

Impact of maternal stress and nutrition on behavioural and physiological outcomes in young lambs

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Abstract

The pre-natal period is of critical importance in defining how individuals respond to their environment throughout life. Stress experienced by pregnant females has detrimental effects on offspring behaviour, health and productivity. The sheep (*Ovis aries*) has been used as a model to inform human studies; however, in a farming context, the consequences for the lamb of stress experienced by the ewe have received less attention. The stressors that pregnant ewes are most frequently exposed to include sub-optimal nutrition and acute and chronic stressors related to husbandry and the environment. This review focuses upon the young sheep, from around 100 days old until adulthood and uses material identified from a systematic survey of the literature relating to production-relevant maternal stressors and lamb outcomes. Overall, the results demonstrated that stressors imposed upon the ewe altered progeny behavioural and physiological responses. However, detailed analysis of the literature shows several deficiencies in the field, as a whole, which greatly limit the ability to draw conclusions as to how welfare may be affected by pre-natal challenges in commercial sheep. These deficiencies included a lack of consistency in response due to the variety of both stressors imposed and responses measured. Key gaps in knowledge include the impact of ewe disease during pregnancy on outcomes for their progeny and more generally how different commercially relevant stressors interact. Furthermore, there is a need to develop a systematic series of behavioural and physiological measures that can be integrated to provide a holistic and practically applicable picture of offspring welfare.

Keywords: animal welfare, gestation, practical application, offspring response, sheep, stress

Introduction

Previous research has shown that sub-optimal maternal nutrition, stress or ill health during pregnancy can affect how offspring develop before birth, with implications for their later biology (Sinclair *et al* 2016). In farm animals, maternal state may therefore be an important contributor to health, welfare and productivity of progeny, and paying closer attention to gestation management could contribute to improvements in these parameters on farms (Rutherford *et al* 2012).

The long-term consequences of changes in the fetal environment have been well-recognised since the first reports (Barker *et al* 1989) describing epidemiological data linking birth weight and later health in humans. In other epidemiological studies, the children born to mothers who were pregnant during the Dutch famine in 1944–1945 experienced increased incidence of *inter alia*, type II diabetes and cardiovascular disease (Lumey *et al* 2011). Other negative human health outcomes have also been seen following stress during pregnancy (eg King *et al* 2012). These adverse effects are generally classified under the developmental origins of health and disease hypothesis (Gluckman & Hanson 2004) and are likely mediated by epigenetic, non-

Mendelian inheritance (Ford & Long 2012). Amongst the variety of animal models used to investigate underlying mechanisms, the sheep (*Ovis aries*) has proved popular since it is similar to the human in respect of maternal and fetal sizes, organ development and maturity at birth (Luther *et al* 2005). To date, however, less emphasis has been placed upon the consequences of disturbances to the fetal environment for the health and welfare of the offspring than on endpoints associated with cardiovascular disease and diabetes. This is of increasing relevance as the current status of legislation on the welfare of fetal animals (for a review, see Campbell *et al* 2014) does not reflect current understanding particularly in the context of post-natal consequences.

In cattle (*Bos taurus*), Arnott *et al* (2012) identified a wide variety of stressors the dam could be exposed to during gestation and which may perturb the uterine environment with adverse consequences for the subsequent welfare and health of the offspring. The stressors identified included under-nutrition, social stress imposed by management practices, such as stocking density, acute stress from handling and transport and thermal stress by being maintained outside the thermoneutral zone. Arnott *et al* (2012)

employed the systematic review process advocated by Sargeant *et al* (2006) to minimise systematic and random errors in study selection. Previously, we (Rooke *et al* 2015) applied the approach of Arnott *et al* (2012) and identified stressors applied to the ewe during gestation which were practically relevant. In Rooke *et al* (2015), the subject material was limited (because of the large number of studies) to measurements of lamb vigour and well-being up to the age of 100 days (weaning) and therefore interpretation of lamb responses in the context of welfare was relatively straightforward. Here, we focus on studies where responses were measurements of behaviour in situations which could be considered fear-inducing or studies where responses in stress physiology were reported. The age at which offspring responses were measured ranged; mainly from weaning to adulthood.

In examining the literature, we have adopted the hypothesis that a stressor or insult to which the ewe was exposed to during pregnancy will influence environmental responsiveness of the offspring, measured either as changes in behaviour or in hypothalamo-pituitary-adrenal (HPA) axis responsiveness, such that adverse consequences for the offspring will ensue. In the review, the term stress(or) applies to any potentially adverse event the ewe is exposed to during pregnancy and fear is defined as a reaction to the perception of actual danger as assessed by fear tests (Forkman *et al* 2007).

Materials and methods

Comprehensive details of the methodologies employed are given in Arnott *et al* (2012) and Rooke *et al* (2015). The following describes first, how relevant information was identified and the review process for all studies (ie both studies reported in Rooke *et al* [2015] and reviewed here). Subsequently, material specific to the present review is described.

Overall review process

Searches

The online database ISI Web of Knowledge was used to search the literature from 1970 as described by Rooke *et al* (2015). The search terms used were designed to combine words relating to sheep and to pre-natal stress and final terms were (pre-natal or perinatal or maternal or fetal or foetal or gestation*) and (stress or programm* or nutrition*) and (sheep or ovine or ewe*). The initial search was carried out in November 2009 and updated until July 2015. Following removal of duplicates, the initial search yielded 3,669 references. After screening for relevance by inspection of title and abstract (2,388 obviously irrelevant references discarded), the remaining references were examined in more detail. Studies measuring solely fetal outcomes were excluded. References were thus retained if post-natal outcomes were measured on the offspring in response to manipulation of maternal nutrition or the application of a stressor to the dam.

Quality assessment

As recommended by Sargeant *et al* (2006), a quality assessment of studies was made. References were selected for inclusion using the following criteria (Arnott *et al* 2012): treatment intervention adequately described; inclusion of a suitable control; use of a large enough sample size; appropriate statistical methods; avoidance of data repetition (eg where components of a single study are reported in several papers); exclusion of conference abstracts/proceedings. Studies remaining at this point formed the raw material for detailed review (n = 98).

Review process

The remaining studies were first classified into the following categories according to outcomes measured in offspring: welfare; birth weight/growth; reproduction; physical defects; others not encompassed by the above groups. Based on the welfare-related aims of the overall review process, and the relatively large number of references, a decision was taken that the first two categories (welfare outcomes and birth-weight/growth) would form the raw material for review. A more detailed inspection of the offspring outcomes considered to potentially influence welfare yielded the following: behavioural changes; adverse effects on bodyweight and rate of weight gain at key stages of life; survival itself and relevant changes in physiological state, such as the ability to thermoregulate, immunoglobulin G (IgG) status of the neonate and changes in the HPA axis. Detailed analysis revealed that outcomes could be classified into two groups. In one group (n = 83), the outcomes were largely directly relevant to neonatal survival and included the ability to thermoregulate and behavioural indicators of both lamb vigour and ewe-lamb bonding. These studies formed the subject matter of the previous review (Rooke *et al* 2015). The second group which included behavioural responses together with studies which measured some aspect of HPA function potentially relevant to welfare, are considered in this review (n = 21; 6 studies contained subject material relevant to both reviews).

Classification of subject matter for review

Studies were initially classified according to the pre-natal treatment applied using the nine hazard categories identified by Arnott *et al* (2012). In the current review, nutrition; social environment, husbandry practices; environmental parameters; infectious environment and maternal health; and artificial challenges (involving exogenous manipulation of HPA axis function) were relevant. Due to the relatively small number of studies, the pre-natal treatments were classified as (i) nutritional (N; either under- or over-nourishment); (ii) stress (S; whether behavioural or physiological) or other (O).

The offspring outcomes were classified as either physiological or behavioural responses to challenge. Physiological outcomes were classified as either (i) changes in baseline adrenocorticotrophic hormone (ACTH) or cortisol concentrations which could be considered as indicators of chronic stress status in contrast to (ii) assessment of HPA axis responsiveness to challenge with either: corticotropin-releasing hormone (CRH) plus arginine vasopressin (AVP); ACTH; or social isolation which could be considered as responses to acute stress. Responses were measured by

Table 1 Summary of studies included in review giving treatments imposed during gestation on ewes and their timing (days, where day = 0 is mating) and the age (months) and gender of young sheep (F, female; M, male; MC, castrate male) when tested.

Study	Reference	Ewe		Young sheep	
		Treatment*	Timing during gestation	Age	Gender
<i>Under (UN) or over (ON) nutrition</i>					
N1	Bloomfield <i>et al</i> 2003	UN: 0.02 or 0.04 requirement	105–115 or 105–125	30	F
N2	Erhard <i>et al</i> 2004	UN: 0.5 requirement	0–95	18	F, M
N3	Corner <i>et al</i> 2005	UN: 0.6 requirement	64–132	12	F
N4	Gardner <i>et al</i> 2006	UN: 0.5 requirement	1–30	12	F, M
N5	Chadio <i>et al</i> 2007; Simitzis <i>et al</i> 2009	UN: 0.5 requirement	0–30; 30–100	2, 3, 4, 5, 6, 10	F, M
N6	Hernandez <i>et al</i> 2009	UN: 0.5–0.8 requirement	–60–30	4, 18	F, M
N7	Hernandez <i>et al</i> 2010	UN: 0.5–0.8 requirement	–60–30; –60–0; 0–30	4, 18	F, M
N8	Long <i>et al</i> 2010	UN: 0.5 requirement	28–147	12	F
N9	Wallace <i>et al</i> 2011	UN: 0.75 requirement; ON: 2.2 requirement	7–147	9, 18, 24	F, M
N10	Long <i>et al</i> 2012	ON: 1.5 requirement	–60–147	20	F, M
N11	Oliver <i>et al</i> 2012	UN: 0.5–0.8 requirement	–60–30	4, 10, 18	F, M
N12	Donovan <i>et al</i> 2013	UN: 0.5–0.8 requirement	–60–30	18	F, M
<i>Stress</i>					
S1	Sloboda <i>et al</i> 2002, 2007	Betamethasone	104; 104–125	6, 12, 24, 36	F, MC
S2	Roussel <i>et al</i> 2004	Isolation (1 h; 2× weekly)	112–147	1, 8	F, M
S3	Roussel-Huchette <i>et al</i> 2008	Isolation (1 h; 2× weekly) Isolation and transport	115–147	4	F, M
S4	Coulon <i>et al</i> 2011	Aversive handling	115–147	1	F, M
S5	Long <i>et al</i> 2013	Dexamethasone	103–104	16, 28 (F2) 6	F
S6	Coulon <i>et al</i> 2014, 2015	Isolation; mixing; transport; dog handling; sham shearing; delayed feeding (total n = 16 per ewe)	94–103	1	F, M
<i>Other</i>					
O1	Erhard & Rhind 2004	Sewage sludge containing parachlorobenzoates	0–147	5	F, M
O2	Fisher <i>et al</i> 2010	Lipopolysaccharide	135; 135–137	5, 18	F, M (5 months only)
O3	Gutleb <i>et al</i> 2011	Parachlorobenzoates	0–147	1	F, M

* UN and ON are expressed as a proportion of the requirement of the ewe and conceptus for energy.

changes in plasma ACTH and cortisol concentrations. Changes in ACTH and cortisol concentrations in response to CRH and AVP are indicative of both pituitary and adrenal responsiveness whereas cortisol response to ACTH specifically quantifies adrenal responsiveness.

Behavioural outcomes measured were classified into those that assessed either emotional reactivity or cognitive flexibility. Emotional reactivity outcomes were measured as

either responses to novel environments (isolation; novel arena or confinement in a weight crate) or to novel stimuli (novel object; startle stimulus or human proximity). Cognitive flexibility outcomes were measured, for example, by the ability of the offspring to learn that the position of a food reward in a T-maze had changed, usually quantified by how many attempts the offspring required to complete two consecutive runs successfully.

Table 2 Changes in basal concentrations of cortisol and ACTH in young sheep which had dams exposed to gestation treatments (responses are with reference to controls); (identity of studies given in Table 1).

Study	Cortisol	ACTH
N1	Decrease	Tendency to increase
N4	No difference	Tendency to increase in males
N5	No difference	No difference
N7	No difference	
N8	No difference	No difference
N9	No difference	Under-nutrition; no difference: Over-nutrition; increase at 9, 18 months
N10	Increase	No difference
N11	No difference	Increase in males at 18 months only
S1	Increase in males at 12 months only	Increase in males at 24 months only
S2	Increase at 1 month; no difference at 8 months	
S5	Increase in F1 and F2	No difference in F1; increase in F2
O2	No difference	

Results

The studies which make up the subject matter of the review are summarised in Table 1 and grouped as: alterations to maternal nutrition (N1–N12); exposure of the ewe to behavioural or physiological stress (S1–S6); others (O1 to O3) which included the potentially toxic effects of para-chloro benzoates and bacterial lipopolysaccharide. In general, the nature of the treatment imposed on the ewe (Table 1) varied within each of the three groups in regard to both timing and severity as did the age at which responses were assessed (ranging from 1 to 36 months in age). Due to the range in ages at which responses were measured, the maturity of offspring studied ranged from weaning to the mature adult. Therefore, the terms young sheep or offspring rather than lamb will be used from here onwards. Across the studies (Tables 2 to 6 describe outcomes for each of the response types), gestation treatments imposed on the ewe produced significant changes in offspring response in 47 out of 71 (67%) of the outcomes. When these outcomes were separated into physiological (Tables 2 and 3) and behavioural (Tables 4 to 6), fewer significant responses were noted for physiological (55%; 21 of 38) than behavioural (84%, 26 of 33) outcomes (Chi-squared, 4.0; $P < 0.05$). Overall, therefore, stress treatments imposed on the ewe did induce changes in behavioural and physiological outcomes in the young sheep and behavioural responses may be more sensitive indicators than physiological responses.

Physiological outcomes

Chronic responses of the young sheep were defined as changes in baseline (pre-test) cortisol or ACTH concentrations (Table 2). There were few differences in baseline

hormone concentrations when ewes were under-nourished. Only in the study of Bloomfield *et al* (2003) did offspring cortisol and ACTH concentrations change as a result of maternal under-nutrition and, indeed, the decrease in cortisol only occurred when severe under-nutrition was applied to the ewe for a short period of time (10 days) in late gestation. Other studies in which ACTH was increased by under-nutrition occurred only in males (Gardner *et al* 2006; Oliver *et al* 2012) at 12 and 18 months of age, respectively and, thus, their practical impact would be limited because most male lambs would have been sent for slaughter at a younger age. Additionally, most studies were carried out with intact males and therefore there must be doubt over their application to castrates. When ewes were treated with betamethasone (Sloboda *et al* 2002), dexamethasone (Long *et al* 2013) or isolated at weekly intervals (Roussel *et al* 2004), all in late gestation, then responses were more pronounced. Possibly, therefore, timing and nature of challenge is important as offspring basal hormone concentrations were only perturbed when the challenges were (i) applied in late gestation (after 100 days) and (ii) acute (including severe UN for 10 days; Bloomfield *et al* 2003). The study of Long *et al* (2013) is notable as there were increases in cortisol concentration not only in young sheep born to ewes administered a single dose of dexamethasone (F1 generation) but were also inherited and exhibited by their progeny (F2 generation).

A different pattern of response was noted when young sheep were acutely challenged with CRH/AVP or cortisol to assess HPA function (Table 3). There was little evidence for differences due to treatment in offspring cortisol concentration to either ACTH or a behavioural

Table 3 Responses of young sheep which had dams exposed to gestation treatments to physiological or behavioural challenges.

Study	Challenge		
	CRH/AVP	ACTH	Social isolation
N1	Increase in ACTH after 10 but not 20 day ewe feed restriction		
N4	Increase in ACTH and cortisol in males but decrease in females		
N5	Increase in ACTH and cortisol at 2 months old only		
N7			Decrease in cortisol
N8	No difference	No difference	Decrease in cortisol
N9	No difference		
N10	Increase in ACTH	No difference	No difference
N11	Increase in ACTH in 18 month old females only		
S1	Increase in ACTH in 24 month old males; increase in cortisol in 6, 12 month old males but decrease at 36 months		
S2			No difference
S5	ACTH and cortisol decreased in both F1 and F2 generation	No difference	
O2		Increase in cortisol	

Responses are given as differences in the increase in plasma adrenocorticotrophic hormone (ACTH) or cortisol concentration (expressed as area under curve) relative to young sheep from control ewes. Challenges were arginine vasopressin (AVP)/corticotropin-releasing hormone (CRH); ACTH; or isolation (identity of studies given in Table 1).

Table 4 Behavioural responses when exposed to a novel environment of young sheep which had dams exposed to gestation challenges (responses are changes with reference to controls); (identity of studies given in Table 1).

Study	Novel environment		
	Social isolation	Arena	Weigh crate
N2	No difference		Males displayed increased activity in crate
N3		Fewer young sheep produced high-pitched bleats	
N5	No difference		
N7	Fewer attempts to escape from enclosure (4 months old only)		
S2	Increase in number of jumps (8 months old only)	Less time spent close to arena entrance	
S3	Increase in number of jumps (1 month old only)	Decrease in number of jumps (1 and 3 months old)	
S6	No overall difference due to pre-natal stress. Stress reactivity of ewe influenced response in absence of pre-natal stress		
O1		Increase in time spent exploring arena in males only	Increased number of vocalisations and decrease in activity in crate
O3			Increases/decreases in activity observed in crate; response depended on parachlorobenzoate

Table 5 Behavioural responses, when exposed to a novel stimulus, of young sheep which had dams exposed to gestation challenges (responses are changes with reference to controls); (identity of studies given in Table 1).

Study	Object	Novel stimulus	
		Startle	Human
N2	Increased latency to approach object	Locomotion activity increased in males but decreased in females	
N3			No difference
N5	No difference		
S2	Spent more time close to object and more time sniffing object	Increased activity in response to stimulus (8 months old only)	
S3		Spent more time within 2 m of object	
S4		Tendency for increase in flight distance from stimulus	Reduced vocalisation
S6		Pre-natal stress treatment lambs spent more time distant from object. Pre-natal stress offspring of high stress reactive ewes spent less time close to object than offspring of low stress reactive ewes; no maternal reactivity effect in non-stressed offspring	Pre-natal stress effects only in offspring of high stress reactive ewes: presence of human reduced locomotor activity, vocalisation, exploration Presence/absence of human did not change responses in offspring of low stress reactive ewes
O1	Increase in time spent exploring (males only)		

Table 6 Behavioural responses relating to cognitive flexibility of young sheep which had dams exposed to gestation challenges (responses are changes with reference to controls); (identity of studies given in Table 1).

Study and test	Response	Comment
<i>T maze</i>		
N2	Initial side preference	Reduction in right side choice in treatment offspring; only significant in females
	Task reversal	Only male treatment offspring failed to improve learning speed between reversals
N6	Initial side preference	Reduction in left side preference in male singletons; reduction in right side preference in female twins
	Task reversal	No differences
<i>Blind maze</i>		
S4	Latency to solve	No differences
	Re-test response	No differences
S6	Latency to solve	Pre-natal stress offspring slower to complete test
	Re-test response	Pre-natal stress offspring slower to complete test

challenge. However, the studies of Long *et al* (2010, 2012) are notable again as the only studies in which CRH/AVP, ACTH and behavioural challenges were applied to the same groups of young sheep. Although the pattern of response differed between studies (probably because different nutritional treatments were imposed upon the ewe), offspring responses to physiological challenge differed from those to social isolation.

Most studies assessed the effect of CRH/AVP challenge on offspring HPA axis function. Changes in HPA axis outcomes were noted in seven of nine studies; however, both increases and decreases in ACTH and cortisol response were noted. This variability in response is likely due, in part, to the age at which the animals were tested. Sloboda *et al* (2002, 2007) re-tested the same young sheep which had dams challenged with betamethasone in late

gestation at 6, 12, 24 and 36 months of age. While there was no effect of treatments imposed on responses to CRH/AVP challenge at 6 months of age, at 12 months, maternal betamethasone administration increased offspring cortisol response but at 24 months of age an increase in ACTH responsiveness and at 36 months a decrease in cortisol response was recorded; thus, HPA function as defined by response to CRH/AVP challenge, was clearly dependant upon the age of the animal at test. Finally, Long *et al* (2013) reported decreases in ACTH and cortisol response to CRH/AVP challenge in both the daughters and grand-daughters of ewes challenged with dexamethasone.

Behavioural outcomes

Emotional reactivity

Overall, in nine of the ten studies listed in Tables 4 and 5, young sheep which were exposed to pre-natal treatments exhibited responses to behavioural challenge; the exception being the study of Chadio *et al* (2007). The hypothesis most commonly stated in these studies was that pre-natal treatments increased emotional reactivity, further interpreted as increased fearfulness. In the studies reviewed, a consistent pattern does not emerge. The two practically important pre-natal treatments, under-nutrition and imposition of various stressors on the ewe, had different characteristics. Under-nutrition was, in general, applied continuously for more than 30 days and from an early stage of gestation. This contrasted with pre-natal stress treatments which were imposed in the last third of gestation and although an individual treatment may have been imposed at regular intervals (weekly), each individual challenge was applied typically for only an hour or less, with this acute exposure differing from the chronic (long-term) nature of under-nutrition. Within pre-natal stress treatments, different treatments probably imposed different severities of challenge. For example, Roussel-Hachette *et al* (2008) considered that isolation and transport of the ewe was more severe than isolation alone, while Coulon *et al* (2015) concluded that different stress treatments applied randomly were a more severe challenge than the same stress treatment applied at regular intervals (eg Roussel-Hachette *et al* 2008). Finally, the nature of the control treatment may be important when interpreting across different studies: Coulon *et al* (2011) employed a positive control (gentle handling of the ewe), while the controls in most other studies consisted of no stress treatment.

The effects of under-nutrition on offspring emotional reactivity were not consistent across studies. Erhard *et al* (2004), in the most detailed study, found evidence for increased emotional reactivity in offspring of under-nourished ewes; treatment lambs took longer to approach a novel object and in males only, activity was increased when confined in a weigh crate after exposure to a sudden stimulus. Simitzis *et al* (2009), however, reported no differences and both Corner *et al* (2005; reduction in high-pitched bleats in a novel arena) and Hernandez *et al* (2010; reduction in escape attempts during social isolation) interpreted the behavioural changes they observed as reductions in emotional reactivity.

When pre-natal stress treatments were applied, there were indications of changed emotional reactivity. Pre-natal stress treatments, at eight months of age, increased the number of jumps during social isolation, the time spent in proximity to and sniffing a novel object and activity after exposure to a startle stimulus (Roussel *et al* 2004). In Roussel-Hachette *et al* (2008), pre-natal stress treatment reduced the number of lambs which produced high-pitched bleats in a novel arena test but increased the time lambs spent close to an umbrella used as a startle stimulus. Coulon *et al* (2011) compared gentle and aversive maternal handling treatments and concluded from an increase in passive responses (reduced locomotor activity and vocalisation) in a human approach test and increased flight distances in response to a novel object/startle stimulus that emotional reactivity of young sheep was increased. Similarly, when random stress treatments were applied to the dam, Coulon *et al* (2015) found that pre-natal stress increased the time spent distant from the novel object. The differences between the studies of Roussel *et al* (2004) and Roussel-Hachette *et al* (2008) and those of Coulon *et al* (2011, 2015) may be that as noted above either the treatment was more severe (Coulon *et al* 2015) or a positive control was used (Coulon *et al* 2011).

Other factors that could have influenced response were the severity of the test used to evaluate the young sheep or the stress reactivity of the ewe upon which stress treatments were imposed. Roussel *et al* (2004) found no differences in offspring response when ewes were selected for low and high reactivity but noted that the ewes habituated to the repeated stress treatments imposed. In contrast, Coulon *et al* (2015) found that young sheep born to ewes selected for high-stress reactivity (based on behavioural and cortisol response to social isolation) were more reactive in human approach and object tests than young sheep born to ewes selected for low reactivity; as the ewe treatments were randomly imposed there was less opportunity for ewes to habituate. Differences in offspring response between different tests imposed have also been related to the severity of the test. These have been ascribed to a ceiling effect where responses of both control and treatment offspring to a more severe test masked treatment differences. Thus, Erhard *et al* (2004) noted greater between-treatment differences when a startle stimulus was applied than in an isolation test and similarly Coulon *et al* (2014) found responses were greater in a human approach/novel object test than in a social isolation test.

Cognitive flexibility

Table 6 summarises four studies which tested aspects of cognitive flexibility of young sheep born to ewes exposed to different treatments. Since the methods used to test the offspring from under-nourished dams (Erhard *et al* 2004; Hernandez *et al* 2009; T-maze) differed from those that had mothers exposed to stress treatments (Coulon *et al* 2011, 2015; maze with fixed blind alleys), then responses differed between studies. For under-nutrition, Erhard *et al* (2004) reported a sexually dimorphic response where male sheep born to under-nourished ewes were slower to learn a reversal task than control males but there were no differences between females.

However, Hernandez *et al* (2009) found no differences between treatment groups, but at six months of age, females were quicker to learn than males. Similarly, while Coulon *et al* (2011) found no differences in cognitive ability between groups, Coulon *et al* (2015) found that young sheep from dams exposed to stress were slower to complete a maze test both during learning the maze and upon subsequent re-test. In a test of judgment bias, Coulon *et al* (2015) also concluded that young sheep from dams exposed to stress had a more pessimistic bias than control sheep and suggested that this could indicate a poorer state of welfare. In contrast to emotional reactivity, offspring of high emotional reactivity ewes were not different in cognitive flexibility to those from low emotional reactivity ewes.

Discussion

Animal welfare implications of pre-natal challenges in sheep

In the current review, the responses of young sheep from approximately weaning to maturity, after exposure of ewes to challenge during pregnancy, have been summarised. As the focus of the review is on the offspring, the consequences of pregnancy challenges for the dam which are an important welfare concern are not discussed here. Overall, there were lasting consequences of maternal challenges for the offspring, despite the variety of challenges imposed on the ewe and the variability of the timing and nature of the tests imposed on the young sheep. The key question for this review is whether these responses have implications for the welfare of the offspring throughout their lifespan and indeed for their own progeny. In assessing welfare implications, the conclusions of the current review cannot be viewed in isolation but must be integrated with the conclusions of the preceding review which addressed responses of the lamb from birth to weaning (Rooke *et al* 2015). The question of whether pre-natal insults increase the risk of adverse welfare outcomes in commercial sheep flocks also requires that some consideration is given to likely exposure scenarios. Applications of risk assessment to animal welfare issues are complex (Smulders & Algers 2009; EFSA 2012, 2014) and are often hampered by a lack of relevant data. Put most simply, a risk assessment requires information on two aspects. Firstly, the characterisation of the biological effect that any identified and defined hazard has on a target population, and secondly, the extent of exposure of that population to the hazard. In relation to gestation treatments, hazards can be viewed as being applied to two target populations; the ewe and her developing fetal progeny. This review, and the previous one (Rooke *et al* 2015), reveal the current level of understanding of the first issue (hazard effects).

Exposure to hazards

Overall, the existing literature provides only very weak understanding of hazard effects. In relation to the second (exposure), remarkably little is known about the severity and exposure prevalence of putative hazards for pregnant ewes. This means that it is only possible to speculate about exposure scenarios. Due to the seasonality of herbage

growth, breeding ewes kept outdoors in winter in temperate production systems (Robinson *et al* 2002) are likely to experience periods of under-nutrition in early to mid gestation. Droughts will have similar effects in other production systems. However, in the UK, nutrition is normally increased in late gestation by either housing and feeding ewes supplementary feed or by timing pregnancy such that increased nutritional demands in late gestation are met by increased pasture availability in spring. Under-nutrition, which was severe in nature and which was applied late in gestation, would not normally be encountered in practice in the UK; the most likely scenario would be extreme weather events, such as snowfall or flooding, which would prevent access to grazing thus causing acute under-nutrition. Ewes encounter a variety of aversive events during pregnancy, the frequency and severity of which are dependant upon individual farm management. These events include: movement and handling using sheep dogs to unfamiliar grazing or housing indoors; unfamiliar housing itself; mixing with unfamiliar ewes; transport; restraint and social isolation; shearing. The number of different aversive event types encountered will vary depending on the system. For example, ewes maintained outdoors throughout gestation will be exposed to fewer aversive events imposed by management than ewes housed in late gestation. However, the very nature of farm management means that to the ewe some aversive events are unpredictable and therefore ewes would be unable to habituate to them. Thus, of the protocols used to impose stress treatments on ewes, the random protocol described by Coulon *et al* (2014) more closely resembles that which would be encountered in practice, although it is unlikely that ewes would be exposed to all the events described by Coulon *et al* (2014). The protocols used previously by Roussel *et al* (2004), Roussel-Huchette *et al* (2008) and Coulon *et al* (2011) in which the same aversive treatment was repeated at weekly intervals, would have the potential for habituation. An exposure scenario which has not been addressed in the review for practical reasons is that of disease, although offspring responses after exposure of the ewe to para-chlorobenzoates and lipopolysaccharides (Fisher *et al* 2010; Gutleb *et al* 2011) do highlight the importance of stressors other than under-nutrition, housing and management practices.

The reports considered in this review do encompass the relevant target population and exposure scenarios. However, since in practice, the ewe is likely to experience more than one exposure scenario (eg both under-nutrition and handling) then the absence of studies, which have examined interactions between different exposure scenarios, although necessary to understand the effects of individual exposure scenarios, is an important omission.

Relevance and quality of data

In the risk assessment process, the identification of relevant data is critical. In this review, the tests employed on young sheep could be broadly divided into two classes: measurement of either physiological or behavioural responses. Since physiological responses are measured to investigate mecha-

nistic relationships and report changes in selected (but relevant, eg the HPA axis) body systems then, first, the responses of many body systems which may be relevant are not reported and, secondly, using specific tests precludes any measurement of integrated responses by the animal. Given that behaviour reflects an integration of neurobiological and endocrine changes, it is likely that behavioural tests will be more relevant to assessing animal welfare. It was indeed found that behavioural tests detected more differences in response between treatments than physiological tests, likely for the above reasons and also because many behavioural outcome tests are typically performed. Therefore, more weight should be given to behavioural responses of the offspring in assessing welfare outcomes, but this also requires a clear hypothesis-driven rationale for test selection.

The most important variables in interpreting the relevance of responses to welfare are the age at which the young sheep is tested and its gender. The age of the animal at test is important as plasticity of response was noted in studies where offspring were tested across a range of ages (Sloboda *et al* 2002, 2007; Chadio *et al* 2007; Oliver *et al* 2012). Tests should therefore be carried out at ages relevant to expected major welfare challenges. The welfare challenges expected will differ and be largely dependant on the fate of the young sheep. The majority of males will be destined for slaughter at around six months of age and the major challenges encountered by them will be weaning, subsequent management (eg housing for fattening), transport to the abattoir and slaughter. Thus, for this group of young sheep, tests carried out between three and six months of age are likely to be relevant. The second main group will comprise largely females retained for breeding. For breeding females, major challenges will occur later in life during repeated breeding cycles beginning from as early as six months of age but more likely 18 months of age; therefore, tests later than six months of age are more relevant. The type of test and direction of response is important in this context. The responses reported in Tables 4 and 5 for emotional reactivity are largely interpreted in relation to fearfulness with those tests involving social isolation being considered to be reliable indicators of fearfulness (Forkman *et al* 2007). Increased reactivity is usually interpreted as increased fearfulness but interpretation of reduced reactivity is more ambiguous being alternatively attributed to either reduced fearfulness or an increase in passive fear response. For young sheep, the major adverse welfare-relevant response is likely to be increased fearfulness in response to the situations noted above. In the papers reviewed, only five tested young sheep at the relevant time. When ewes had been previously under-nourished (Chadio *et al* 2007; Simitzis *et al* 2009; Hernandez *et al* 2009, 2010), a significant response, decreased escape attempts by the offspring was observed by one study (Hernandez *et al* 2010). Thus, there is little evidence for increased fearfulness as a result of chronic maternal under-nutrition when tested at an appropriate age. In response to stressors imposed on the ewe, Roussel *et al* (2004) and Roussel-Huchette *et al* (2008) did not find a consistent pattern of change. As pointed out by Coulon *et al* (2015) the stress protocols imposed allowed the

possibility of habituation by the ewe and this should be taken into account in interpreting these studies. Further, in all the above studies, males had not been castrated and therefore the relevance of the results can be questioned as many males destined for slaughter will be castrated. Thus, overall, current evidence does not suggest increased fearfulness in young sheep destined for slaughter as a result of maternal stress imposition, although this conclusion must be heavily qualified because of the small number of studies carried out at the relevant age and the inherent limitations of the studies.

For breeding ewes, especially when grazing, increased emotional reactivity may not be an adverse consequence of pre-natal stress as it may enhance the ability of the ewe to deal with challenges, such as predators in the grazing environment, although as discussed above for the young sheep increased reactivity may not be an advantage when the ewe is exposed to novel environments and challenges, such as housing and transport. Considering responses measured at the relevant age (more than 12 months in age; Erhard *et al* 2004; Corner *et al* 2005; Chadio *et al* 2007; Hernandez *et al* 2009, 2010) and only responses for females, there were few significant responses when the dam was under-nourished. Thus, there is little evidence for adverse effects of under-nutrition on emotional reactivity. Only Roussel *et al* (2004), measured responses of offspring at a relevant age in response to maternal stress challenges and recorded an increase in emotional reactivity at eight months of age. Overall, therefore, under-nourishment of the ewe appeared to have little adverse consequences for young sheep or breeding females but there was insufficient evidence to make any conclusions in respect of stress challenges imposed on the ewe as only offspring of betamethasone-challenged ewes were tested at the relevant age (Sloboda *et al* 2007; Long *et al* 2013). One factor that future research should consider, is the inherent stress responsiveness of the ewe subjected to pregnancy challenges as Coulon *et al* (2015) found that reactivity of young sheep was increased when the dam was classified as having high stress responsiveness. It is possible also that since Long *et al* (2013) found that basal cortisol concentrations were increased in the grand-daughters of ewes exposed to dexamethasone, that high stress responsive ewes (Coulon *et al* 2015) may themselves have been exposed to stress-related events during pregnancy.

Suggestions for future research work and strategies

Overall, it is clear that pre-natal challenges, either in the form of sub-optimal maternal nutrition or maternal stress have the potential to alter, after weaning, aspects of biology that could have implications for welfare. A similar conclusion was drawn following a review of the same literature in relation to pre-weaning lamb outcomes (Rooke *et al* 2015). However, in many studies, it is difficult to draw clear conclusions in relation to the relative welfare status of pre-natally challenged animals versus controls. As it stands now, the research literature in this area does not form a solid basis upon which advice to farmers could be based. Partly, this is of course because the field is relatively young and direct practical relevance is often not the primary motiva-

tion of those conducting the study. Based on these reviews, along with others recently conducted in cattle (Arnott *et al* 2012), poultry (Dixon *et al* 2016) and pigs (Otten *et al* 2015), where similar issues exist, it is possible to provide some suggestions for a way forward. These relate to: i) the choice of treatments; ii) the choice of outcome measures; and iii) possible factors that may modify the effects of pre-natal challenge on welfare outcomes.

Choice of treatments

Work in this area is conducted for two reasons. Firstly, many studies are used to examine basic biology or to inform human relevance. Secondly, studies are conducted to inform considerations of sheep health, welfare and production in commercial practice. The former is much more common, whilst the latter is more useful from an applied perspective. Basing treatments on practically relevant factors runs the risk that the differences between treated and control animals will be too small for statistical significance to be achieved. However, such a finding adds valuable information about animal management and welfare outcomes. The reality is that such 'negative findings' may be harder to publish and do little for the career progression of the researchers involved. However, a good example from recent work with applied relevance (not included in the current review because neonatal outcomes were measured) is that of Averós *et al* (2015) who kept ewes at three different stocking densities (1, 2 or 3 m² per ewe) during gestation and examined the impact of this housing on progeny. Although main effects of stocking density were not significant there were interactions between maternal stocking density and post-natal stress (early separation from dam) such that negative effects of post-natal stress on the offspring were exacerbated by reduced stocking density.

Beyond the specific choice of treatment, the general lack of attempted replication is an important problem. Demonstration of the repeatability of a finding is the cornerstone of science, yet pre-natal stress studies are rarely conducted in the same way twice, and this greatly limits the robustness of conclusions about the reproducibility or generalisability of findings. As with other areas of research (Ioannidis 2005) many of the findings will likely be false. Indeed, Ioannidis (2005) identified characteristics of a research field that increase the likelihood of individual findings being false, and several of these are potential problems in the field of pre-natal stress and animal welfare, including: small studies, small effect sizes, a large number of tested relationships and a high level of flexibility in study design, choice of outcome and methods of analysis. There are likely to be various structural and institutional factors which limit attempts at replicating key findings. Funding bodies and journals are not keen on studies which repeat other work, indeed a replication study can even be criticised on ethical grounds, and career progression is similarly not rewarded by studies that confirm previous findings. This means that conclusions are often drawn on the findings of single studies. These conclusions are particularly precarious in light of the fact that pre-natal stress studies often measure multiple outcomes, with statistical accounting for this being rare.

Despite the fact that the ewe is likely to encounter multiple stressors during pregnancy, for example, under-nutrition and handling, none of the reviewed studies investigated interactions between nutrition and stressors. Further, while Rooke *et al* (2015) found no disease-related studies, the one study reported here (Fisher *et al* 2010) did report changes in lamb physiology following maternal exposure to endotoxins. Both these areas (interactions between stressors and responses to disease challenge) are worthy of further experimentation.

Choice of outcome measures

Pre-natal stress studies often involve measurement of many different outcomes, across various areas of biology. Across the literature, as a whole, there is a lack of consistency in choice and application of outcome measures. Even where the same parameter is assessed individual studies often vary in the exact approach taken. This is particularly notable in relation to tests of emotionality where, unlike the situation in rodents where tests are generally highly standardised (eg open field, elevated-plus maze), there is still substantial variability in test parameters, including arena size and design, and also the nature of outcome measures recorded. Furthermore, there is also a limited degree of prior validation work which allows for variable interpretation of these outcome measures. Another common issue is that many outcome measures are not measured at multiple time-points either in the same or different cohorts of animals. This means that the time-course of biological changes induced by pre-natal challenge is uncertain. A broader issue, which hampers clear animal welfare conclusions, is that individual measures are rarely integrated into some clear understanding of whether the welfare state of the animals involved is overall better or worse as a consequence of the experiences of their mother. Even if it is believed that a physiological or behavioural measure assessed does indeed represent altered emotionality, it is often not clear what the implications of such alterations are for the lifetime welfare of affected animals.

Experimental design factors which may modify offspring outcomes from ewe treatments

Variable post-natal environments

Whilst discussing experimental design issues in developmental plasticity studies, Groothuis and Taborsky (2015) noted that most theoretical frameworks for understanding pre-natal effects rely on a comparison of outcomes under different post-natal conditions. Yet, such a comparison is almost never made in research studies involving captive species and animal welfare issues. In interpreting the studies reviewed one must consider that the welfare relevance of any change in responsiveness may be situation-specific. Thus, increased responsiveness in an environment that induces fear and changed HPA axis responsiveness may not have adverse consequences for welfare where the sheep may be exposed to predation, eg in a hill environment, but will be relevant in situations such as transport, lairage or slaughter of the animal. Similarly, it may be necessary to distinguish between responses which

arise from permanent programming of the HPA axis from those which are expressions of developmental flexibility. Whilst for some species, such as pigs, where the range of environments encountered under production conditions is relatively narrow, sheep are managed in different systems, varying from a semi-wild extensive existence to a more intensive lowland system. Changes that are seen in a research context in housed animals may actually be neutral or even beneficial in an extensive setting.

Maternal effects

In risk assessment terms, it is important to consider whether different ewe breeds or ages can be considered as the same or different target populations for the purposes of drawing conclusions about animal welfare impact. Over 100 different breeds of sheep are used in the UK alone (English Beef and Lamb Executive [EBLEX] 2014) and this range would be extended by consideration of different countries. These breeds vary widely in their productivity and reproductive characteristics, as well as in key aspects of their health, overall robustness and behaviour. Yet it is rare (with some exceptions: Burt *et al* 2007; Rooke *et al* 2010) that studies attempt to expose different breeds to the same treatment. A very tentative conclusion from these studies is that animals selected for lean tissue growth are more sensitive to pre-natal effects as the birth-weight of lambs born to the selected breed in each study was reduced, to a greater extent, by under-nutrition of the ewe. However, more direct testing of this in future studies would be welcome and an important contribution to elucidating the true industry relevance of this area.

Within breeds, differences between ewes in reactivity may also be important determinants of response although evidence is not consistent. While Roussel *et al* (2004) found no effect of maternal reactivity on offspring response, Coulon *et al* (2014) reported that pre-natally stressed offspring of high emotional reactivity ewes were more affected. These differences in response may be related to either breeds used or the methods used to characterise the ewes.

From a practical perspective, another factor which may alter the effects of a standard challenge is maternal parity. Parity effects could occur in two different ways. Firstly, it is possible that previous experience has a mediating effect on how ewes respond to environmental factors. For instance, younger ewes may find handling and housing more stressful during their first pregnancy compared to later. Secondly, body reserves may differ over several breeding seasons altering the impact of a standard level of nutrition. Finally, there are well-known effects of parity on maternal care (Dwyer & Smith 2008; Munoz *et al* 2009). As noted above (in relation to variable ewe temperament: Coulon *et al* 2015) and previously (eg body reserves: Rooke *et al* 2015), other types of variation in ewe biology will likely modulate the impact on fetal lambs.

Animal welfare implications and conclusion

The data gathered together here and in a related review (Rooke *et al* 2015) suggest, as expected from other species, that the nutrition and stress state of pregnant ewes can affect many aspects of their progeny's biology, at birth and throughout their life. In some cases, these changes may even carry-over into subsequent generations. Furthermore, some of the identified changes clearly have implications for animal welfare. However, detailed analysis of the literature shows several deficiencies in the field as a whole which greatly limit the ability to i) draw conclusions about how welfare may be affected by pre-natal challenges in commercial sheep, or ii) suggest ways that these effects could be avoided, or even how maternal treatment during gestation might contribute to improving the welfare of farmed sheep. Suggestions have been made relating to how experimental designs could be improved to aid translation to applied relevance. Particularly in respect of both behavioural and physiological outcomes, there is a need for measures that can be integrated to give a global picture of offspring welfare (ie a stated conclusion that progeny welfare is overall better or worse for the animals concerned).

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