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Published in:
Physiology and Behavior

DOI:
10.1016/j.physbeh.2016.02.001

First published: 01/01/2016

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

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Abstract

Dairy animals are subjected to a number of potential stressors throughout their lives, including daily interactions with humans. The quality of these interactions may have direct consequences for the animal undergoing the experience, but if such events occur during gestation it may also affect the developing fetus. This study examined the effects of differential handling during mid-gestation in 40 twin-bearing Saanen x Toggenburg primiparous goats. Between days 80 and 115 of gestation (gestation=150 days), goats were subjected to aversive (AVS, n=13), gentle (GEN, n=13) or minimal (M, n=14) handling protocols for 10 minute periods twice daily. The control (M) group did not receive handling treatments and all goats received normal husbandry procedures outside treatment periods. Salivary cortisol measured during the treatment period was higher in AVS goats (mean cortisol (sem) in pg/µl: AVS: 176.7 (18.2), GEN: 119.6 (11.1), M: 126.5 (13.7); P=0.007). Data collection was focussed on mother-offspring behaviors 2h post-partum, placental morphology and colostrum quality. AVS goats were the only treatment group to suffer fetal loss (16% loss vs 0% in GEN and M, P=0.05). Treatment also influenced placental morphology with a tendency for fewer cotyledons evident in placentae from the aversive treatment (AVS: 87.9 (7.8), GEN: 107.1 (7.9), M: 112.1 (9.3), P=0.093), and significantly fewer medium sized cotyledons (AVS: 67.6 (7.8), GEN: 89.3 (6.4), M: 84.3 (5.4), P=0.042). GEN goats displayed more grooming and nosing behaviors towards their young during the first 2 h post-partum (Grooming: GEN: 89.3% (7.1), AVS: 72.6% (7.7), M: 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). There was an overall trend for kids from mothers experiencing the AVS treatment to take longer to stand, reach the udder and suck compared to kids from GEN and M treatment groups. Treatment significantly affected latency to perform play behavior, with kids from AVS goats taking on average 25 min longer to play for the first time than kids from GEN and M treatment groups (P<0.001). The results show that handling during gestation affects placental morphology, fetal survival and post-partum maternal behaviors, and influences kid behavioral development. Such results have important animal welfare implications, demonstrating that negative handling of pregnant females results in poorer placental quality with potential for fetal loss. It also demonstrates the beneficial effects of positive handling on enhancement of maternal behaviors.

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

1. Introduction

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

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

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

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

2. Materials and methods

2.1 Ethical Statement

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

2.2 Animals, housing and feeding

Forty mixed breed (Saanen x Toggenburg) primiparous goats were used in this study. Following an ultrasound scan at approximately 60 days post service 36 were confirmed as bearing twins, and four as single-bearing. In the barn used for the experiment the goats were initially housed as one single group (as they had been prior to selection). All goats were familiar to each other. The research barn was naturally ventilated with deep straw bedding. Following acclimatisation to the new barn, goats were randomly allocated to one of three handling treatment groups (aversive, gentle and minimal) and put in one of three identical pens per treatment group (4-5 goats per pen, 2.5m wide, 5.0m long) based on body weight (Fig 1). Mean body weight was 40kg±0.86 (range 30.6-53.2kg). Their condition score (as determined using the Langston University method http://www.luresext.edu/goats/research/bcshowto.html) averaged 2.27±0.04 (range 1.75-2.75). Three singleton goats were allocated to the control group (minimal) and one to the aversive treatment group, this decision was based on body weight distribution within groups. Goats remained in these smaller pens during the treatment period. Pens had barred partitions so groups could make contact with each other but only within treatment (Fig 1). Upon completion of the treatment period these partitions were lifted so that each treatment group had a larger area for the remainder of gestation. Goats remained in these larger pens (7.5m wide, 5.0m long; 2.7-2.9m^2 available per goat) for kidding and post-partum data collection. Thus immediately pre-, during and post the treatment period goats remained within treatment group. Post-kidding and data collection goats and kids were moved to large post-kidding pens (7.5m wide, 5.0m long). Goats were fed a complete gestation and lactation diet as concentrate (13.2 MJ ME/kg DM, 20% CP, Harbro Ltd) which was fed in quantities according to calculated requirements for maintenance and stage of gestation and lactation. Silage hay and fresh water were available ad libitum. As the handling part of the experiment was the treatment, it was important that the shed accommodated these treatments with the least amount of effects transferring between groups, thus all three treatment groups were located in separate areas within the shed. Appropriate partitions were placed between the treatment groups (with minimum disruption to ventilation). Two handling pens were constructed at either end of the shed with the pen intended for the aversive treatment located in an outside arena. Artificial lighting provided an 8:16h light:dark regime with lights on at 8am in addition to any natural light that entered the building via ventilation openings. Staff were present 24 h a day during kidding when artificial lighting was provided continuously. To acclimatise the goats to this regime, artificial lighting provision was gradually increased one week prior to kidding due dates. Temperature and relative humidity (RH) within the shed was monitored via data loggers (Tinytag Gemini data loggers. Tinytag©) and averaged 5.3°C±0.06 and 83.3% RH ±2.00 during gestation and 11.2°C ±0.04 and 78.2% RH ±0.13 during kidding.

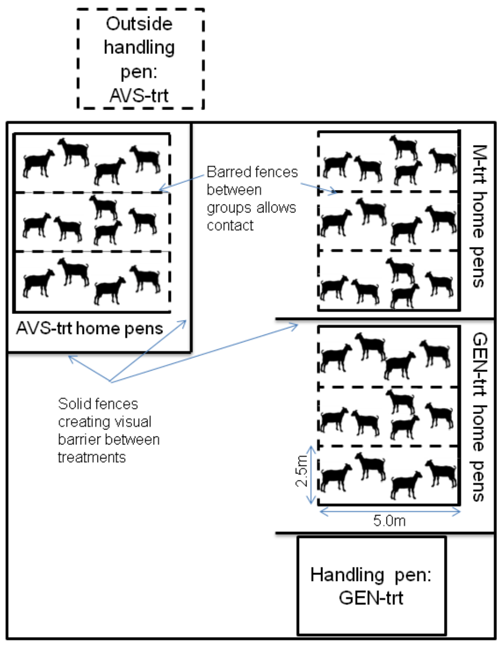

Fig 1. Diagram (not to scale) of experimental barn showing the pen arrangement and group sizes during the treatment period. Solid-sided partitions maintained a visual barrier between treatment groups, whilst barred partitions between pens within treatment allowed groups of goats to make contact. These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

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

2.2.1.1 *Cortisol and glucocorticoid metabolite analysis*

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

Although saliva sampling is generally considered a non-invasive method to assess HPA-axis activation, it did involve gentle restraint of the goats and therefore faecal samples were also collected from the home pens to complement the saliva sampling at a group level. Samples were as fresh as possible, collected in labelled zip-lock plastic bags and frozen at -20°C until analysis. Faecal GM extraction and analysis was carried out following the methodology described by Palme et al. (2013). Briefly, 0.5g of faeces was transferred to a 15ml tube and 5ml 80% methanol was added. The tube was vortexed for 30 min on a multivortexer and centrifuged for 15 min at 2,500 x *g*. The supernatant was then diluted 1:10 in assay buffer (Trishydroxyaminomethane, Sodium chloride, Bovine serum albumin, Tween 80, pH7.5) and faecal GM concentration was measured using enzyme immunoassay (EIA) (Kleinsasser et al., 2010), read on a spectrophotometer (Thermo Scientific Multiskan FC Microplate Photometer) at 450nm. Faecal GM concentration was standardized to the weight of the fresh faeces used for the extraction (ng of faecal GM per g of fresh faeces). Quality control samples were included on every plate for intra- and inter-assay coefficients (CV = 18% and 11% respectively).

2.2.2 Kidding data collection

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

2.2.2.1 Behavioral observations

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

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

2.2.2.2 Kid temperature, weight and body size measurements

Thirty-min after birth first born kids were marked for birth order using colored sticky tape placed above the hock of the left hind leg, for all kids the navel was disinfected with iodine solution and rectal temperature (T30) recorded using a digital thermometer (BF-169 Flexible tip digital thermometer, Farlin Infant Products Corporation, Taiwan). Rectal temperature was measured again 2 h after birth (T2h) and repeated 24 h after birth (T24h). At 24 h of age kids were weighed, sexed and crown to rump length was measured (the length from the crown of its head to the base of its tail). From these measurements, ponderal index (PI; body weight (kg)/crown-rump length $(m)^3$) and body mass index (BMI; body weight/crown-rump length^2) were calculated for each kid.

2.2.2.3 Placentae collection, dissection and cortisol extraction

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

2.2.2.4 Colostrum collection and analysis

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

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

2.3 Statistical analysis

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

3.0 Results

3.1 Glucocorticoid concentration during treatment period

Five goats (three from the M, two from AVS) returned salivary cortisol levels for one of their samples above the level of detection 999 pg/μl and these outliers were excluded from analysis. AVS goats had significantly higher salivary cortisol concentrations over the treatment period than goats from the GEN and M groups (mean cortisol (sem) in pg/μl: AVS: 176.7 (18.2), GEN: 119.6 (11.1), M: 126.5 (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, P=0.048), with a general elevation over the five-week treatment period, peaking at week 4 (average cortisol (ng/g) ± sem: Week 1: 128.3 (11.1), Week 2: 112.4 (9.2), Week 3: 156.0 (20.0), Week 4: 163.7 (18.9), Week 5: 139.4 (12.7)), however there were no effects of treatment ($F_{2,6}$=1.09, P=0.394).

3.2 Fetal loss and litter size

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

3.3 Placental traits

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

Table 2. Placental traits and cortisol levels (means and standard error of the difference (sed)) comparing data from Minimal (n=14), Aversive (n=12) and Gentle (n=13) handling treatment groups. 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

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

3.4 Maternal behavior in the first 2 h post-partum

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

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

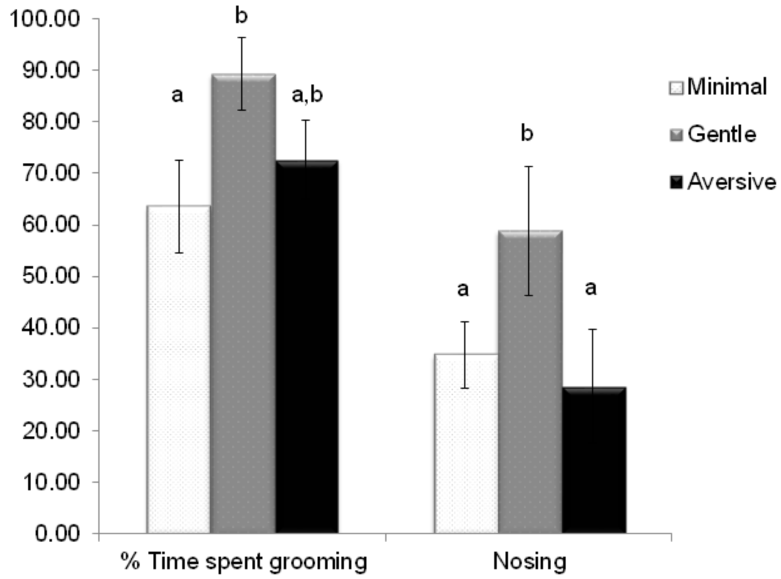

Fig 2. Plot demonstrating the effects of prenatal handling treatments on mean percentage of observation period goats spent grooming their kids (±sem) and mean number of times goats nosed their kids (±sem) during the first 2h observation period after the birth of the second kid. Bars with different letter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

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

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

Negative maternal behavior was displayed rarely, with butting, biting or pushing of kids restricted to only three goats, with only one incident each (data not shown). Actively withdrawing from kids whilst they were at their mother's head was also rarely exhibited, as were behaviors that prevented the kid from sucking. There were no differences between treatment groups in these negative maternal behaviors (Table 3).

3.5 Colostrum IgG

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

3.6 Kid behaviors

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

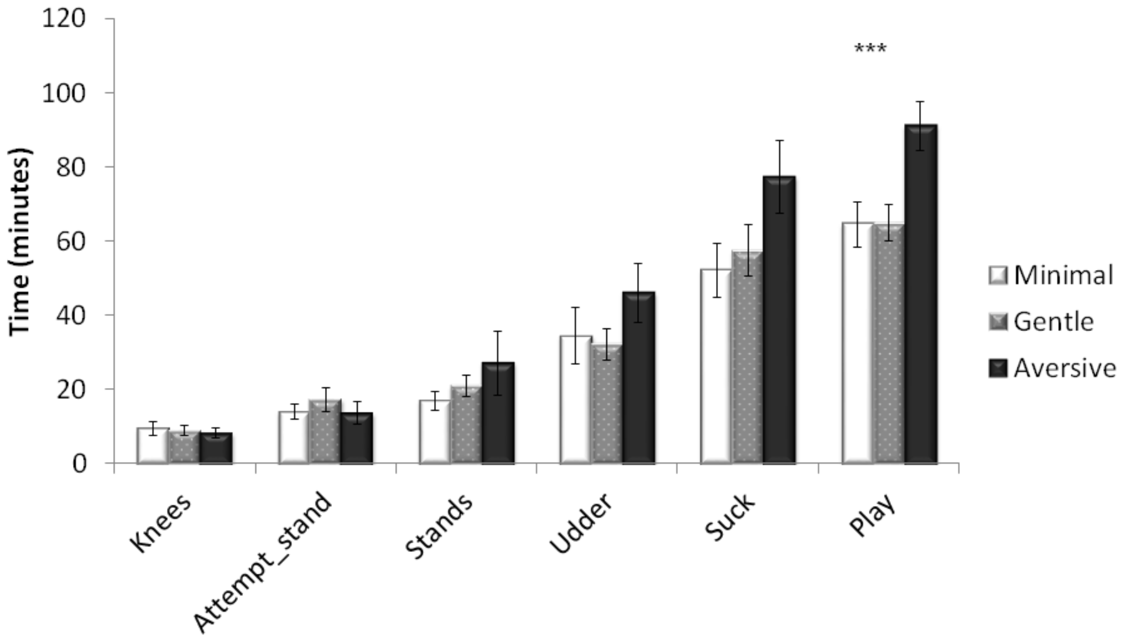

Fig 3. Influence of prenatal handling treatments on latency for kids to perform first time behaviors. Data presented are means (±sem) adjusted for twin and birth interval. *** P<0.001

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

3.7 Kid weight, shape and temperature

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

3.8 Correlations

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

5. Discussion

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

5.1 *Fetal loss, placental morphology and cortisol*

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

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

5.2 Mother-offspring behavior

5.2.1 Maternal behavior

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

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

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

5.2.2 Kid Behavior

The process of birth stimulates the neonate and a sustained period of arousal promotes exploration of the mother's body and perception of sensory cues to facilitate finding the udder (Nowak and Poindron, 2006). As with most precocial and semi-precocial neonates, those that are quick to get to their feet, reach the udder and suck colostrum are those that are most likely to survive (Fraser, 1990; Edwards and Broom 1982; Dwyer, 2003). The lack of energy reserves and the need to maintain homeothermy means colostrum ingestion is a priority (Mellor and Stafford, 2004). The AVS-treatment influenced kid behavioral development with kids from goats experiencing AVS-handling demonstrating a trend towards increased latencies to reach the udder and suck successfully. Play behavior (solitary locomotor-rotational play) was displayed by many of the kids within the first 2 h post-partum. However, the latency to display such behavior was much longer in kids from goats experiencing the AVS-handling treatment, which subsequently influenced the frequency of play behavior within the observation period. Early play behavior in sheep (Dwyer & Lawrence, 2005) is known to be independent of maternal behavior, thus this delay in the onset of play may result from some impact of prenatal stress on the neurological or physical development of the kid, rather than as a result of maternal responsiveness. The fetal brain would have been developing during the time the prenatal stress was applied and it is well-established that prenatal stress can have significant effects on offspring cognitive development (Weinstock, 2008). In this study there appeared to be an increasing deficit in the behaviors of AVS kids as behaviors became more complex and required greater coordination. In addition, mammalian play is believed to occur only when animals have sufficient nutrition and other physiological requirements are satisfied (Graham and Burghardt, 2010). Thus, the delayed sucking success in the AVS kids may have impaired their ability to display play responses.

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

6. Conclusions

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

Acknowledgements

The research leading to these results has received funding from the European Union’s Seventh Framework Programme for research, technological development and demonstration under grant number 266213; project Animal Welfare Indicators (FP7-KBBE-2010-4). Scottish Government also supports several of the authors. We are grateful to Yeoman's farm for the supply of dairy goats for participation in this study and for the assistance of farm and technical staff during the project.

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