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1 **Positive and negative gestational handling influences placental traits and mother-offspring**
2 **behavior in dairy goats**

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17
18 **Abstract**

19
20 Dairy animals are subjected to a number of potential stressors throughout their lives, including daily
21 interactions with humans. The quality of these interactions may have direct consequences for the
22 animal undergoing the experience, but if such events occur during gestation it may also affect the
23 developing fetus. This study examined the effects of differential handling during mid-gestation in 40
24 twin-bearing Saanen x Toggenburg primiparous goats. Between days 80 and 115 of gestation
25 (gestation=150 days), goats were subjected to aversive (AVS, n=13), gentle (GEN, n=13) or minimal
26 (M, n=14) handling protocols for 10 minute periods twice daily. The control (M) group did not
27 receive handling treatments and all goats received normal husbandry procedures outside treatment
28 periods. Salivary cortisol measured during the treatment period was higher in AVS goats (mean
29 cortisol (sem) in pg/μl: AVS: 176.7 (18.2), GEN: 119.6 (11.1), M: 126.5 (13.7); P=0.007). Data
30 collection was focussed on mother-offspring behaviors 2h post-partum, placental morphology and
31 colostrum quality. AVS goats were the only treatment group to suffer fetal loss (16% loss vs 0% in
32 GEN and M, P=0.05). Treatment also influenced placental morphology with a tendency for fewer
33 cotyledons evident in placentae from the aversive treatment (AVS: 87.9 (7.8), GEN: 107.1 (7.9), M:
34 112.1 (9.3), P=0.093), and significantly fewer medium sized cotyledons (AVS: 67.6 (7.8), GEN: 89.3
35 (6.4), M: 84.3 (5.4), P=0.042). GEN goats displayed more grooming and nosing behaviors towards

36 their young during the first 2 h post-partum (Grooming: GEN: 89.3% (7.1), AVS: 72.6% (7.7), M:
37 63.4% (9.0), $P=0.045$. Nosing frequency: GEN: 58.8 (12.5), AVS: 28.6 (11.1), M: 34.7 (6.5), $P=0.021$).
38 There was an overall trend for kids from mothers experiencing the AVS treatment to take longer to
39 stand, reach the udder and suck compared to kids from GEN and M treatment groups. Treatment
40 significantly affected latency to perform play behavior, with kids from AVS goats taking on average
41 25 min longer to play for the first time than kids from GEN and M treatment groups ($P<0.001$). The
42 results show that handling during gestation affects placental morphology, fetal survival and post-
43 partum maternal behaviors, and influences kid behavioral development. Such results have important
44 animal welfare implications, demonstrating that negative handling of pregnant females results in
45 poorer placental quality with potential for fetal loss. It also demonstrates the beneficial effects of
46 positive handling on enhancement of maternal behaviors.

47

48 Keywords: Goats; prenatal stress; handling; placenta; behavior

49

50 **1. Introduction**

51

52 It has become increasingly evident that an animal's early life experiences can have both short- and
53 long-term consequences for its behavioral and physiological responses, health and wellbeing. This
54 phenomenon is known as "early-life programming" (Barker et al. 1993; Seckl, 1998) and if such
55 experiences are deemed stressful, and occur at a period of time when specific tissues are at a
56 sensitive stage of development, the impact can be detrimental. Studies of prenatal stress (PNS) have
57 largely been focussed in altricial species under laboratory conditions investigating paradigms that
58 are not necessarily relevant across species (Rutherford et al. 2012). The main intention of such
59 studies is translational; using rodents to model conditions in humans. Extrapolating studies in
60 rodents to other mammals may result in a number of inaccurate conclusions, particularly when
61 looking at the effects of PNS on brain development as the maturation of the rodent brain peaks
62 much later in pregnancy than it does in more precocial species. The growing body of literature on
63 early-life programming demonstrates that the effects of PNS are highly sensitive to species, sex,
64 relevance and timing of the stressor (for reviews: Braastad, 1998; Charil et al. 2010; Rutherford et al.
65 2012).

66

67 Farm animals can experience a number of stressors throughout their lives including social (e.g. high
68 stocking densities, dynamic mixing), isolation or handling stress (e.g. restraint, gathering). It is
69 becoming increasingly evident that when pregnant livestock experience such stressors there can be

70 substantial risks of undesirable early-life programming effects for their developing offspring as well
71 as direct cognitive and emotional impacts on the mother. For example, in pigs, disrupted hierarchies
72 and social defeat experienced by sows subjected to dynamic mixing (a social stressor) during
73 gestation resulted in substantial PNS effects; offspring experienced greater stress and pain reactivity
74 (Rutherford et al. 2009), poorer growth rates and transgenerational effects were observed whereby
75 female offspring of PNS mothers showed abnormal maternal care (Rutherford et al. 2014), including
76 increased savaging behavior (Jarvis et al. 2006). Pregnant sheep and goats can experience a number
77 of stressors in the months preceding parturition; they may be gathered from a largely remote
78 existence under extensive conditions and brought inside to experience higher stocking densities and
79 more forced social interactions with conspecifics and humans. In goats Vas et al. (2013)
80 demonstrated that reduced space accompanied by increased stocking densities resulted in greater
81 incidences of defensive and offensive behavior (Vas et al. 2013), and increased fearfulness in the
82 offspring when subjected to social and isolation tests (Chojnacki et al. 2014). Similar results were
83 reported in sheep by Averós et al. (2015) demonstrating increased emotional reactivity and fear
84 responses in lambs from mothers experiencing high stocking densities during pregnancy.

85

86 One potential stressor of particular relevance to livestock species is the interactions they experience
87 with humans. Dairy goats are subjected to daily interactions with stockworkers and it is the quality
88 of those interactions which could influence the affective state of the animal and have important
89 implications for its well-being. Coulon et al. (2011) found that aversively handled pregnant sheep
90 produced offspring that were more fearful. In contrast Roussel-Huchette et al. (2008) reported a
91 reduction in lamb fear levels when their mothers were exposed to repeated isolation and transport
92 stress during late gestation. There is little consensus in the literature regarding the effects of
93 handling treatments. In addition it is notable that the majority of handling experiments have
94 investigated the effects of negative interactions rather than applying a positive treatment. Hild et al.
95 (2011) and Coulon et al. (2011) are an exception; in sheep they applied a gentle and an aversive
96 handling protocol and focussed on studying subsequent offspring brain and behavioral development.
97 Their results centred on evidence of detrimental effects from the aversive treatment rather than
98 positive outcomes from the gentled treatment. However this aspect of prenatal handling warrants
99 further investigation in different species. It is known that stressful early-life experiences can be
100 mitigated via altered maternal behavior (Nguyen et al. 2008) and if maternal behavior can be
101 enhanced via positive interactions with humans there maybe long-term benefits for offspring.

102

103 Waiblinger et al. (2006) assessed the human-animal relationship in farm animals, stating that there is
104 an emotion-based classification of an animal's perception of humans which results in three main
105 categories: frightening (resulting in fear or avoidance responses in human presence), neutral
106 (neither a fear response or a positive reaction such as approach), or pleasant (resulting in an
107 approach response or human presence can be reassuring under adverse conditions). The aim of the
108 current study was to create a paradigm that evokes these negative, positive and neutral perceptions
109 in pregnant dairy goats in order to investigate the influence different affective states have on the
110 mothers as well as their developing offspring.

111

112 **2. Materials and methods**

113

114 2.1 Ethical Statement

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116 This study was reviewed and approved by the SRUC Ethical Review Committee (approval ID: ED AE
117 50-2012). All animal management procedures were adhered to by trained staff.

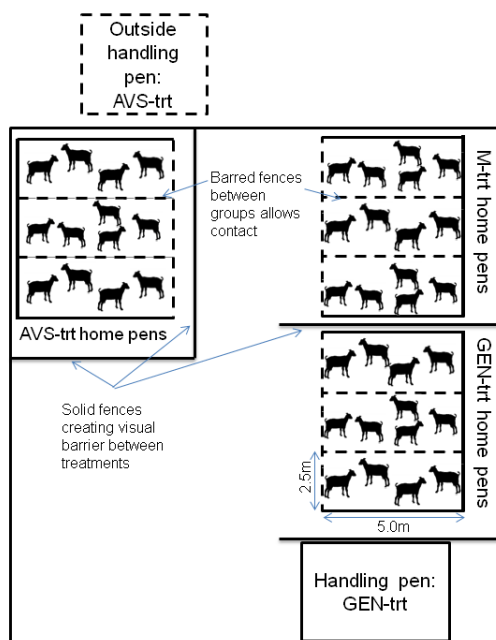
118

119 2.2 Animals, housing and feeding

120

121 Forty mixed breed (Saanen x Toggenburg) primiparous goats were used in this study. Following an
122 ultrasound scan at approximately 60 days post service 36 were confirmed as bearing twins, and four
123 as single-bearing. In the barn used for the experiment the goats were initially housed as one single
124 group (as they had been prior to selection). All goats were familiar to each other. The research barn
125 was naturally ventilated with deep straw bedding. Following acclimatisation to the new barn, goats
126 were randomly allocated to one of three handling treatment groups (aversive, gentle and minimal)
127 and put in one of three identical pens per treatment group (4-5 goats per pen, 2.5m wide, 5.0m
128 long) based on body weight (Fig 1). Mean body weight was $40\text{kg}\pm 0.86$ (range 30.6-53.2kg). Their
129 condition score (as determined using the Langston University
130 method <http://www.luresext.edu/goats/research/bcshowto.html>) averaged 2.27 ± 0.04 (range 1.75-
131 2.75). Three singleton goats were allocated to the control group (minimal) and one to the aversive
132 treatment group, this decision was based on body weight distribution within groups. Goats remained
133 in these smaller pens during the treatment period. Pens had barred partitions so groups could make
134 contact with each other but only within treatment (Fig 1). Upon completion of the treatment period
135 these partitions were lifted so that each treatment group had a larger area for the remainder of
136 gestation. Goats remained in these larger pens (7.5m wide, 5.0m long; $2.7\text{-}2.9\text{m}^2$ available per goat)

137 for kidding and post-partum data collection. Thus immediately pre-, during and post the treatment
 138 period goats remained within treatment group. Post-kidding and data collection goats and kids were
 139 moved to large post-kidding pens (7.5m wide, 5.0m long). Goats were fed a complete gestation and
 140 lactation diet as concentrate (13.2 MJ ME/kg DM, 20% CP, Harbro Ltd) which was fed in quantities
 141 according to calculated requirements for maintenance and stage of gestation and lactation. Silage
 142 hay and fresh water were available ad libitum. As the handling part of the experiment was the
 143 treatment, it was important that the shed accommodated these treatments with the least amount
 144 of effects transferring between groups, thus all three treatment groups were located in separate
 145 areas within the shed. Appropriate partitions were placed between the treatment groups (with
 146 minimum disruption to ventilation). Two handling pens were constructed at either end of the shed
 147 with the pen intended for the aversive treatment located in an outside arena. Artificial lighting
 148 provided an 8:16h light:dark regime with lights on at 8am in addition to any natural light that
 149 entered the building via ventilation openings. Staff were present 24 h a day during kidding when
 150 artificial lighting was provided continuously. To acclimatise the goats to this regime, artificial lighting
 151 provision was gradually increased one week prior to kidding due dates. Temperature and relative
 152 humidity (RH) within the shed was monitored via data loggers (Tinytag Gemini data loggers.
 153 Tinytag©) and averaged $5.3^{\circ}\text{C} \pm 0.06$ and $83.3\% \text{ RH} \pm 2.00$ during gestation and $11.2^{\circ}\text{C} \pm 0.04$ and
 154 $78.2\% \text{ RH} \pm 0.13$ during kidding.



155
 156 **Fig 1.** Diagram (not to scale) of experimental barn showing the pen arrangement and group sizes
 157 during the treatment period. Solid-sided partitions maintained a visual barrier between treatment
 158 groups, whilst barred partitions between pens within treatment allowed groups of goats to make
 159 contact. These barred partitions were removed on completion of the treatment period and goats
 160 kidded in larger pens within treatment.

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2.2. Experimental setup

2.2.1 Gestation treatments and data collection

Handling treatments were undertaken for each group daily for two 10 minute periods, five days a week and were similar to handling treatments applied by other authors studying prenatal handling stress in sheep (Hild et al. 2011; Coulon et al. 2011). The handling period was applied during the middle part of gestation between days 80-115. For the remaining period until kidding the goats were not disturbed apart from daily husbandry routines.

The gentle handling treatment (GEN) involved each group of goats being moved to a handling pen located at one end of the shed. The pen was enriched with straw bedding and straw bales. Goats were allowed to move voluntarily to the handling pen where they received a small food reward (taken from their daily ration) in a trough. Once in the handling pen a trained handler entered the pen and sat down, making no direct eye contact with the goats and speaking in a soft voice. Handlers interacted with any goats that approached and initiated contact. They could pet, stroke and scratch the goats. Handling periods were predictable, occurring at set time points after morning feeding (1030-1130) and in the afternoon (1400-1500), and goats were always handled in the same pen order. Handlers wore white overalls with faces uncovered.

The aversive handling treatment (AVS) involved each group of goats being moved to a handling pen located outside the home shed. The handling arena was barren (concrete) with barred, high fenced penning to prevent escape. A trained handler entered the pen and the handling was unpredictable and erratic. The handler spoke in a loud tone, made direct eye contact, moved the animals about the pen in an erratic manner, occasionally isolating one member from the rest of the group. The presence of a dog outside of the handling pen occurred randomly. Handling times were unpredictable occurring at no set time points on treatment days. Handlers wore red overalls, hats and snoods to cover their faces. No physical contact was made with the goats, all movements and separations were achieved by hand gestures and loud vocalizations by the handler.

The control group of goats received minimal handling throughout (M) – i.e. standardized husbandry (feeding, bedding, any medical treatments if necessary etc.) which was common to all treatment

194 groups and the staff wore regular blue overalls, also worn for all treatment groups when not
195 performing handling treatments. These husbandry routines took approximately 40 min per day.

196

197 2.2.1.1 *Cortisol and glucocorticoid metabolite analysis*

198

199 Saliva cortisol and faecal glucocorticoid metabolites (11-oxoaetiocholanolone EIA) (hereafter faecal
200 GM) were analysed to determine whether treatments differentially activated the HPA-axis and
201 whether goats habituated to the treatments over the five-week treatment period. Saliva samples
202 were collected at the same time of day once a week from all goats. The sampling was carried out 15
203 min prior to the treatment session and then 15 min after the end of the 10 minute treatment period.
204 For the control group samples were taken at the same time points. Each pen was moved calmly to a
205 separate sampling area close to their home pen and each goat was offered a large cotton bud (MP
206 Cotton buds; Millpledge Veterinary, Nottinghamshire, UK) on which to chew until it became
207 saturated with saliva (approximately 60 s per goat). Cotton buds were then placed in Salivette tubes
208 (SARSTEDT AG & Co., Nümbrecht, Germany), sealed, and centrifuged for 5 min at $2,600 \times g$. The
209 supernatant was pipetted off, into a clean container, and frozen at -20°C until assayed. In
210 preparation for assay, the samples were thawed on ice, centrifuged at $2,300 \times g$ for 5 min at room
211 temperature, and pipetted into a clean container. The supernatant was then used to measure
212 salivary cortisol by radioimmunoassay (RIA) using Coat-a-count cortisol kits (Siemens Medical
213 Solutions Diagnostics, Newbury, UK).

214

215 Although saliva sampling is generally considered a non-invasive method to assess HPA-axis
216 activation, it did involve gentle restraint of the goats and therefore faecal samples were also
217 collected from the home pens to complement the saliva sampling at a group level. Samples were as
218 fresh as possible, collected in labelled zip-lock plastic bags and frozen at -20°C until analysis. Faecal
219 GM extraction and analysis was carried out following the methodology described by Palme et al.
220 (2013). Briefly, 0.5g of faeces was transferred to a 15ml tube and 5ml 80% methanol was added.
221 The tube was vortexed for 30 min on a multivortexer and centrifuged for 15 min at $2,500 \times g$. The
222 supernatant was then diluted 1:10 in assay buffer (Trishydroxyaminomethane, Sodium chloride,
223 Bovine serum albumin, Tween 80, pH7.5) and faecal GM concentration was measured using enzyme
224 immunoassay (EIA) (Kleinsasser et al., 2010), read on a spectrophotometer (Thermo Scientific
225 Multiskan FC Microplate Photometer) at 450nm. Faecal GM concentration was standardized to the
226 weight of the fresh faeces used for the extraction (ng of faecal GM per g of fresh faeces). Quality

227 control samples were included on every plate for intra- and inter-assay coefficients (CV = 18% and
228 11% respectively).

229

230 2.2.2 *Kidding data collection*

231

232 Kidding occurred in the home pens and kidding assistance was only given according to the following
233 protocol: 1 h after the appearance of fluids but no appearance of parts of the kid, and/or 2 h after
234 parts of the kid were seen at the vulva with no other obvious progress being made. Assistance was
235 rarely required: minor assistance to correct presentation was given to two kids from the M
236 treatment and six kids required manual delivery (n=2 per treatment group). The time of birth, the
237 interval between littermates and the degree of assistance required were recorded for all goats.
238 Abandonment and/or rejection of kids was rare, however one kid was rejected by its mother,
239 following a 2 h interval between the birth of twin kids and a manual delivery, and was removed from
240 the trial to be hand-reared.

241

242 2.2.2.1 *Behavioral observations*

243

244 During kidding, goats were kept under 24 h surveillance by observers. This was complemented by
245 continuous video recording via closed-circuit (CCTV) cameras positioned above pens (infra-red
246 cameras, RF concepts, Ireland) connected to GeoVision Digital Surveillance System software (ezCCTV
247 Ltd, Herts, UK) and by eye-level digital recordings using a hand-held camcorder (Canon Legria)
248 mounted on a tripod. Goat and kid vocalizations (Table 1) were recorded live using a Psion
249 Workabout handheld computer (Psion PLC, London, UK) and Observer data collection software
250 (Noldus Information Technology, Netherlands). Live observations involved continuous focal sampling
251 for the first 30 min after the birth of each kid, followed by three 10-min periods, every 20 min, over
252 the following 90 min. Live observations allowed accurate recording of latency for kids to perform
253 specific behaviors (Table 1) which were confirmed by video recordings. These digital video
254 recordings were used to further exam each kid's behavior including number of times kids
255 approached the udder, sucking attempts (both successful and unsuccessful), number of times each
256 kid stood and fell down, as well as latency and number of play bouts (Table 1). Each kid's behavior
257 was observed for 2 h continuously from its birth. Maternal behavior and mother-young interactions
258 (for definitions see Table 1) were also recorded continuously for 2 h from the birth of the second
259 twin from video records.

260

261 **Table 1.** Goat dam and kid behaviors

Behavior	Description
Dam behaviors	
Grooming	Goat licks and nibbles kid
Noses	Goat touches any part of the kid with its muzzle but does not groom
Leaves	Goat leaves the vicinity of kid (defined as an adult goat’s body length in any direction from the kid). “Leaves” is different to withdraw as kid not actively at head and goat does not need to be orientated towards kid before leaving.
Approaches	Goat starts away from the vicinity of the kid, orientates itself towards the kid, and then actively enters the vicinity of the kid.
Presents udder	Goat crouches, turns one hind leg out to aid sucking
Withdraws	Goat moves backwards away from her kid whilst kid is at her head (2+steps)
Butts, Pushes	Goat knocks kid down or away with a rapid downward or sideways motion of the head
Prevention of sucking attempts	Goat movements that occur within 5s of the kid moving towards the udder
• Backing	Goat steps backwards as the kid moves forwards
• Circling	Goat steps sideways, moving hindquarters only away from the kid
• Forwards	Goat steps forwards over or past the kid
Low-pitched vocalization	Goat emits a low pitched rumble sound with her mouth closed
High-pitched vocalizations	Goat emits a high-pitched bleat with her mouth open
Kid behaviors	
Shakes head	Kid lifts head up off the ground and shakes it from side to side
To knees	Kid rolls onto sternum, pushes front half of body up off the ground whilst balancing on knees.
Attempts to stand	Kid supports its weight on any one foot (usually on knees with one or both hindlegs standing, rarely pushing front half of body up with one or both front legs).
Stands	Kid supports its weight on all four feet for at least five seconds
Reaches udder	Kid, whilst standing, moves actively towards udder region, nudging goat with head within 10 cm of udder.
Unsuccessful suck	Kid with head under goat in immediate vicinity of udder, prevented from sucking by goat movement, or fails to get teat into mouth.
Suck	Kid with head under goat, has teat in its mouth, making sucking movements of head or sucking noises, may be wagging tail, usually standing still and unlike with unsuccessful suck, can sometimes see swallowing movements.
Bleat	Kid makes a high or low pitched vocalisation
Plays	Kid performs locomotor play - jumping or pivoting, often with random hind leg kicks and exuberant head tosses

262

263 *2.2.2.2 Kid temperature, weight and body size measurements*

264

265 Thirty-min after birth first born kids were marked for birth order using colored sticky tape placed

266 above the hock of the left hind leg, for all kids the navel was disinfected with iodine solution and

267 rectal temperature (T30) recorded using a digital thermometer (BF-169 Flexible tip digital
268 thermometer, Farlin Infant Products Corporation, Taiwan). Rectal temperature was measured again
269 2 h after birth (T2h) and repeated 24 h after birth (T24h). At 24 h of age kids were weighed, sexed
270 and crown to rump length was measured (the length from the crown of its head to the base of its
271 tail). From these measurements, ponderal index (PI; body weight (kg)/crown-rump length (m)³) and
272 body mass index (BMI; body weight/crown-rump length²) were calculated for each kid.

273

274 *2.2.2.3 Placentae collection, dissection and cortisol extraction*

275

276 Placentae were collected when delivered and any debris carefully removed (i.e. straw). Any
277 remaining amniotic fluid was blotted dry before placentae were weighed. Each cotyledon was
278 dissected free from the membranes and classified as either small (<1 cm diameter), medium (1-5cm)
279 or large (>5 cm) and categorized based on shape; either raised (spherical) or long and flat. Once
280 placed in their categories the cotyledons were weighed. One of each size was then selected (three in
281 total) and placed in 50ml tubes and frozen at -20°C for glucocorticoid (GC) analysis. For laboratory
282 analysis samples were thawed and the three cotyledons (small, medium, large) from each placenta
283 were weighed. A 0.5g sample (approximately) was cut from each cotyledon and homogenized in 1ml
284 of chilled phosphate buffered saline (PBS, pH 7.4) in a FastPrep machine (Thermo Savant FastPrep
285 120 Cell Disrupter System). The samples were vortexed, then centrifuged for 2 min before pipetting
286 0.5 ml of the supernatant into 15 ml plastic tubes. Then 5ml of diethyl ether (Fisher, UK) was added
287 to each tube prior to vortexing for 10s and freezing at -80°C overnight. The solvent layer (diethyl
288 ether containing cortisol) was decanted into a new glass tube, where it was dried using nitrogen
289 (Techne Dri-Block DB-3A Sample Concentrator). Samples were then reconstituted in 250 ml of assay
290 buffer (PBS (Sigma) + 0.1% Bovine serum Albumin, Sigma), vortexed and assayed. An indirect ELISA
291 using an in-house protocol developed by co-author Al-Dujaili (Al-Dujaili et al. 2009; 2012)
292 determined cortisol concentrations using a spectrophotometer (Thermo Scientific Multiskan FC
293 Microplate Photometer) with a filter of 595nm. Placental cotyledon cortisol was expressed as ng of
294 cortisol per g of original tissue used.

295

296 *2.2.2.4 Colostrum collection and analysis*

297

298 At 2 h after the birth of the last kid, the goat and her kids were moved to post-kidded pens. If kids
299 had not sucked they were assisted to suck. Colostrum samples were then collected from both teats
300 (approximately 2ml from each teat) and frozen at -20°C for subsequent analysis of immunoglobulin

301 (IgG) concentration. Colostrum IgG levels were measured using a pre-prepared quantitative double
302 antibody sandwich Goat IgG ELISA test kit following manufacturer's instructions (Biopanda Reagents,
303 NI) and quantified using a spectrophotometer, filter 450nm (Thermo Scientific Multiskan FC
304 Microplate Photometer).

305

306 2.3 Statistical analysis

307

308 To determine the effects of treatment on saliva cortisol and faecal GM levels during gestation
309 Generalized Linear Mixed Models (GLMM) were used. Average cortisol (pg/ μ l) was fitted as the
310 response variate using a Poisson distribution with a Logarithm function. Week of treatment (i.e. 1-5),
311 sampling time point (i.e. pre- or post- treatment) and treatment were fitted as fixed effects, with
312 goat and pen fitted as random effects to account for repeated measures from saliva and faecal
313 sampling. GLMMs also determined the effects of treatment on both maternal and kid measurements
314 taken post partum. Where data were skewed a Poisson distribution with a Logarithm function was
315 used. Goat was fitted as a random factor to take into account litter effects. Where differences were
316 found, post-hoc comparisons were made using Fishers' Least Square Differences (LSD) tests. For
317 placental traits, placental cortisol and colostrum IgG level, treatment was fitted as the fixed effect
318 with litter size as a covariate. For mother offspring behavior treatment was fitted as the fixed effect
319 with litter size as a covariate and birth interval with litter size fitted as an interaction. Where twin
320 births occurred maternal behavior analysis commenced only after the birth of the second kid. A Chi-
321 square test was used to explore categorical outcome variables and where expected counts were less
322 than five, a Monte Carlo simulation was included and as a result of small sample size, the likelihood-
323 ratio chi-square (based on maximum-likelihood theory) was applied (Yuan et al. 2007). For kid data
324 fixed effects included in the model were treatment, sex, birth interval and twin (i.e. whether or not
325 the kid had a live-born twin) or litter size (for weight and shape parameters) and sex by treatment
326 interactions. Spearman's rank correlations were used to identify relationships between covariates.
327 All analyses were made using Genstat 16 software. Significance was considered to be $P < 0.05$ but
328 some tendencies ($P < 0.1$) are presented.

329

330 3.0 Results

331

332 3.1 Glucocorticoid concentration during treatment period

333

334 Five goats (three from the M, two from AVS) returned salivary cortisol levels for one of their samples
335 above the level of detection 999 pg/ μ l and these outliers were excluded from analysis. AVS goats
336 had significantly higher salivary cortisol concentrations over the treatment period than goats from
337 the GEN and M groups (mean cortisol (sem) in pg/ μ l: AVS: 176.7 (18.2), GEN: 119.6 (11.1), M: 126.5
338 (13.7); $F_{2,387}=5.04$, $P=0.007$). There was a significant influence of time on faecal GM levels ($F_{4,24}=2.82$,
339 $P=0.048$), with a general elevation over the five-week treatment period, peaking at week 4 (average
340 cortisol (ng/g) \pm sem: Week 1: 128.3 (11.1), Week 2: 112.4 (9.2), Week 3: 156.0 (20.0), Week 4: 163.7
341 (18.9), Week 5: 139.4 (12.7)), however there were no effects of treatment ($F_{2,6}=1.09$, $P=0.394$).

342

343 3.2 Fetal loss and litter size

344

345 AVS goats were the only treatment group to experience fetal loss: two goats gave birth to singletons
346 when scanned as carrying twins, and one goat did not deliver any kids, whereas all GEN and M goats
347 delivered the number of kids they had been scanned as carrying ($\chi^2_2=5.44$, $P=0.05$). There were two
348 incidences of stillbirth, one from each of the GEN and M groups respectively.

349

350 3.3 Placental traits

351

352 The results for treatment differences in placental traits are presented in Table 2 and are adjusted for
353 litter size. There was a tendency for treatment to affect total cotyledon number ($F_{2,34}=2.37$, $P=0.093$)
354 with significant differences between treatment groups found in the number of medium sized
355 cotyledons ($F_{2,34}=3.17$, $P=0.042$). Differences were with placentae from the AVS goats having fewer
356 medium raised cotyledons compared to other treatment groups (Table 2). Treatment also influenced
357 the number of small cotyledons ($F_{2,34}=3.71$, $P=0.036$), specifically small-raised cotyledons ($F_{2,34}=4.56$,
358 $P=0.018$). Goats experiencing minimal handling treatments had a greater number of small raised
359 cotyledons compared to the handled treatment groups (Table 2). Cortisol concentrations were only
360 significantly different in the small cotyledons ($F_{2,34}=3.50$, $P=0.042$), with cotyledons from goats
361 experiencing the AVS treatment having lower cortisol levels than the other treatment groups (Table
362 2).

363

364

365 **Table 2.** Placental traits and cortisol levels (means and standard error of the difference (sed))
 366 comparing data from Minimal (n=14), Aversive (n=12) and Gentle (n=13) handling treatment groups.
 367 Data presented are adjusted for litter size.

	MINIMAL	AVERSIVE	GENTLE	sed	F-stat	P-value
Placental weight (g)	552.1	637.0	608.4	59.75	1.12	0.327
Placental efficiency (LW:PW)	8.72	7.96	8.29	1.16	0.58	0.561
Total number of cotyledons	112.1 ^a	87.9 ^b	107.1	11.76	2.37	0.093
Number of small cotyledons	23.66 ^a	13.87 ^b	13.49 ^b	4.31	3.71	0.036
• Small_raised	22.57 ^a	13.63 ^b	12.29 ^b	3.84	4.56	0.018
• Small_long	0.09	0.05	0.04	1.13	0.14	0.870
Number of medium cotyledons	84.27 ^(b)	67.61 ^{a (a)}	89.31 ^b	8.81	3.17	0.042
• Medium_raised	77.17 ^(b)	59.57 ^{a (a)}	78.38 ^b	8.98	2.67	0.083
• Medium_long	5.90	5.18	7.31	4.28	0.43	0.648
Number of large cotyledons	4.12	6.10	4.26	1.79	1.57	0.208
• Large_raised	1.45	2.12	1.63	1.18	0.48	0.619
• Large_long	1.59	2.26	2.31	1.17	0.08	0.776
Total cotyledons wgt (g)	174.4	180.2	171.1	20.87	0.09	0.913
Small cotyledons wgt (g)	5.91	4.40	3.88	1.65	1.29	0.276
Medium cotyledons wgt (g)	154.2	148.4	147.9	19.72	0.07	0.935
Large cotyledons wgt (g)	13.40	25.99	18.93	7.20	1.59	0.219
Cortisol levels in cotyledons (ng/g)						
• Small	84.40 ^(b)	36.54 ^{a (a)}	126.94 ^b	119.2	3.50	0.042
• Medium	110.30	78.10	108.38	101.5	0.37	0.690
• Large	124.40	82.40	99.60	61.95	0.74	0.669
• Average	225.60	172.60	153.90	50.46	1.56	0.209

368 LW refers to the litter weight and PW to the placental weight. Superscripted letters indicate where differences
 369 lie. Values with different superscripts in bold differ at the P<0.01 level; values with different superscripts in
 370 italics differ at the P<0.05 level; values with different superscripts within brackets tend to differ (P<0.10).
 371

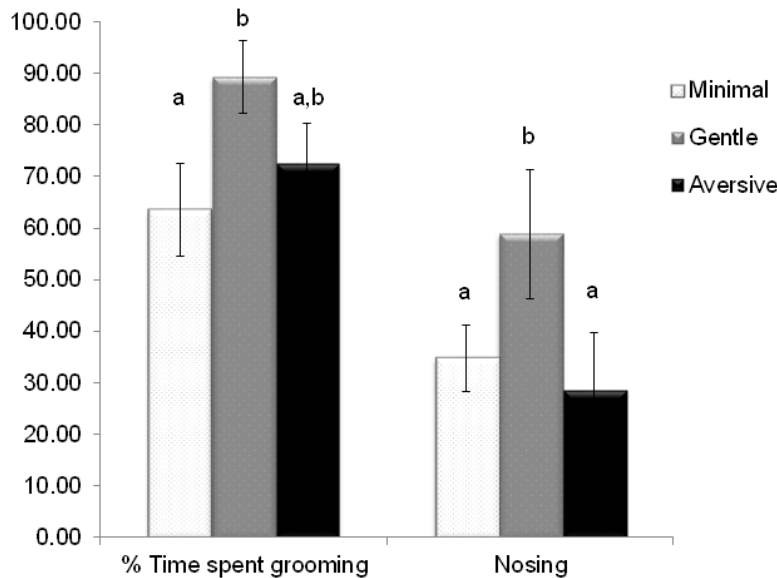
372 3.4 Maternal behavior in the first 2 h post-partum

373

374 For live-born twins average birth interval was 22.14 min (\pm 4.30) with no significant difference
 375 between treatment groups ($F_{2,27}=1.45$, $P=0.251$).

376

377 There was a significant treatment difference in grooming ($F_{2,34}=3.10$, $P=0.045$) and nosing
 378 ($F_{2,34}=3.85$, $P=0.021$) behavior towards kids, with GEN goats displaying more of these behaviors
 379 during the first 2 h observation period post-partum compared to M and AVS goats (Fig 2). The
 380 number of times goats left their kids in the observation period was influenced by treatment
 381 ($F_{2,34}=3.91$, $P=0.034$) with GEN goats rarely leaving their kids compared with AVS and M treatment
 382 goats (Table 3). Consequently there was also an influence on number of approaching incidences
 383 ($F_{2,34}=5.46$, $P=0.009$ - Table 3). There were no significant differences in the amount of low or high
 384 pitched vocalizations emitted by the goats from different treatments. There were no treatment
 385 effects on goat responses to kid sucking attempts (Table 3).



386

387 **Fig 2.** Plot demonstrating the effects of prenatal handling treatments on mean percentage of
 388 observation period goats spent grooming their kids (\pm sem) and mean number of times goats nosed
 389 their kids (\pm sem) during the first 2h observation period after the birth of the second kid. Bars with
 390 different letter superscripts differ at the $P < 0.05$ level within behavior. See text for details. Data
 391 presented are adjusted for litter size and birth interval (for twins).

392

393 **Table 3.** Differences in maternal behaviors (displayed as mean totals in the first 2h post-partum)
 394 performed by goats from the three prenatal handling treatments. Data adjusted for litter size. Birth
 395 interval as an interaction with litter size was fitted as a co-variate. Grooming and nosing behaviors
 396 shown in Fig 2.

	MINIMAL	AVERSIVE	GENTLE	sed	F-stat	P-value
Low-pitched vocalizations	462.40	322.10	375.40	64.51	0.77	0.472
High-pitched vocalizations	12.05	12.03	10.83	8.30	0.27	0.764
Presents udder	0.50	2.90	0.99	1.69	0.15	0.864
Approaches	11.94 ^a	10.96 ^a	2.58 ^b	1.98	5.46	0.009
Leaves	7.32 ^a	8.37 ^a	2.11 ^b	2.20	3.91	0.034
Withdraws*	2.69	1.95	-0.89	2.75	0.62	0.542
Prevention of sucking attempts						
Circles	9.99	10.39	15.40	4.06	1.88	0.153
Backs	2.74	4.92	4.16	5.06	0.66	0.517
Forwards	5.04	4.00	6.72	3.60	0.69	0.509

397 Values with different superscripts in bold differ at the $P < 0.01$ level. (*Data presented for withdraws are
 398 adjusted means but the behavior was performed rarely. True means are: M: 3.57, AVS: 2.75 and GEN: 0.69)

399

400 Negative maternal behavior was displayed rarely, with butting, biting or pushing of kids restricted to
 401 only three goats, with only one incident each (data not shown). Actively withdrawing from kids
 402 whilst they were at their mother's head was also rarely exhibited, as were behaviors that prevented

403 the kid from sucking. There were no differences between treatment groups in these negative
404 maternal behaviors (Table 3).

405

406 3.5 Colostrum IgG

407

408 There was a great deal of variation in the levels of colostrum IgG between goats with no significant
409 differences between treatment groups (mean IgG (sem) in mg/ml: GEN: 75.86 (28.4), AVS: 98.46
410 (25.0), M: 65.57 (4.0); $F_{2,34}=0.80$, $P=0.460$).

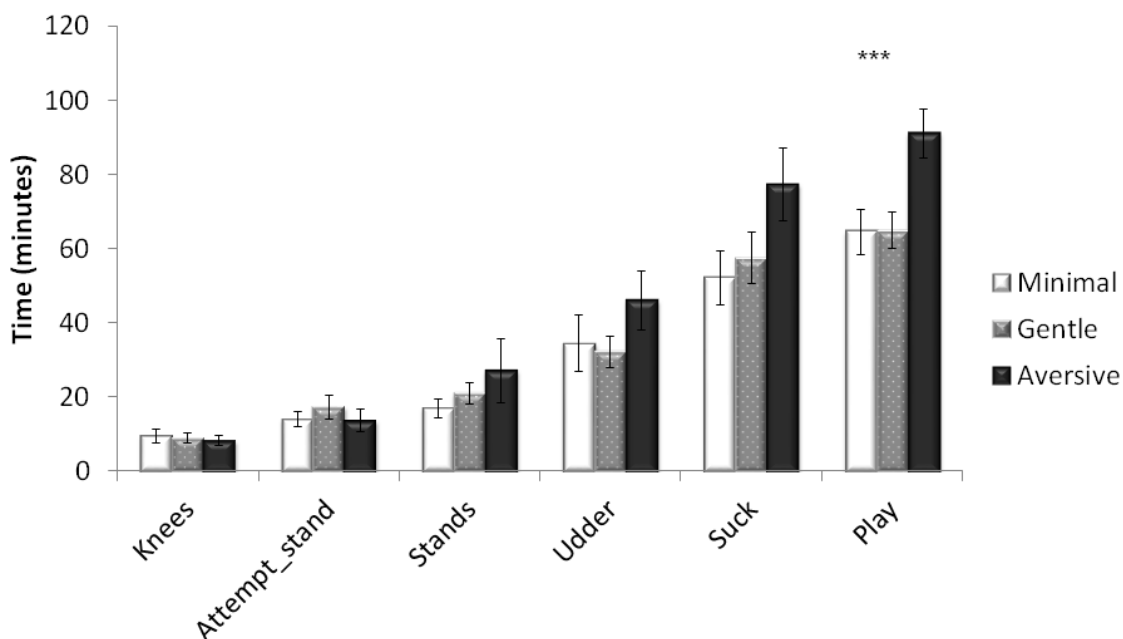
411

412 3.6 Kid behaviors

413

414 There were no significant treatment differences in latency for kids to perform first time, landmark
415 behaviors (getting to knees, attempting to stand, standing, reaching the udder and sucking).
416 However there was a significant influence of treatment on more coordinated behaviors, particularly
417 play ($F_{2,62} = 14.27$; $P<0.001$). Kids from mothers experiencing the AVS treatment showed a trend for
418 taking longer to perform udder contact and sucking and were significantly slower to show play
419 behavior compared to kids from mothers experiencing the GEN- and M- treatments (Fig 3). Sucking
420 assistance was given to 12% of kids from the M treatment group, 20% from the GEN treatment
421 group and 33% of kids from the AVS treatment group ($X^2_2=2.93$, $P=0.231$).

422



423

424 **Fig 3.** Influence of prenatal handling treatments on latency for kids to perform first time behaviors.
425 Data presented are means (\pm sem) adjusted for twin and birth interval. *** $P<0.001$

426

427 Regardless of treatment male kids were consistently slower than female kids to suck successfully
428 (mean latency (sem) in min: female kids: 53.4 (5.6), male kids: 69.5 (7.4), $F_{1,52}=8.18$, $P=0.007$). The
429 number of times kids performed different behaviors and vocalizations during the first 2 h post-
430 partum are summarised in Table 4. There were no significant differences between treatment groups,
431 although kids from the GEN treatment group tended to play more frequently than kids from the AVS
432 treatment group (Table 4). Regardless of treatment group, sex influenced frequency of locomotor
433 play with females more playful than males (mean total number of play bouts (sem): female kids:
434 11.1 (3.3), male kids: 5.8 (3.4); $F_{1,52}=5.18$, $P=0.027$).

435

436 3.7 Kid weight, shape and temperature

437

438 Birth weight, body mass index, ponderal index and kid temperature are summarised in Table 5.
439 There were no significant differences between treatment groups in weight or shape measures. Kids
440 experiencing the longest birth intervals had lower rectal temperatures 2 h after birth than kids born
441 after shorter intervals ($F_{1,41}=4.45$, $P=0.041$). When birth assistance was factored into the model,
442 those kids that were delivered manually had the lowest rectal temperatures (mean rectal
443 temperature (sem) °C: No birth assistance: 38.7 (0.1), presentation correction only: 38.4 (0.7),
444 manual delivery: 37.7 (0.3), $F_{2,53}=6.29$, $P=0.004$).

445

446 3.8 Correlations

447

448 Grooming and nosing behaviors by the mother correlated with latencies for kids to perform certain
449 landmark behaviors, specifically latency to reach the udder (grooming: $r_s = -0.378$, $P<0.001$, nosing:
450 $r_s = -0.419$, $P<0.001$) and latency to suck successfully (grooming: $r_s = -0.302$, $P=0.012$, nosing: $r_s = -$
451 0.345 , $P=0.004$).

452

453 5. Discussion

454

455 This study has demonstrated that handling pregnant dairy goats in an unpredictable and aggressive
456 manner, for only 20 min per day, over a 5 week period in mid-gestation, significantly affects
457 placental development, fetal loss and aspects of maternal care. It also affects kid behaviors,
458 including the latency to perform certain behaviors for the first time and reduces the frequency of

459 expression of play behavior. Conversely, gentle handling increased the expression of maternal care
460 immediately after birth.

461

462 5.1 *Fetal loss, placental morphology and cortisol*

463

464 The impact of the treatments on fetal survival could be considered the most significant result in
465 terms of animal welfare and production performance, although results should be regarded with
466 caution given the relatively small numbers of animals affected. Elevated salivary cortisol levels in
467 goats from the AVS treatment demonstrated that aversively handled goats were experiencing higher
468 levels of physiological stress during the treatment period than the other groups and this stress
469 response could be one possible explanation for fetal loss. It appears that dairy goats are particularly
470 sensitive to fetal loss (10-30% - Norwegian dairy goats - Engeland et al. 1999; 20-50% in Angora dairy
471 goats – Van Rensburg, 1971) with several of these authors suggesting that advancing age, difficulty
472 in conceiving, low social status and triplet pregnancies are risk factors for fetal loss. In addition,
473 several studies have associated increased maternal blood corticosteroid levels in goats subsequently
474 aborting compared to those that maintained a normal pregnancy (Wentzel et al. 1975; Romero-R et
475 al. 1998), suggesting that abortions, particularly those without a disease aetiology, may be related to
476 a stressful situation. In the present study fetal loss was found only in goats experiencing AVS
477 handling, providing further supportive evidence that goats are highly sensitive to stress. Maternal
478 glucocorticoids are expected to increase as pregnancy progresses (Liggins, 2000); overall the faecal
479 GM results reflected this effect in the current study. However there were no treatment effects in the
480 faecal GM, intended to determine whether the treatment influenced the stress physiology of
481 animals in a more chronic manner (as shown in other studies (Palme et al. 2000; Möstl et al. 2002)).
482 The saliva samples obtained from the goats did show AVS goats with elevated cortisol levels over the
483 treatment period. In addition there were behavioral indicators that the AVS goats found the
484 handling stressful including excessive defecation in the handling arena (an indicator of fear and
485 stress – Fraser, 1974; Smulders et al. 2006), which was not observed in the GEN group during their
486 treatment period. Therefore we could speculate that there was an acute stress response that caused
487 high enough levels of cortisol in the AVS treatment groups which could result in an upregulation of
488 11 β -hydroxysteroid dehydrogenase type 2 (11 β -HSD2), an enzyme responsible for converting
489 cortisol into its inactive form of cortisone (Burton and Waddel, 1999; Matthews, 2002), which may
490 affect fetal survival or lead to modifications in placental function (Jonker, 2004). The concentrations
491 of this enzyme were not measured however cortisol was measured in placental cotyledons and
492 interesting results were found with goats from the AVS treatment showing significantly lower levels,

493 specifically when compared to those from the GEN treatment. Such low levels could further support
494 activation of the 11 β -HSD2 enzyme in this treatment group as a result of an excess level of
495 glucocorticoids caused by the prenatal stressor. However, such conclusions must be regarded with
496 caution as without measuring the expression of this enzyme directly we can only infer such a
497 conclusion.

498

499 Placental morphology was influenced by maternal stress suggesting that placental capacity to
500 transfer nutrients and oxygen to the fetus may have been affected. Ruminant placentae have
501 discrete areas of attachment, the placentomes, which are formed by interaction between uterine
502 caruncles and chorionic cotyledons. Normal fetal growth and development are dependent on the
503 normal growth and development of placentomes (Kelly, 1992; Igwebuike and Ezeasor, 2013). Initially
504 such structures are bulbous or raised in shape and become flatter in late pregnancy (Kelly, 1992).
505 Goats in the AVS-treatment group had the lowest number of medium sized cotyledons and tended
506 to have the lowest number of cotyledons overall, regardless of litter size. As cotyledon numbers are
507 usually fixed in ruminants by day 56 of gestation (Kelly, 1992), before handling treatments were
508 imposed, the tendency for a difference in overall cotyledon number was unexpected. The average
509 size of goat cotyledons, however, is known to increase linearly over gestation (Igwebuike and
510 Ezeasor, 2013) which may represent a response to increased nutritional demands of the fetus(es)
511 during development. It has been demonstrated that size of placentomes (and consequently the
512 cotyledons), rather than morphology (i.e. raised or flat) influences vascular function of the placenta
513 (Vonnahme et al. 2008) and it has been hypothesized that prenatal stress can accelerate the
514 morphological changes from less developed to more developed placentomes in an attempt to
515 “rescue” the fetus via increased vascularity, therefore greater blood flow and nutrient transfer
516 (Vonnahme et al. 2006, 2008). Goats from the M treatment had significantly greater numbers of
517 small cotyledons compared to handled goats. If the previous “rescue” hypothesis was considered it
518 would confirm that M treatment groups were not suffering prenatal stress, however it suggests that
519 both positive and negative handling interactions during pregnancy are having an affect. The only
520 cotyledon size category where the AVS treatment had a greater number (though not significant) was
521 in the large sized group. It is possible that the differences in the number of different sized cotyledons
522 could reflect a compensatory mechanism in the AVS-treatment goats to support their developing
523 offspring in expectation of a challenging post-natal environment. Such a strategy is evident in
524 prenatal stress studies, where stressors applied in mid to late pregnancy can result in increased birth
525 weight, presumably as a result of altered placental function (e.g. Roussel et al. 2004; Corner et al.
526 2007). There were no such significant influences of treatment on birth weight or size (i.e. ponderal

527 index and body mass index) in the current study. The relatively small sample size in this study only
528 allows inferences of possible reasons for the differences between treatment groups and a larger
529 sample size could have seen a greater effect on placental traits allowing a more robust conclusion
530 about the strategy adopted by the goats.

531

532 *5.2 Mother-offspring behavior*

533

534 *5.2.1 Maternal behavior*

535

536 Much of the discussion so far has detailed the negative aspects of handling treatments applied
537 during mid-gestation; however this study has demonstrated that positive handling of pregnant dairy
538 goats results in greater attentiveness towards their offspring during the first 2 h post-partum.
539 Specifically goat mothers from the GEN treatment spent a greater proportion of time grooming their
540 kids and showed a higher frequency of nosing behaviors towards their kids in the 2 h after the birth
541 of the second kid compared to goats from the M and AVS treatments. This result is in contrast to
542 Hild et al. (2011) who applied similar GEN and AVS treatments to pregnant sheep and found
543 increased maternal care in the AVS group compared to GEN. It is not clear why this discrepancy is
544 found, although Hild et al were working with a different species and applying the stressor during the
545 latter part of gestation, both factors that could offer some explanation regarding the discrepancies.

546

547 However the tendency for GEN goats to spend more time with their kids than the AVS and M goats
548 further demonstrates the effect of positive handling on maternal attentiveness. Grooming the
549 neonate is an important component of the behavioral repertoire of small ruminants whereby
550 focussed interest in the newborn involves intense licking behavior starting at the head to clear
551 placental membranes and working along the whole body whilst emitting low-pitched vocalizations
552 (Nowak et al. 2000; Dwyer, 2014). Such focused attention establishes the mother-young bond,
553 facilitates sucking success by the offspring and thus promotes survival (Dwyer and Lawrence, 2005).
554 The correlations between maternal attentiveness (i.e. grooming and nosing behavior) with latency to
555 reach the udder and suck successfully observed in the current study further support the well-
556 established relationship between positive maternal behavior and offspring sucking success. There is
557 also evidence in other studies that positive postnatal maternal care can have long-term effects on
558 offspring cognition, development and future reproductive success (for review see Champagne and
559 Curley, 2009).

560

561 Hemsworth and colleagues over a number of studies have clearly demonstrated the sequential links
562 between the attitudes that stockhandlers have towards their livestock, their subsequent behavior
563 towards them, the impact this has on animal fear levels and finally the consequences of increased
564 fear for production and reproduction (Hemsworth et al., 1995; Hemsworth and Coleman, 2011). In
565 addition, in pigs, they found that the proportion of physical interactions that were negative was
566 significantly related to both total litter size and number born alive (Hemsworth et al. 1989),
567 suggesting both prenatal and perinatal influences of the human-animal relationship on neonatal
568 mortality. Many interactions between humans and animals on farm can be negative, involving
569 necessary but aversive husbandry procedures (e.g. vaccinations, foot trimming, shearing). Few,
570 other than feeding, can be considered positive (Waiblinger et al. 2006), however this study
571 demonstrates that a high quality human-animal relationship can be beneficial in terms of increased
572 maternal care. Maternal behavior in the AVS treatment was not dissimilar to that displayed by the
573 control population receiving minimal handling and the very rare displays of negative maternal
574 behavior were not treatment specific. Thus the effect of prenatal handling on maternal behavior was
575 an enhancement resulting from gentling, rather than suppression from aversive handling.

576

577 *5.2.2 Kid Behavior*

578

579 The process of birth stimulates the neonate and a sustained period of arousal promotes exploration
580 of the mother's body and perception of sensory cues to facilitate finding the udder (Nowak and
581 Poindron, 2006). As with most precocial and semi-precocial neonates, those that are quick to get to
582 their feet, reach the udder and suck colostrum are those that are most likely to survive (Fraser, 1990;
583 Edwards and Broom 1982; Dwyer, 2003). The lack of energy reserves and the need to maintain
584 homeothermy means colostrum ingestion is a priority (Mellor and Stafford, 2004). The AVS-
585 treatment influenced kid behavioral development with kids from goats experiencing AVS-handling
586 demonstrating a trend towards increased latencies to reach the udder and suck successfully. Play
587 behavior (solitary locomotor-rotational play) was displayed by many of the kids within the first 2 h
588 post-partum. However, the latency to display such behavior was much longer in kids from goats
589 experiencing the AVS-handling treatment, which subsequently influenced the frequency of play
590 behavior within the observation period. Early play behavior in sheep (Dwyer & Lawrence, 2005) is
591 known to be independent of maternal behavior, thus this delay in the onset of play may result from
592 some impact of prenatal stress on the neurological or physical development of the kid, rather than
593 as a result of maternal responsiveness. The fetal brain would have been developing during the time
594 the prenatal stress was applied and it is well-established that prenatal stress can have significant

595 effects on offspring cognitive development (Weinstock, 2008). In this study there appeared to be an
596 increasing deficit in the behaviors of AVS kids as behaviors became more complex and required
597 greater coordination. In addition, mammalian play is believed to occur only when animals have
598 sufficient nutrition and other physiological requirements are satisfied (Graham and Burghardt,
599 2010). Thus, the delayed sucking success in the AVS kids may have impaired their ability to display
600 play responses.

601

602 It is important to discount variables that might influence behavioral development such as birth
603 difficulty and inability to properly thermoregulate. The immediate postnatal period for all neonates
604 is characterized by thermal instability with newborns extremely vulnerable to hypothermia (Dwyer,
605 2008). Goat kids are considered to be more sensitive to cold than lambs as they are less insulated
606 and display slower metabolic rates per unit live weight (Wentzel et al., 1979; Muller and
607 McCutcheon, 1991). Thermoregulation depends on rapid ingestion of colostrum. Birth interval
608 influenced the thermoregulatory abilities of neonates, with kids that had experienced manual
609 delivery showing the lowest rectal temperatures 2 h after birth. Hypoxia and reduction in core body
610 temperatures will both influence sucking success, however birth interval was accounted for within
611 the statistical models and manual delivery was only experienced by six kids in total, two from each
612 treatment group. Therefore these factors cannot explain the longer latencies to display specific
613 behaviors and play in the AVS-treatment kids.

614

615 **6. Conclusions**

616 This study shows that the quality of human interactions with the mother during pregnancy affects
617 the placenta, maintenance of the pregnancy and post-partum maternal behaviors. In addition, some
618 aspects of offspring behavioral development are affected. Such results have important animal
619 welfare implications, demonstrating that negative handling of pregnant females results in altered
620 placental morphology and potential for fetal loss, whereas positive handling seems to enhance
621 expression of maternal care. It also demonstrates that prenatal handling stress can delay behavioral
622 development in neonates, which may reflect a cognitive deficit that could impact upon neonatal
623 survival.

624

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