, but no ring
227 was applied. All animals received analgesia (see *Animals*) following the end of the experiment as
228 per the CSIRO Chiswick farm's standard husbandry procedures before being returned to the
229 paddock.
230

Behavioural assessment

232 Video cameras were used to continuously record the behaviour of lambs in the study. For each
233 pen, one camera (Dahua IR Dome 4 MP network camera: DH-IPC-HDBW2431RP-ZAS-27135-
234 S2, Zhejiang Dahua Vision Technology Co., Ltd, Hangzhou, China) was mounted on roofing
235 rafters at each end of the pen. Each camera provided a view of the entire area available to the
236 lambs. The cameras were connected to digital video recorders and captured by SmartGuard
237 software from Pacific Communications (PACOM), Macquarie Park, NSW, Australia. The

238 assessment of the behaviour post-treatment focused on the acute pain related behaviours and
239 postural behaviours associated with ring tail docking. The pain related behaviour assessment was
240 conducted from video footage, the person assessing behaviour was a female research officer with
241 previous training and experience in assessment of pain-related behaviours and was blinded to
242 lamb treatment application. Assessment took place for one full minute at five-minute intervals
243 for the first 45 mins post-procedure, according to the ethogram shown in Table 1. Postural
244 behaviours recorded were normal upright, abnormal upright, unknown upright, normal lying,
245 abnormal lying, and unknown lying (Table 1). The most prominent posture displayed in the one-
246 minute observation period was recorded. Acute pain behaviour was assessed every five minutes
247 for one full minute and a count of all behaviours observed in that minute was taken. The
248 ethogram used in this study was based on validated behaviour patterns (Molony et al. 2002)
249 described in previous studies (Grant 2004; Kent et al. 1998; Marini et al. 2021) of behavioural
250 responses of lambs following ring tail docking. Grimace scales were not applied due to their
251 limited validity in lambs (Evangelista et al. 2022) and the woolly faces of the Merino lambs used
252 preventing accurate classifications.
253
254 **Emotional reactivity test**
255 Following induction to the animal housing facility, lambs and ewes were given one day to
256 habituate to the pens without additional disturbance. During the following two days lambs were
257 habituated to the emotional reactivity test arena in groups of four. Lambs were kept in the arena
258 for ten mins, twice daily, to habituate them to the novel environment and brightly coloured feed
259 bowls containing feed pellets.
260
261 One hour after treatment, lambs were individually moved into the test arena, in their treatment
262 order, for behavioural testing. The behavioural test was divided into several consecutive phases:
263 'Isolation', 'Novelty', 'Startle'. For the Isolation phase, individual lambs were moved into the
264 arena; lambs were tested for 90 s without any threat present. After 90 s of isolation had passed, a
265 novel object (brightly coloured umbrella) was introduced into the arena to begin the Novelty
266 phase. The closed umbrella was placed through a hole in the arena on the opposite side to the
267 food bowl location (Fig. 2), and held there until the lamb approached within approximately 20
268 cm. Once the lamb approached the closed umbrella, the umbrella was opened, the Novelty phase
269 was ceased, and the next phase began. If the lamb didn't approach the umbrella within 3 mins,
270 the test was ceased, and the lamb was removed from the arena and returned to its pen.
271
272 If the tested lamb approached the closed umbrella and the umbrella opened, the Startle phase of
273 the test began immediately. The lambs' immediate startle responses were recorded, then the lamb
274 was kept in the arena with the open umbrella for 90 s to assess their behaviour as a potential
275 measure of attention bias. During this time, lambs were recorded for their latency to eat as well
276 as attention towards the umbrella, assessed as duration looking towards the umbrella within the
277 field of binocular vision. After 90 s the lamb was removed from the arena and returned to its pen.

278

279 Throughout all phases of the emotional reactivity test, the lamb's behaviour was recorded using
280 GoPro cameras as described in section 2.2. Footage from the cameras was used to undertake
281 behaviour assessments using The Observer XT software (Noldus, The Netherlands). The footage
282 for two lambs were missing due to technical errors (one Ring Male and one Ring Female). Data
283 from these animals were excluded from the analysis of emotional reactivity. The person
284 performing the video observations post-treatment was blinded to treatment. Live pen side
285 observations were also recorded for latency to eat and number of bleats during each phase of the
286 test, as well as latency to approach the umbrella. The behaviours recorded during each phase of
287 testing are defined in Table 2.

288

289 **Statistics**
290 Statistical analysis was conducted using R and R studio software (R version 4.0.2 "Taking Off
291 Again") (Team 2022). Data were tested for normality through visual inspection of residual plots
292 and the Shapiro-Wilks test. A P-value of < 0.05 was considered statistically significant and $0.1 >$
293 $P \geq 0.05$ was considered a statistical tendency. Data are presented as means ± standard error of
294 the mean unless otherwise stated. There were no data exclusions.

295

296 **Pain behaviours**
297 The packages *lme4* (Bates et al. 2015)*, ggpubr* (Kassambara & Kassambara 2020)*, dplyr*
298 (Wickham et al. 2019) and *car* (Fox & Weisberg 2018) were used. The frequency of occurrence
299 for individual postures was too low for data analysis, therefore categories were combined as
300 upright (normal upright, abnormal upright, unknown upright), lying (normal lying, abnormal
301 lying, unknown lying) and abnormal postures (abnormal upright, abnormal lying). Lying and
302 abnormal postural behaviours were analysed using a non-linear mixed effects model, fixed
303 factors included Treatment ($n = 20$ per group), Sex, Cohort, Pen and the interaction of Treatment
304 x Sex, with Lamb included as a random effect. Sum of all abnormal postures was not normally
305 distributed and was analysed using a Generalized linear mixed model with Poisson distribution,
306 fixed effects and the random effect fitted were the same as other postures. The acute pain
307 behaviour categories were combined for analysis. Acute pain behaviour counts were analysed
308 using a Generalized linear mixed model with a Quasi-Poisson distribution due to over dispersal.
309 Fixed effects included Treatment ($n = 20$ per group), Sex, Cohort, Pen and Time as well as the
310 interaction of Treatment x Sex, with lamb included as a random effect.

311

312 **Emotional reactivity**
313 Statistical analyses were divided by test phase. All animals completed the Isolation test phase
314 and were given an opportunity to approach the umbrella in the Novelty phase. Latency to
315 approach the umbrella was analysed using a Cox proportional hazards model with the *survival*
316 (Therneau 2022; Therneau & Grambsch 2000) package, fitting sex and treatment as fixed effects.
317 Latencies were deemed as censored results if the lamb failed to approach the umbrella within

318   180 s. Reactions to the closed umbrella entering the test arena and to the umbrella opening were
319   analysed using Fisher's Exact Tests. The remaining Novelty test data analysis included only
320   those that did not approach the umbrella within 180 s ($n = 30$), while only lambs that approached
321   the umbrella moved on to the Startle phase ($n = 48$). Reactions to the umbrella opening in the
322   Startle phase were analysed using Fisher's Exact Tests.
323
324   Principal components analysis (PCA) was used to condense correlated behavioural measures
325   within each phase of testing into their principal components, using the *psych* (Revelle 2022)
326   package. Variables were normalised using the scale function in R, then the scaled variables were
327   checked for multicollinearity by analysing the correlation matrix. Variables with any correlations
328   above 0.90, or with most correlations below 0.30, were excluded from the analysis (Field et al.
329   2012). Sampling adequacy was confirmed by conducting Bartlett's sphericity tests (P<0.05) and
330   calculating Kaiser-Meyer-Olkin (KMO) measures of sampling adequacy. The KMO statistic
331   varies between 0 and 1, where a value close to 1 is considered ideal, while variables with a score
332   < 0.5 were deemed unacceptable (Field et al. 2012). Factors included in the analyses were
333   subjected to varimax rotation. The number of rotated components used in further analyses were
334   selected based on the number needed to explain more than 75% of the total variance and by
335   checking the PCA model residuals (less than half with absolute values > 0.05), fit (> 0.9) and
336   communality (all communalities > 0.7).
337
338   The selected rotated components within each test phase were then used to determine the effect of
339   sex and treatment on the independent dimensions underlying the lambs' behavioural reactivity.
340   The rotated components were analysed using linear mixed effects models with the package *nlme*
341   (Pinheiro & Bates 2022; Pinheiro & Bates 2000), fitting fixed effects of Treatment, Sex, Cohort,
342   Pen and the interaction of Treatment x Sex and a random effect of Lamb (Field et al. 2012). All
343   of the rotated components for the isolation phase were transformed to maintain the assumption of
344   normality of the model residuals. A log transformation was applied after adding an integer of 2.7,
345   2.6 and 2.3 for the 3 rotated components respectively, such that the minimum value for each
346   rotated component was ≈ 1. The second rotated component for the novelty phase and the third
347   rotated component for the startle phase were log transformed to ensure normality of model
348   residuals after adding integers of 2.4 and 2.1 respectively. Interactions and fixed factors were
349   removed from the models using a backward elimination approach, considering the lowest Akaike
350   Information Criterion (AIC) as an indicator of model fit.
351

352   **Results**
353   **Pain behaviours**
354   Only the results of mean sum of abnormal postural behaviour and lying behaviour are presented,
355   as upright behaviour is the opposite of lying and all abnormal postures are mutually exclusive.
356   For lying behaviours there was a treatment ($F_{1,69} = 19.0$), cohort ($F_{4,69} = 7.2$), and pen ($F_{3,69} = $
357   3.7) effect observed ($P < 0.05$), but no effect of the interaction of treatment x sex ($F_{1,69} = 0.5$, P =

358   0.47). Lambs in the Sham group were observed lying fewer times than the Ring lambs (Figure

359   3). Lambs in cohort 2 were observed lying more than cohort 1 (t_{69} = 2.3, Mean count = 5.2 ± 0.5

360   vs 4 ± 0.5 respectively, P = 0.02). Cohort 5 lambs lay down fewer times than cohort 1 (t_{69} = -2.8,

361   Mean count = 2.5 ± 0.5 vs 4 ± 0.5 respectively, P = 0.005).

362

363   Ring lambs displayed more observed abnormal postures compared to Sham lambs (z = -3.8, P =

364   .0001, Figure 4). Each subsequent cohort (2 to 5) after cohort 1 either tended to display or

365   displayed more abnormal postures following tail-docking. Cohort 2 and 3 displayed an additional

366   mean of 1 ± 0.4 observed abnormal postures (z = 2.8 and 2.4, P < 0.05), and cohort 4 and 5

367   displayed an additional mean of 0.7 ± 0.4 (z = 1.7 and 1.8, P < 0.08), observed abnormal

368   postures compared to cohort 1 mean count of 0.5 ± 0.3. Pen influenced the behaviour display of

369   lambs with animals in pen 2 and 3 displaying fewer observed abnormal postures compared to

370   pen one, with a decrease in mean count of -0.6 ± 0.2 (z = -2.2, P = 0.02) and -0.7 ± 0.3 (z = -2.4,

371   P = 0.01) respectively.

372

373   Acute pain behaviours were assessed every 5 mins for the 45-min period following treatment.

374   There was an effect of cohort ($\chi^2(4)$ = 119.3, P < 0.001), pen ($\chi^2(3)$ = 7.9, P < 0.05) and time

375   ($\chi^2(8)$ = 678.3, P < 0.001) as well as sex ($\chi^2(1)$ = 54.2, P < 0.001) and treatment ($\chi^2(1)$ = 1450.3,

376   P < 0.001) and an effect of their interaction ($\chi^2(4)$ = 119.3, P < 0.05). There was a difference in

377   overall mean count of acute behaviours displayed between male and female lambs that were tail

378   docked (t = -4.9, P < 0.001, Fig. 5). Lambs in the Sham group displayed significantly fewer acute

379   behaviours than the tail docked lambs (t = -7.2, P < 0.001). There were no differences in

380   behaviour seen between sham male and female lambs (P = 0.5).

381

382   **Emotional reactivity**

383   **Isolation phase**

384   In the Isolation test phase (*n* = 78), six variables fit the criteria for inclusion in a PCA. Sampling

385   adequacy was verified as 'good' by the overall KMO (0.73) and values for individual variables

386   ranging from 0.5 to 0.82. Bartlett's test of sphericity indicated that correlations between items

387   were sufficiently large to conduct a PCA ($\chi^2(15)$ = 259, P < 0.001). Three components were

388   retained for further analysis. Table 3 shows the factor loadings on each variable after rotation.

389   Based on the items that cluster on each component, we labelled component 1 as fearful

390   responses, component 2 as exploration and component 3 as a social response for easier

391   interpretation, however other interpretations of these behaviour profiles should also be

392   considered. For all 3 principal components, linear mixed effects models indicated no significant

393   effect of treatment, sex or their interaction on behavioural reactivity. The AIC supported

394   inclusion of cohort only in all models.

395

396   **Novelty phase**

397 For latency to approach the umbrella ($n = 78$), the Cox proportional hazards model fitting
398 treatment and sex as fixed effects was not significant (Likelihood ratio test = 3.38, df = 2, P =
399 0.2). There was no effect of treatment group on reaction to the closed umbrella entering the arena
400 (number of lambs with a High reaction in FRing = 7, MRing = 5, FSham = 7, MSham = 5;
401 Fisher's Exact Test, P = 0.905).
402
403 For the Novelty phase including only animals that failed to approach the umbrella ($n = 30$), five
404 variables fit the criteria for inclusion in a PCA. Sampling adequacy was verified as 'good' by the
405 overall KMO (0.71) and values for individual variables ranging from 0.5 to 0.86. Bartlett's test
406 of sphericity indicated that correlations between items were sufficiently large to conduct a PCA
407 ($\chi^2(10) = 80$, P < 0.001). Three components were retained for further analysis. Table 4 shows the
408 factor loadings on each variable after rotation. Based on the items that cluster on each
409 component, we labelled component 1 as high activity fearful responses, component 2 as
410 exploration and component 3 as an active response.
411
412 For the first rotated component, the AIC supported inclusion of a treatment by sex interaction in
413 the model, however the interaction term was not significant ($\chi^2(1) = 2.3$, P = 0.13). There tended
414 to be a treatment effect ($\chi^2(1) = 3.0$, P = 0.084), whereby Ring animals scored higher on the
415 'fearful' index than Sham animals (Figure 6; Least squares means 0.30 ± 0.26 and -0.38 ± 0.26
416 respectively, averaging over the levels of sex). For the second rotated component, the AIC
417 supported inclusion of sex in the model only, for which there tended to be a sex effect ($\chi^2(1) =$
418 3.7, P = 0.056), whereby Female animals scored higher on the 'explorative' index than Male
419 animals (Least square means 0.96 ± 0.12 and 0.67 ± 0.10 respectively). For the third rotated
420 component, the AIC supported the null model, with no significant effect of treatment, sex or the
421 treatment by sex interaction.
422
423 **Startle phase**
424 For the Startle phase including only animals that approached the umbrella ($n = 48$), there was no
425 effect of treatment group on reaction to the umbrella opening (number of lambs with a High
426 reaction in FRing = 3, MRing = 7, FSham = 6, MSham = 4; Fisher's Exact Test, P = 0.564) or
427 jump response to the umbrella opening (number of lambs that jumped in FRing = 5, MRing = 3,
428 FSham = 4 MSham = 2; Fishers Exact Test, P = 0.777). Five variables fit the criteria for
429 inclusion in a PCA. Sampling adequacy was considered mediocre, with an overall KMO of 0.61
430 and values for individual variables ranging from 0.5 to 0.64. Bartlett's test of sphericity indicated
431 the correlations between items were sufficiently large to conduct a PCA ($\chi^2(10) = 33$, P < 0.001).
432 Three components were retained for further analysis. Table 5 shows the factor loadings on each
433 variable after rotation. Based on the items that cluster on each component, component 1 was
434 labelled as fearful responses, component 2 as an attentive response and component 3 as an
435 escape response.
436

437   For the first rotated component, the AIC supported inclusion of treatment only in the model. The
438   effect of treatment was significant ($\chi^2(1) = 5.6$, $P = 0.018$), whereby Ring animals scored higher
439   on the 'fearful' index than Sham animals (Least square means 0.31 ± 0.19 and -0.33 ± 0.20
440   respectively). There was no effect of sex or the sex by treatment interaction on behavioural
441   reactivity. For the second rotated component, the AIC supported the null model, with no
442   significant effects of sex, treatment or the sex by treatment interaction. For the third rotated
443   component, the AIC supported the model fitting cohort only, with no significant effects of sex,
444   treatment or the sex by treatment interaction.
445

Discussion

447   Pain research in livestock often focuses on the impact that castration has on male animals, with
448   limited research looking at the different impacts that painful husbandry procedures may have on
449   female lambs compared to males. The aim of the current research was to examine how the sex of
450   lambs may impact their experience of pain in response to the procedure of tail-docking. During
451   the 45 minutes after ring tail-docking, female lambs that were tail-docked displayed more acute
452   pain related behaviours when compared to their male counterparts. This display of different
453   behaviours between sexes was not seen in Sham group lambs. A difference in abnormal postural
454   behaviour was also seen between Sham and Ring lambs however no sex differences were
455   observed for these behaviours. An emotional reactivity test was applied as a measure of
456   emotional state in the lambs. The Ring lambs displayed more fearful behaviours during the
457   Novelty and Startle phases of the test compared to Sham lambs, but against our expectations, no
458   sex effect was observed.
459

460   Previous research on the impact of painful husbandry procedures on lambs have included both
461   sexes without direct comparison between them (Grant 2004; Price & Nolan 2001; Shutt et al.
462   1987; Small et al. 2020). In this study, the direct comparison of the tail docking procedure
463   between the sexes found that female lambs displayed more pain avoidance behaviours in the
464   hour following tail docking compared to the male lambs, indicating a higher sensitivity to the
465   pain caused by the procedure. Lamb age is an important factor in the stress response caused by
466   pain, with sex differences not being observed in very young lambs (1 -7 days of age) (Guesgen et
467   al. 2011; Mellor & Murray 1989; Turner et al. 2006), but by 8 weeks of age female lambs'
468   cortisol response to tail docking is significantly greater than that of male lambs (Turner et al.
469   2006). Lambs in Australia commonly undergo painful husbandry procedures between 4–12
470   weeks of age, therefore, sex is likely to impact the pain response of the lambs in commercial
471   settings. This also has implications for experimental testing of new pain-relief pharmaceuticals to
472   ensure they are validated as effective for both sexes.
473

474   Lambs undergoing ring tail docking were observed lying down more often than the sham lambs,
475   which is consistent with reporting of behaviour in other studies following ring tail docking
476   (Lester et al. 1996; Molony et al. 1993; Small et al. 2020). The observation of increased lying is

477   indicative of the discomfort caused by the procedure and is specific to lambs undergoing ring
478   castration and tail docking. Lying behaviour reduces in ring castrated and tail-docked lambs
479   when they are provided pain relief, whereas surgically castrated and tail-docked lambs tend to
480   display standing behaviours as a sign of discomfort (Small et al. 2014). There is also growing
481   evidence that the pain of tail docking and castration procedures lasts beyond current
482   commercially available analgesic coverage (Mellor & Stafford 2000) and potentially beyond
483   wound healing (Johnston et al. 2022), with neuroma formation reported in docked lambs' tails
484   (French & Morgan 1992; Larrondo et al. 2019). The presence of neuromas can lead to persistent
485   pain after the tail wound is healed, however, it is difficult to assess the chronic pain that painful
486   husbandry procedures may cause in lambs (Mellema et al. 2006). The lambs tail-docked in this
487   study displayed behaviors indicative of acute pain and are therefore at risk of this pain
488   developing into chronic pain or from suffering from allodynia and hyperalgesia (Johnston et al.
489   2022; Viñuela-Fernández et al. 2007). There is also evidence of sex differences on the
490   neuroimmune mediation and subsequent chronification of pain, with females more likely to
491   develop chronic pain (as reviewed by Midavaine et al. 2021; Mogil 2020; Price & Ray 2019). As
492   well as evidence of discrepancy of analgesic efficacy between sexes, with females not
493   responding to analgesia as well as males (Mogil 2020; Price & Ray 2019). This has implications
494   for the ewes that are retained long term within the sheep industry. If female lambs are more
495   sensitive to pain and the current analgesic options available are potentially inadequate, there may
496   be ewes in the national flock suffering from undetected and untreated chronic pain, thus
497   negatively impacting their welfare. Furthermore, as female sheep reach maturity, their stress
498   responses and thus affective experience of pain may vary across their oestrus cycle (Komesaroff
499   et al. 1998), an additional factor to consider for any necessary flock pain management.
500
501   The emotional reactivity tests were able to detect some differences between the treatment groups,
502   with Ring animals more likely to show putatively fearful behaviours than Sham animals,
503   particularly during the Startle phase, but there were minimal effects of sex observed. Previous
504   tests of emotional reactivity and fear in lambs and adult sheep have found some sex differences
505   of varying magnitudes (Atkinson et al. 2022; Boissy et al. 2005; Roussel et al. 2008;
506   Vandenheede & Bouissou 1993; Viérin & Bouissou 2003). This suggests the emotional
507   reactivity of males and females does differ, but is likely to be dependent on age, breed, and the
508   exact behavioural reaction being assessed. The lack of sex or 'sex within treatment' effect in the
509   current study contrasted with the sex differences seen in the display of acute pain behaviours.
510   The females may have been experiencing more pain, but the novelty of the testing situation may
511   have been distracting, directing attention away from pain (Bushnell et al. 2013) to their new
512   surroundings. This suggestion is supported by the female's tendency to sniff their environment
513   more in the Novelty subtest compared to males.
514
515   The observation of the Ring lambs showing more behaviours associated with fearfulness than
516   Sham lambs is consistent with our predictions of greater emotional reactivity in animals

517   experiencing pain. Sheep are prey animals where their stoical behaviour can often prevent overt
518   displays of pain and distress (Gougoulis et al. 2010) but this doesn't preclude the experience of
519   pain. Emotional reactivity tests provide further insight into the affective state of the sheep, where
520   the tail-docking in this study led to greater fear-related behaviour consistently across both the
521   Novelty and Startle phases. During the Startle phase, Ring animals showed greater vigilance and
522   a longer latency to eat indicative of higher arousal and more vocalisations suggesting a negative
523   valence. A negative state while experiencing pain has also been demonstrated in laying hens
524   suffering from keel bone damage (Armstrong et al. 2020; Wei et al. 2022) and in dairy calves
525   following disbudding (Neave et al. 2013). In the Novelty phase, Ring animals tended to score
526   higher on a 'fearful' score comprising high loadings of attention to the umbrella, latency to eat
527   and vigilance. While these were interpreted as representing a fearful response, they might also be
528   considered as increased interest or curiosity in the novel object. Hypervigilance while in pain
529   may also have evolutionary advantages, where observations in squid (Crook et al. 2014) and
530   mice (Lister et al. 2020) showed that induced pain resulted in a reduced predation risk or
531   increased attention to a perceived predator threat respectively. Despite observing an effect of
532   pain on emotional reactivity, pain did not appear to impact startle or jump responses to the
533   umbrella entering the arena or opening. This contrasts with previous studies in sheep and other
534   species that have demonstrated the startle response can be potentiated by negative affect (Doyle
535   et al. 2015; Grillon 2008).

536

537   The emotional reactivity test sequence employed in this study was limited by requiring approach
538   responses from animals to proceed through the entire testing sequence. Thirty out of the 78
539   lambs did not approach the novel object (umbrella), preventing recording of responses during the
540   last phase of testing. Comparable limitations have been observed in previous applications of the
541   test sequence (Erhard et al. 2004) or similar test sequences (Roussel et al. 2008) where not all
542   individuals will complete the full testing regime restricting the dataset, and, thus, losing
543   potentially informative animals. There are many behavioural tests that have been validated for
544   measuring emotional reactivity in sheep, assessing different aspects of temperament (reviewed in
545   Dodd et al. (2012)). It is important to consider that tests eliciting the greatest fear/reactivity or
546   test situations that have additive effects (e.g., a novel object presented in a novel environment)
547   may lead to floor effects and reduced sample sizes. Additionally, prior experiences of the
548   animals occurring in close temporal association such as handling and application of treatment
549   procedures may compound and further affect specific behavioural test outcomes.

550

551   The attention bias test paradigm developed by Lee et al. (2016) presents a method for assessing
552   affective state that does not require an approach response or the meeting of learning criterion, but
553   this method was deemed unsuitable for use in young lambs during our pilot study, due to an
554   inconsistent response to the predator threat stimuli. Porter & Bouissou (1999) found that 3-4-
555   week-old lambs did not display differential avoidance of or attraction toward images of a ewe
556   versus a dog, while adult ewes responded negatively to the dog image. Young (2006) found no

557 differences in restlessness displayed by 1-6-week-old castrated lambs in the presence of a live
558 dog compared to a cardboard box or live goat. Together, these findings suggest the young lambs
559 may not have had enough experience with dogs to have made a negative association with them
560 and different threatening stimuli should be used to assess attention bias in young animals.
561 Alternatively, the Startle phase of the main study might itself be interpreted as an attention bias
562 test, where the umbrella poses a potential threat to the lambs. Duration looking toward the threat
563 is a key measure of attention bias (Crump et al. 2018) that loaded highly on the second principal
564 component, for which no treatment effect was observed. On the other hand, general fearfulness
565 was impacted by treatment in both the Startle and Novelty phases. This may indicate attention
566 bias is not impacted by pain or lamb sex, or perhaps the Startle phase may be more correctly
567 interpreted as a novel object test instead, considering that the umbrella is not innately
568 threatening.
569
570

571 ## Conclusions
572 The current study aimed to assess sex differences in pain using behavioural responses and an
573 emotional reactivity test as a measure of emotional state. Lambs that underwent tail docking
574 displayed more behavioural indicators of pain, with female lambs displaying more pain
575 avoidance behaviours than male lambs, indicating higher sensitivity. This study also
576 demonstrated that lambs in pain are experiencing a negative affective state, with more tail
577 docked lambs displaying behaviour associated with fearfulness during Novelty and Startle
578 phases of the emotional reactivity test, compared to the control lambs. However, no sex
579 differences were seen during the emotional reactivity test. Female sheep make up a majority of
580 Australia's flock and the implication of increased sensitivity to; and the potential chronification
581 of pain should be investigated.
582

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588

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782 **Figures**
783
784 Figure 1. Schematic diagram of the test arena used during the pilot trial, indicating the location of the
785 food, entry door and threat door. Camera boxes were positioned in all corners of the arena, but only the
786 bottom left corner box contained a GoPro camera (shaded grey).
787
788 Figure 2. (A) Schematic diagram and (B) photograph of the test arena used during the main study,
789 indicating the location of the food, entry door and umbrella. The photograph depicts a sheep in the Startle
790 phase of testing. Camera boxes were positioned in all corners of the arena, but only the bottom left corner
791 box (shaded grey in panel (A)) contained a GoPro camera. Lines and numbers indicate the zones (1 – 5)
792 of the arena used during behavioural assessment. Zone 5 was only counted once the umbrella was
793 introduced (novelty and startle phase)
794
795 Figure 3: Mean count of all lying behaviours observed for male and female lambs within the Sham and
796 Ring treatment groups in the 45 minutes post tail docking. There was a difference in behaviour between
797 the treatment groups (P = 0.01), there was no significant difference between sex (P = 0.12) or sex within
798 treatment (P = 0.46).
799
800 Figure 4: Raw data for mean count of all abnormal postural behaviours observed for male and female
801 lambs within the Sham and Ring treatment groups in the 45 minutes post tail docking.
802
803 Figure 5: Raw data for mean count of all acute pain behaviours observed for male and female lambs
804 within the Sham and Ring treatment groups in the 45 minutes post tail docking. The presence of the
805 asterisk indicates a significant difference within treatment.
806
807 Figure 6: Raw mean ± s.e.m. of rotated principal component scores reflecting 'fearfulness' within the
808 Novelty and Startle test phases for Sham and Ring treatment groups post tail docking. P values were
809 derived from linear mixed effects models fitting either sex, treatment and a treatment by sex interaction or
810 treatment only, for each test phase respectively.
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812
813 **Tables**
814
815 Table 1: Description of active pain avoidance behaviours and postures recorded every 5 minutes, for 1
816 minute during the 45 mins post tail-docking. The most prominent posture displayed in the one-minute
817 observation period was recorded. For acute behaviour a count of all behaviours observed in that minute
818 was taken.
819
820 Table 2: Behavioural measures recorded during the phases of the emotional reactivity test; Isolation,
821 Novelty and Startle. The phase(s) of the test in which a given behaviour was recorded is indicated by "Y".
822

823   Table 3. Summary of the principal components analysis results for the Isolation phase of behavioural
824   reactivity testing ($n = 78$). Absolute factor loadings over 0.6 are written in bold.

825   Table 4. Summary of the principal components analysis results for the Novelty phase of behavioural
826   reactivity testing, for sheep that failed to approach the umbrella ($n = 30$). Absolute factor loadings over
827   0.6 are written in bold.

828   Table 5. Summary of the principal components analysis results for the Startle phase of behavioural
829   reactivity testing, for sheep that approached the umbrella ($n = 48$). Absolute factor loadings over 0.6 are
830   written in bold.
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Figure 1

Schematic diagram of the test arena used during the pilot trial

The diagram indicates the location of the food, entry door and threat door. Camera boxes were positioned in all corners of the arena, but only the bottom left corner box contained a GoPro camera (shaded grey).

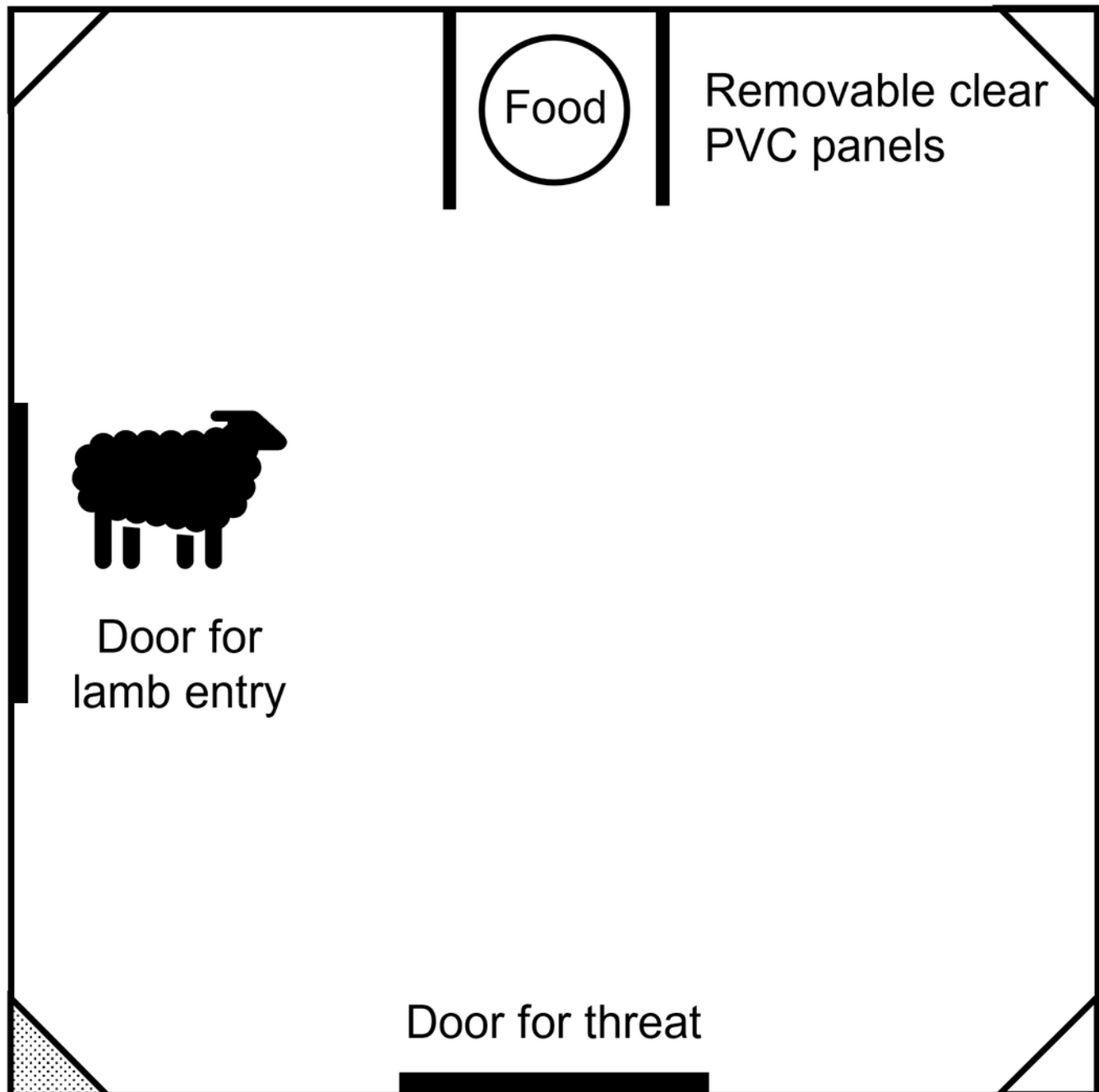



Figure 2

The final setup of the test arena for the emotional reactivity test

(A) Schematic diagram and (B) photograph of the test arena used during the main study, indicating the location of the food, entry door and umbrella. The photograph depicts a sheep in the Startle phase of testing. Camera boxes were positioned in all corners of the arena, but only the bottom left corner box (shaded grey in panel (A)) contained a GoPro camera. Lines and numbers indicate the zones (1 – 5) of the arena used during behavioural assessment. Zone 5 was only counted once the umbrella was introduced (novelty and startle phase)

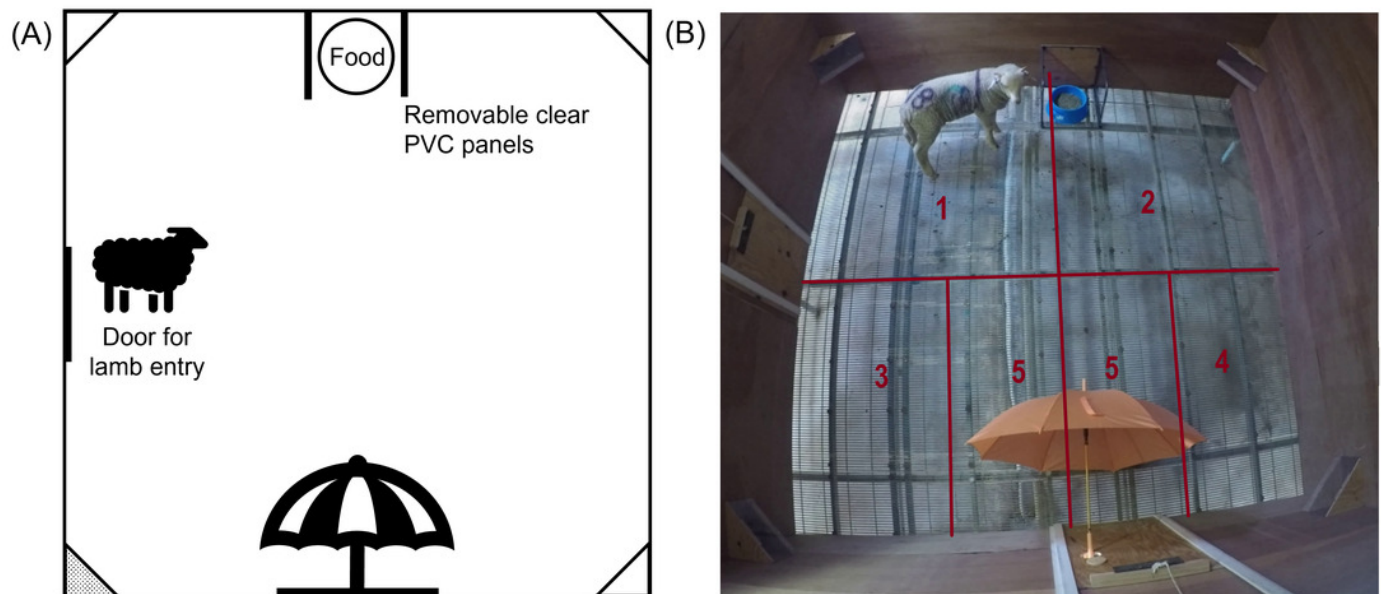



Figure 3

Mean count of all lying behaviours observed for male and female lambs within the Sham and Ring treatment groups in the 45 minutes post tail docking.

There was a difference in behaviour between the treatment groups (P = 0.01), there was no significant difference between sex (P = 0.12) or sex within treatment (P = 0.46).

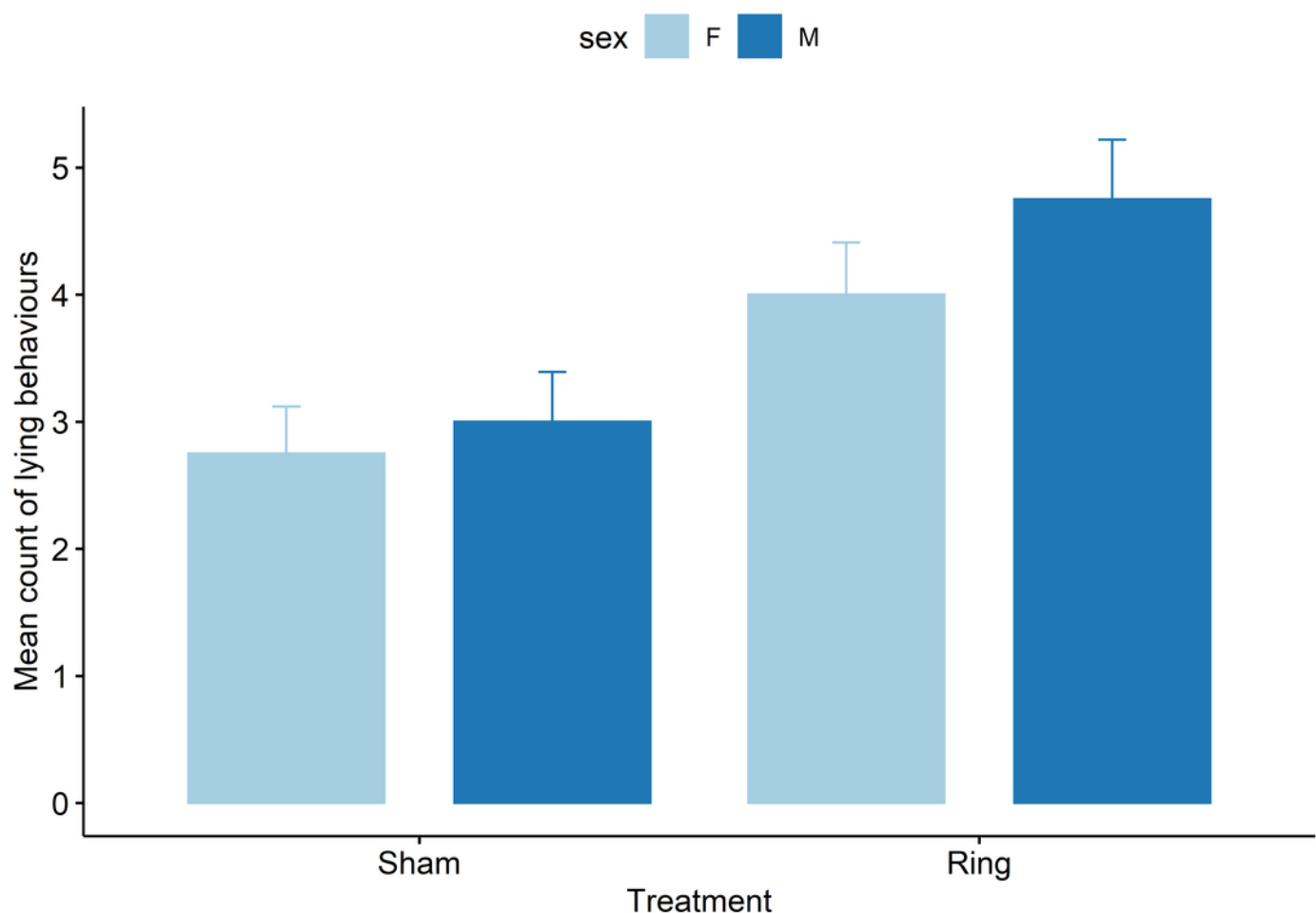



Figure 4

Raw data for mean count of all abnormal postural behaviours observed for male and female lambs within the Sham and Ring treatment groups in the 45 minutes post tail docking.

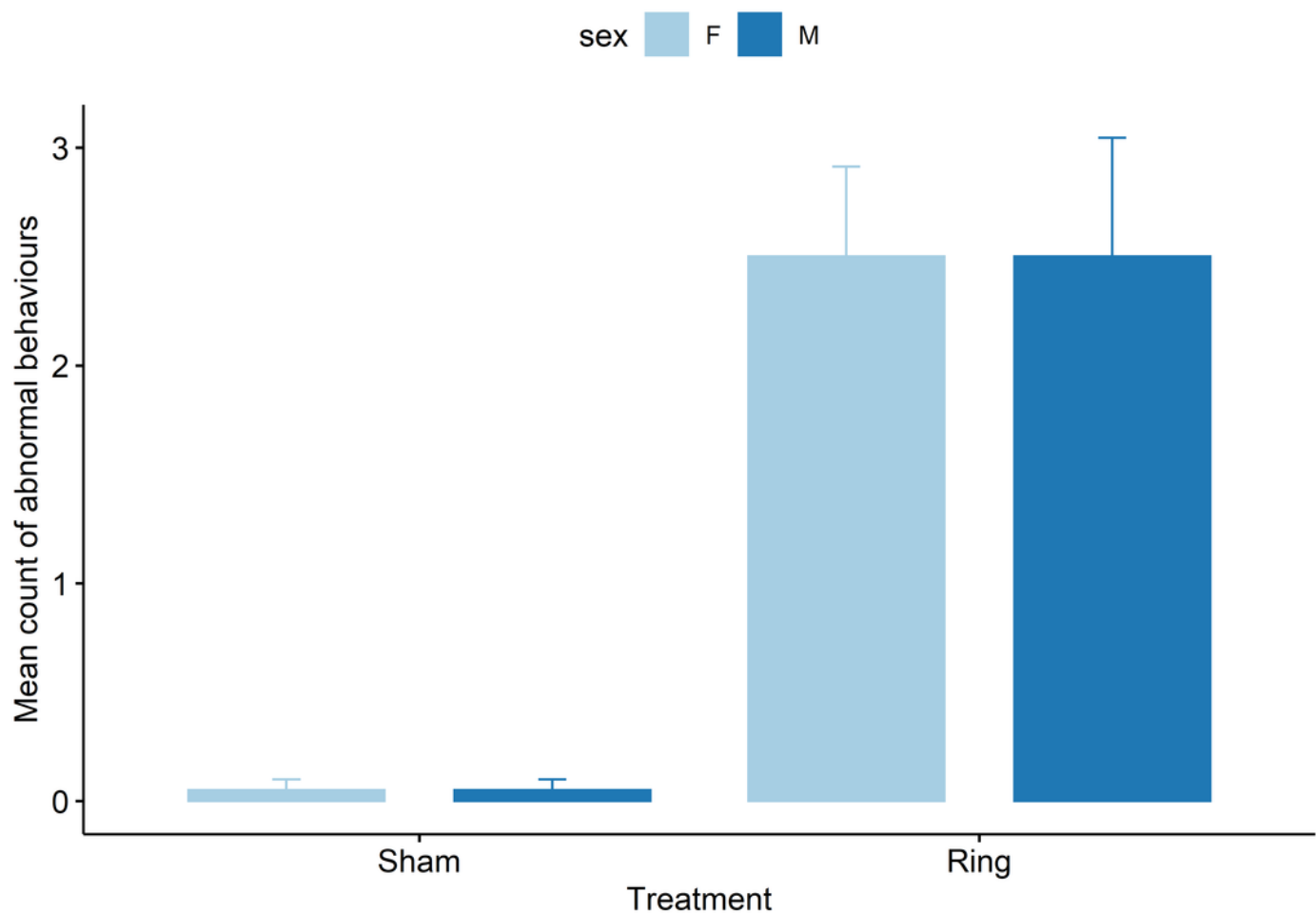



Figure 5

Raw data for mean count of all acute pain behaviours observed for male and female lambs within the Sham and Ring treatment groups in the 45 minutes post tail docking

The presence of the asterisk indicates a significant difference within treatment. P values were derived from the Generalized linear mixed model

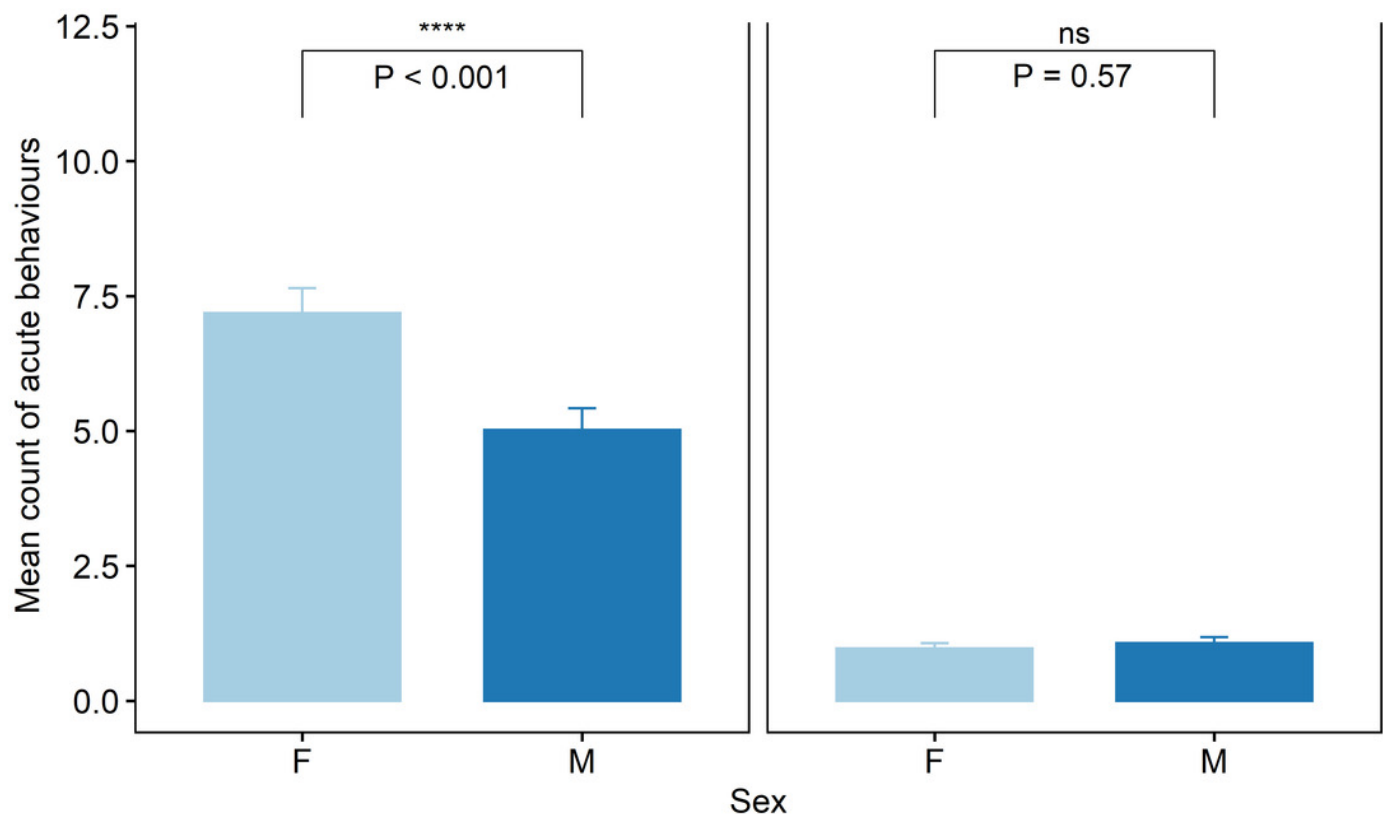
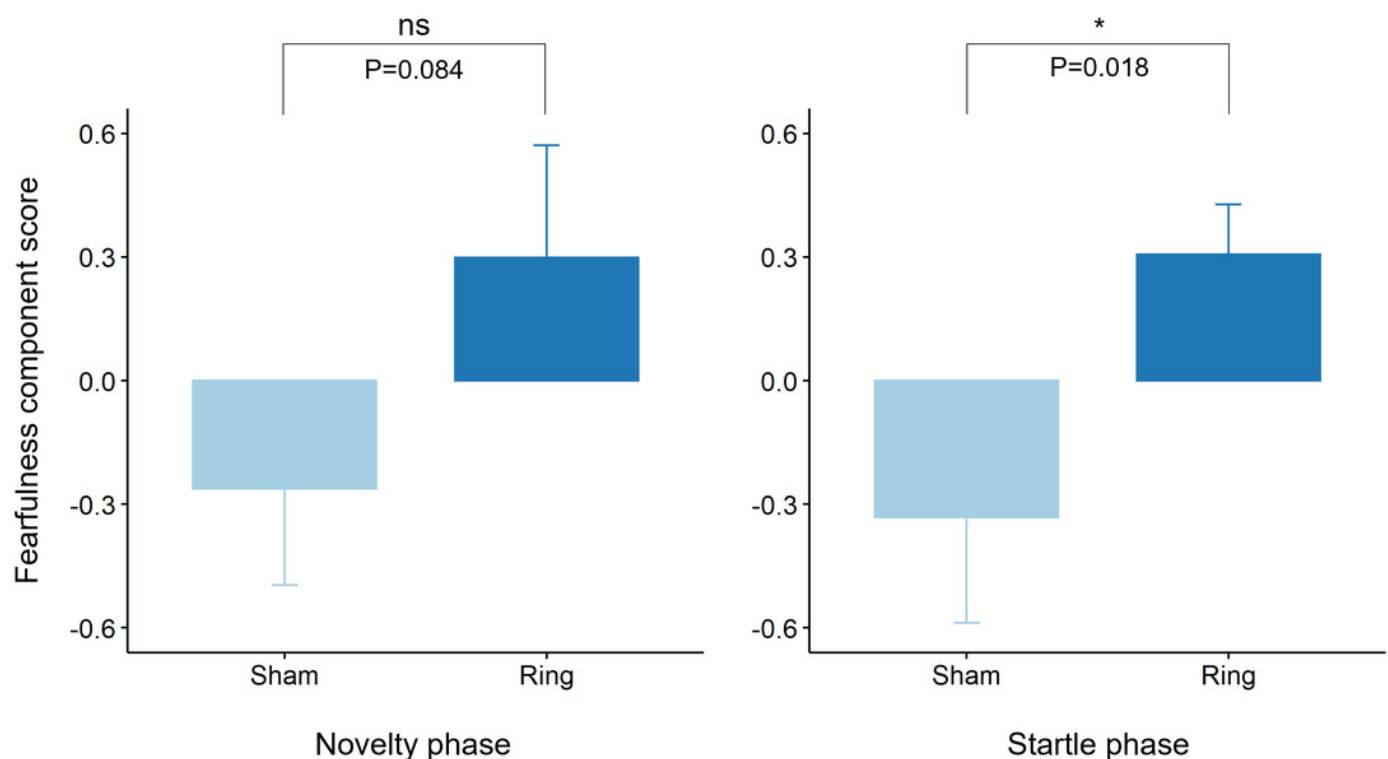



Figure 6

Raw mean ± s.e.m. of rotated principal component scores reflecting 'fearfulness' within the Novelty and Startle test phases for Sham and Ring treatment groups post tail docking

P values were derived from linear mixed effects models fitting either sex, treatment and a treatment by sex interaction or treatment only, for each test phase respectively.

Description of active pain avoidance behaviours and postures recorded every 5 minutes, for 1 minute during the 45 mins post tail-docking. The most prominent posture displayed in the one-minute observation period was recorded. For acute behaviour a count o

1 Table 1: Description of active pain avoidance behaviours and postures recorded every 5 minutes, for 1
2 minute during the 45 mins post tail-docking. The most prominent posture displayed in the one-minute
3 observation period was recorded. For acute behaviour a count of all behaviours observed in that minute
4 was taken.

Acute Behaviour	Abbreviation	Description
Restlessness	Rst	Number of times lamb stood up and laid down. Instances of lamb rising as far as its knees included in the one count.
Foot stamping/kicking	Fsk	Either a front or hind limb (usually hind limb) was lifted and forcefully placed on the ground while standing or was used to kick while standing or lying.
Rolling	Rl	Rolled from lying on one side to the other without getting up. Half rolls where the lamb rolled on its back and then returned to lying on the same side included.
Jumping	Jmp	All four feet off ground simultaneously
Licking/biting wound site	Lbw	Movement of the head beyond the shoulder, including both looking and touching at the source of pain and grooming.
Head shake	Sh	Forceful voluntary shake of the head
Easing quarters	Eq	Abnormally lowers rear quarters (standing) or attempts to keep quarters off the ground (lying).
Tail wiggle	Tw	Lamb moves their tail about in a wiggling motion
Unsteady hindquarters	Uh	Lamb staggers or totters in hindquarters sideways

Postures	Abbreviation	Description
Normal upright	NU	Standing, walking or playing while exhibiting a usual posture or gait; smooth movements
Abnormal upright	AU	Standing exhibiting unusual posture e.g. rounded, hunched appearance; standing stretched; ataxia; jerky movements; walking unsteadily, backwards, on knees
Unknown upright	UU	Lamb is upright but cannot be seen clearly enough to distinguish type of upright
Normal lying	NL	Ventral recumbency, all legs tucked under body or very close to body
Abnormal lying	AL	Twisted lying; ventral recumbency with forelimbs tucked under body, one or both hind limbs partially or fully extended; including dog sitting and lateral lying (lateral recumbency with one shoulder on ground, hind limbs and/or forelimbs fully extended)
Unknown lying	UL	Lamb is lying but cannot be seen clearly enough to distinguish type of lying

Behavioural measures recorded during the phases of the emotional reactivity test; Isolation, Novelty and Startle. The phase(s) of the test in which a given behaviour was recorded is indicated by "Y".

1 Table 2: Behavioural measures recorded during the phases of the emotional reactivity test; Isolation,
2 Novelty and Startle. The phase(s) of the test in which a given behaviour was recorded is indicated by "Y".

Behaviour	Definition	Isolation	Novelty	Startle
Vigilance	Percentage of test phase duration spent with the head at or above shoulder height (%)	Y	Y	Y
Feeding	Percentage of test phase duration eating food (%) and latency to eat from start of test phase (s)	Y	Y	Y
Zones crossed	Number of zones crossed with both front feet (n)	Y	Y	Y
Vocalizations	Number of open-mouthed and closed mouthed bleats recorded separately (n)	Y	Y	Y
Sniff environment	Number of sniffs of the walls, floors or camera boxes (n)	Y	Y	Y
Escape attempts	Number of escape attempts characterized by jumping or rearing towards wall (n)	Y	Y	Y
Umbrella approach	Latency to approach umbrella (s)		Y	
Umbrella reaction	Categorical score of reaction to the umbrella entering or opening; None (no or minimal response e.g. head turn), Low (noticeable startle, steps backwards or moves a short distance while remaining within one zone), Med (quickly moves away but remains within the one zone or walks to another zone) or High (rapidly moves away across multiple zones, may attempt to escape). A jump with all feet off the ground was also scored separately (0/1)		Y	Y
Umbrella attention	Percentage of test phase duration spent with the head oriented directly towards the umbrella (%)		Y	Y

Summary of the principal components analysis results for the Isolation phase of behavioural reactivity testing ($n = 78$).

Absolute factor loadings over 0.6 are written in bold.

1 Table 3. Summary of the principal components analysis results for the Isolation phase of behavioural
2 reactivity testing (*n* = 78). Absolute factor loadings over 0.6 are written in bold.

Isolation behaviour	Varimax rotated factor loadings		
	Component 1 (fearful)	Component 2 (explorative)	Component 3 (social)
Zones crossed	**0.88**	0.19	0.20
High vocalizations	**0.86**	0.37	-0.11
Vigilance %	**0.62**	**0.66**	0.22
Latency to eat	0.43	**0.74**	0.19
Sniff environment	0.17	**0.89**	0.10
Low vocalizations	0.08	0.17	**0.98**
Eigenvalues	2.10	1.97	1.10
% of variance	35	33	18

Summary of the principal components analysis results for the Novelty phase of behavioural reactivity testing, for sheep that failed to approach the umbrella ($n = 30$).

Absolute factor loadings over 0.6 are written in bold.

2   Table 4. Summary of the principal components analysis results for the Novelty phase of behavioural
3   reactivity testing, for sheep that failed to approach the umbrella ($n = 30$). Absolute factor loadings over
4   0.6 are written in bold.

Novelty behaviour	Varimax rotated factor loadings		
	Component 1 (fearful)	Component 2 (explorative)	Component 3 (active)
Attention to umbrella	**0.92**	0.03	0.10
Latency to eat	**0.89**	0.31	0.00
Vigilance %	**0.83**	0.36	0.30
Sniff environment	0.24	**0.94**	0.18
Zones crossed	0.12	0.17	**0.98**
Eigenvalues	2.41	1.14	1.09
% of variance	48	23	22

Summary of the principal components analysis results for the Startle phase of behavioural reactivity testing, for sheep that approached the umbrella ($n = 48$).

Absolute factor loadings over 0.6 are written in bold.

1 Table 5. Summary of the principal components analysis results for the Startle phase of behavioural
2 reactivity testing, for sheep that approached the umbrella ($n = 48$). Absolute factor loadings over 0.6 are
3 written in bold.

	Varimax rotated factor loadings		
Startle behaviour	Component 1 (fearful)	Component 2 (attentive)	Component 3 (escape)
Latency to eat	**0.84**	0.06	0.01
Vigilance %	**0.83**	0.18	0.05
High vocalizations	**0.64**	-0.54	0.16
Attention to umbrella	0.20	**0.89**	-0.13
Escape attempts	0.07	-0.14	**0.99**
Eigenvalues	1.83	1.15	1.02
% of variance	0.37	0.23	0.20

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