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

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Running title: Maternal stress in sheep and progeny welfare

Abstract

The prenatal 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 about how welfare may be affected by prenatal 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.

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 cardio-vascular disease (Lumey *et al* 2011). Other negative human health outcomes have also been seen following stress during pregnancy (e.g. 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 end-points associated with cardio-vascular disease and diabetes. This is of increasing relevance as the current status of legislation on the welfare of fetal animals (see Campbell *et al* 2015 for review) does not reflect current understanding particularly in the context of postnatal consequences.

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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 thermo-neutral zone. Arnott *et al* (2012) employed the systematic review process advocated by Sargeant *et al* (2006) to minimize 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 (i.e. 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 prenatal stress and final terms were (prenatal 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 3669 references. After screening for relevance by inspection of title and abstract (2388 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 (e.g. where

112 components of a single study are reported in several papers)**;** exclusion of conference

113 abstracts / proceedings. Studies remaining at this point formed the raw material for detailed

114 review (n = 98).

115 *Review process*

116 The remaining studies were first classified into the following categories according to

117 outcomes measured in offspring: welfare; birth weight / growth; reproduction; physical

118 defects; others not encompassed by the above groups. Based on the welfare-related aims of

119 the overall review process, and the relatively large number of references, a decision was

120 taken that the first two categories (welfare outcomes and birth-weight / growth) would form

121 the raw material for review. A more detailed inspection of the offspring outcomes considered

122 to potentially influence welfare yielded the following: behavioural changes; adverse effects

123 on body weight and rate of weight gain at key stages of life; survival itself and relevant

124 changes in physiological state such as the ability to thermo-regulate, immunoglobulin G

125 (IgG) status of the neonate and changes in the HPA axis. Detailed analysis revealed that

126 outcomes could be classified in two groups. In one group (n=83), the outcomes were largely

127 directly relevant to neonatal survival and included the ability to thermo-regulate and

128 behavioural indicators of both lamb vigour and ewe-lamb bonding. These studies formed the

129 subject matter of the previous review (Rooke *et al* 2015). The second group which included

130 behavioural responses together with studies which measured some aspect of HPA function

131 potentially relevant to welfare are considered in this review (n=21; 6 studies contained

132 subject material relevant to both reviews).

133 **Classification of subject matter for review**

134 Studies were initially classified according to the prenatal treatment applied using the nine

135 hazard categories identified by Arnott *et al* (2012). In the current review, nutrition; social

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environment, husbandry practices; environmental parameters; infectious environment and maternal health; and artificial challenges (involving exogenous manipulation of HPA axis function) were relevant. Because of the relatively small number of studies, the prenatal treatments were classified as (a) nutritional (N; either under- or over-nourishment); (b) 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 (a) changes in baseline adrenocorticotropic hormone (ACTH) or cortisol concentrations which could be considered as indicators of chronic stress status in contrast to 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 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.

Results

159 The studies which make up the subject matter of the review are summarized in Table 1 and

160 are grouped as: alterations to maternal nutrition (N1 – N12); exposure of the ewe to

161 behavioural or physiological stress (S1 – S6); others (O1 to O3) which included the

162 potentially toxic effects of para-chloro benzoates and bacterial lipopolysaccharide. In general,

163 the nature of the treatment imposed on the ewe (Table 1) varied within each of the three

164 groups in regard to both timing and severity as did the age at which responses were assessed

165 (ranging from 1 to 36 months in age). Because of the range in ages at which responses were

166 measured, the maturity of offspring studied ranged from weaning to the mature adult.

167 Therefore, the terms young sheep or offspring rather than lamb will be used from here

168 onwards. Across the studies (Tables 2 to 6 describe outcomes for each of the response types),

169 gestation treatments imposed on the ewe produced significant changes in offspring response

170 in 47 out of 71 (67%) of the outcomes. When these outcomes were separated into

171 physiological (Tables 2 and 3) and behavioural (Tables 4 to 6), fewer significant responses

172 were noted for physiological (55%, 21 of 38) than behavioural (84%, 26 of 33) outcomes

173 (Chi square, 4.0; $P<0.05$). Overall therefore, stress treatments imposed on the ewe did induce

174 changes in behavioural and physiological outcomes in the young sheep and behavioural

175 responses may be more sensitive indicators than physiological responses.

176 Table 1 near here

177 **Physiological outcomes**

178 Chronic responses of the young sheep were defined as changes in baseline (pre-test) cortisol

179 or ACTH concentrations (Table 2). There were few differences in baseline hormone

180 concentrations when ewes were under-nourished. Only in the study of Bloomfield *et al*

181 (2003) did offspring cortisol and ACTH concentrations change as a result of maternal

182 undernutrition and indeed the decrease in cortisol only occurred when severe undernutrition

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183　　was applied to the ewe for a short period of time (10 days) in late gestation. Other studies in

184　　which ACTH was increased by undernutrition occurred only in males (Gardner *et al* 2006;

185　　Oliver *et al* 2012) at 12 and 18 months of age respectively and thus, their practical impact

186　　would be limited because most male lambs would have been sent for slaughter at a younger

187　　age. Additionally, most studies were carried out with intact males and therefore there must be

188　　doubt over their application to castrates. When ewes were treated with betamethasone

189　　(Sloboda *et al* 2002), dexamethasone (Long *et al* 2013) or isolated at weekly intervals

190　　(Roussel *et al* 2004), all in late gestation, then responses were more pronounced. Possibly,

191　　therefore timing and nature of challenge is important as offspring basal hormone

192　　concentrations were only perturbed when the challenges were (a) applied in late gestation

193　　(after 100 days) and (b) acute (including severe UN for 10 days, Bloomfield *et al* 2003). The

194　　study of Long *et al* (2013) is notable as there were increases in cortisol concentration not only

195　　in young sheep born to ewes administered a single dose of dexamethasone (F1 generation)

196　　but were also inherited and exhibited by their progeny (F2 generation).

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198　　　　　　　　　　　　　　　　　　Table 2 near here

199　　A different pattern of response was noted when young sheep were acutely challenged with

200　　CRH/AVP or cortisol to assess HPA function (Table 3). There was little evidence for

201　　differences due to treatment in offspring cortisol concentration to either ACTH or a

202　　behavioural challenge. However, the studies of Long *et al* (2010, 2012) are notable again as

203　　the only studies in which CRH/AVP, ACTH and behavioural challenges were applied to the

204　　same groups of young sheep. Although the pattern of response differed between studies

205　　(probably because different nutritional treatments were imposed upon the ewe), offspring

206　　responses to physiological challenge differed from those to social isolation.

208   Most studies assessed the effect of CRH/AVP challenge on offspring HPA axis function.

209   Changes in HPA axis outcomes were noted in 7 of 9 studies; however, both increases and

210   decreases in ACTH and cortisol response were noted. This variability in response is likely in

211   part due to the age at which the animals were tested. Sloboda *et al* (2002, 2007) re-tested the

212   same young sheep whose dams had been challenged with betamethasone in late gestation at

213   6, 12, 24 and 36 months of age. While there was no effect of treatments imposed on

214   responses to CRH/AVP challenge at 6 months of age, at 12 months of age, maternal

215   betamethasone administration increased offspring cortisol response but at 24 months of age

216   an increase in ACTH responsiveness and at 36 months of age a decrease in cortisol response

217   was recorded; thus HPA function as defined by response to CRH/AVP challenge was clearly

218   dependant upon the age of the animal at test. Finally, Long *et al* (2013) reported decreases in

219   ACTH and cortisol response to CRH/AVP challenge in both the daughters and grand-

220   daughters of ewes challenged with dexamethasone.

221   **Behavioural outcomes**

222   *Emotional reactivity*

223   Overall, in 9 of the 10 studies listed in Tables 4 and 5, young sheep who were exposed to

224   prenatal treatments exhibited responses to behavioural challenge; the exception being the

225   study of Chadio *et al* (2007). The hypothesis most commonly stated in these studies was that

226   prenatal treatments increased emotional reactivity, further interpreted as increased

227   fearfulness. In the studies reviewed a consistent pattern does not emerge. The two practically

228   important prenatal treatments, undernutrition and imposition of various stressors on the ewe

229   had different characteristics. Undernutrition was in general applied continuously for more

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230 than 30 days and from an early stage of gestation. This contrasted with prenatal stress

231 treatments which were imposed in the last third of gestation and although an individual

232 treatment may have been imposed at regular intervals (weekly), each individual challenge

233 was applied typically for only an hour or less, with this acute exposure differing from the

234 chronic (long-term) nature of undernutrition. Within prenatal stress treatments, different

235 treatments probably imposed different severities of challenge. For example, Roussel-Hachette

236 *et al* (2008) considered that isolation and transport of the ewe was more severe than isolation

237 alone, while Coulon *et al* (2015) concluded that different stress treatments applied randomly

238 were a more severe challenge than the same stress treatment applied at regular intervals (e.g.

239 Roussel-Hachette *et al* 2008). Finally the nature of the control treatment may be important

240 when interpreting across different studies: Coulon *et al* (2011) employed a positive control

241 (gentle handling of the ewe), while the controls in most other studies consisted of no stress

242 treatment.

243   Tables 4 and 5 near here

244   The effects of undernutrition on offspring emotional reactivity were not consistent across

245 studies. Erhard *et al* (2004), in the most detailed study, found evidence for increased

246 emotional reactivity in offspring of under-nourished ewes; treatment lambs took longer to

247 approach a novel object and in males only, activity was increased when confined in a weigh

248 crate after exposure to a sudden stimulus. Simitzis *et al* (2009) however reported no

249 differences and both Corner *et al* (2005; reduction in high pitched bleats in a novel arena) and

250 Hernandez *et al* (2010; reduction in escape attempts during social isolation) interpreted the

251 behavioural changes they observed as reductions in emotional reactivity.

When prenatal stress treatments were applied,, there were indications of changed emotional reactivity. Prenatal stress treatments, at 8 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), prenatal 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 vocalization) 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 prenatal 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

276 imposed have also been related to the severity of the test. These have been ascribed to a

277 ceiling effect where responses of both control and treatment offspring to a more severe test

278 masked treatments differences. Thus, Erhard *et al* (2004) noted greater between-treatment

279 differences when a startle stimulus was applied than in an isolation test and similarly Coulon

280 *et al* (2014) found responses were greater in a human approach / novel object test than in a

281 social isolation test.

282 ***Cognitive flexibility***

283   Table 6 near here

284 Table 6 summarizes four studies which tested aspects of cognitive flexibility of young sheep

285 born to ewes exposed to different treatments. Since the methods used to test the offspring

286 whose dams had been under-nourished (Erhard *et al* 2004; Hernandez *et al* 2009; T-maze)

287 differed from those whose mothers had been exposed to stress treatments (Coulon *et al* 2011,

288 2015; maze with fixed blind alleys), then responses differed between studies. For

289 undernutrition, Erhard *et al* (2004) reported a sexually dimorphic response where male sheep

290 born to under-nourished ewes were slower to learn a reversal task than control males but

291 there were no differences between females.

292 However, Hernandez *et al* (2009) found no differences between treatment groups, but at 6

293 months of age, females were quicker to learn than males. Similarly while Coulon *et al* (2011)

294 found no differences in cognitive ability between groups, Coulon *et al* (2015) found that

295 young sheep whose dams had been exposed to stress were slower to complete a maze test

296 both during learning the maze and upon subsequent re-test. In a test of judgment bias, Coulon

297 *et al* (2015) also concluded that young sheep whose dams had been exposed to stress had a

298 more pessimistic bias than control sheep and suggested that this could indicate a poorer state

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299   of welfare. In contrast to emotional reactivity, offspring of high emotional reactivity ewes

300   were not different in cognitive flexibility to those from low emotional reactivity ewes.

301   **Discussion**

302   **Animal welfare implications of prenatal challenges in sheep**

303   In the current review, the responses of young sheep from approximately weaning to maturity,

304   after exposure of ewes to challenge during pregnancy, have been summarised. As the focus of

305   the review is on the offspring, the consequences of pregnancy challenges for the dam which

306   are an important welfare concern are not discussed here. Overall there were lasting

307   consequences of maternal challenges for the offspring, despite the variety of challenges

308   imposed on the ewe and the variability of the timing and nature of the tests imposed on the

309   young sheep. The key question for this review is whether these responses have implications

310   for the welfare of the offspring throughout their lifespan and indeed for their own progeny. In

311   assessing welfare implications, the conclusions of the current review cannot be viewed in

312   isolation but must be integrated with the conclusions of the preceding review which

313   addressed responses of the lamb from birth to weaning (Rooke *et al* 2015). The question of

314   whether prenatal insults increase the risk of adverse welfare outcomes in commercial sheep

315   flocks also requires that some consideration is given to likely exposure scenarios.

316   Applications of risk assessment to animal welfare issues are complex (Smulders & Algers

317   2009; EFSA 2012, 2014) and are often hampered by a lack of relevant data. Put most simply

318   a risk assessment requires information on two aspects. Firstly, the characterisation of the

319   biological effect that any identified and defined <u>hazard</u> has on a <u>target population</u>, and

320   secondly, the extent of <u>exposure</u> of that population to the hazard. In relation to gestation

321   treatments, hazards can be viewed as being applied to two target populations; the ewe and her

322   developing fetal progeny. This review, and the previous one (Rooke *et al* 2015), reveal the

323   current level of understanding of the first issue (hazard effects).

324   *Exposure to hazards*

325   Overall, the existing literature provides only very weak understanding of hazard effects. In

326   relation to the second (exposure), remarkably little is known about the severity and exposure

327   prevalence of putative hazards for pregnant ewes. This means that it is only possible to

328   speculate about exposure scenarios. Because of the seasonality of herbage growth, breeding

329   ewes kept outdoors in winter in temperate production systems (Robinson *et al* 2002) are

330   likely to experience periods of undernutrition in early to mid gestation. Droughts will have

331   similar effects in other production systems. However in the UK, nutrition is normally

332   increased in late gestation by either housing and feeding ewes supplementary feed or by

333   timing pregnancy such that increased nutritional demands in late gestation are met by

334   increased pasture availability in spring. Undernutrition which was severe in nature and which

335   was applied late in gestation would not normally be encountered in practice in the UK; the

336   most likely scenario would be extreme weather events such as snowfall or flooding which

337   would prevent access to grazing thus causing acute undernutrition. Ewes encounter a variety

338   of aversive events during pregnancy, the frequency and severity of which are dependant upon

339   individual farm management. These events include: movement and handling using sheep

340   dogs to unfamiliar grazing or housing indoors; unfamiliar housing itself; mixing with

341   unfamiliar ewes; transport; restraint and social isolation; shearing. The number of different

342   aversive event types encountered will vary depending on the system. For example, ewes

343   maintained outdoors throughout gestation will be exposed to fewer aversive events imposed

344   by management than ewes housed in late gestation. However, the very nature of farm

345   management means that to the ewe some aversive events are unpredictable and therefore

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346 ewes would be unable to habituate to them. Thus of the protocols used to impose stress

347 treatments on ewes, the random protocol described by Coulon *et al* (2014) more closely

348 resembles that which would be encountered in practice although it is unlikely that ewes

349 would be exposed to all the events described by Coulon *et al* (2014). The protocols used

350 previously by Roussel *et al* (2004), Roussel-Huchette *et al* (2008) and Coulon *et al* (2011) in

351 which the same aversive treatment was repeated at weekly intervals, would have the potential

352 for habituation. An exposure scenario which has not been addressed in the review for

353 practical reasons is that of disease, although offspring responses after exposure of the ewe to

354 para-chlorobenzoates and lipopolysaccharides (Fisher *et al*, 2010; Gutleb *et al*, 2011) do

355 highlight the importance of stressors other than undernutrition, housing and management

356 practices.

357 The reports considered in this review do encompass the relevant target population and

358 exposure scenarios. However, since in practice, the ewe is likely to experience more than one

359 exposure scenario (e.g. both undernutrition and handling) then the absence of studies, which

360 have examined interactions between different exposure scenarios, although necessary to

361 understand the effects of individual exposure scenarios, is an important omission.

362 *Relevance and quality of data*

363 In the risk assessment process, the identification of relevant data is critical. In this review, the

364 tests employed on young sheep could be broadly divided into two classes: measurement of

365 either physiological or behavioural responses. Since, physiological responses are measured to

366 investigate mechanistic relationships and report changes in selected (but relevant e.g. the

367 HPA axis) body systems, then, first, the responses of many body systems which may be

368 relevant are not reported and secondly, using specific tests precludes any measurement of

369 integrated responses by the animal. Given that behaviour reflects an integration of

370 neurobiological and endocrine changes, it is likely that behavioural tests will be more

371 relevant to assessing animal welfare. It was indeed found that behavioural tests detected more

372 differences in response between treatments than physiological tests, likely for the above

373 reasons and also because many behavioural outcome tests are typically performed. Therefore,

374 more weight should be given to behavioural responses of the offspring in assessing welfare

375 outcomes, but this also requires a clear hypothesis-driven rationale for test selection.

376 The most important variables in interpreting the relevance of responses to welfare are the age

377 at which the young sheep is tested and its gender. The age of the animal at test is important as

378 plasticity of response was noted in studies where offspring were tested across a range of ages

379 (Sloboda *et al* 2002, 2007; Chadio *et al* 2007; Oliver *et al* 2012). Tests should therefore be

380 carried out at ages relevant to expected major welfare challenges. The welfare challenges

381 expected will differ and will be largely dependant on the fate of the young sheep. The

382 majority of males will be destined for slaughter at around 6 months of age and the major

383 challenges encountered by them will be weaning, subsequent management (e.g. housing for

384 fattening), transport to the abattoir and slaughter. Thus for this group of young sheep, tests

385 carried out between 3 and 6 months of age are likely to be relevant. The second main group

386 will comprise largely females retained for breeding. For breeding females, major challenges

387 will occur later in life during repeated breeding cycles beginning from as early as 6 months of

388 age but more likely 18 months of age; therefore tests later than 6 months of age are more

389 relevant. The type of test and direction of response is important in this context. The responses

390 reported in Tables 4 and 5 for emotional reactivity are largely interpreted in relation to

391 fearfulness with those tests involving social isolation being considered to be reliable

392 indicators of fearfulness (Forkman *et al* 2007). Increased reactivity is usually interpreted as

393 increased fearfulness but interpretation of reduced reactivity is more ambiguous being

394 alternatively attributed to either reduced fearfulness or an increase in passive fear response.

395 For young sheep, the major adverse welfare relevant response is likely to be increased

396 fearfulness in response to the situations noted above. In the papers reviewed, only five tested

397 young sheep at the relevant time. When ewes had been previously undernourished (Chadio *et*

398 *al* 2007; Simitzis *et al* 2009; Hernandez *et al* 2009, 2010), a significant response, decreased

399 escape attempts by the offspring was observed by one study (Hernandez *et al.,* 2010). Thus

400 there is little evidence for increased fearfulness as a result of chronic maternal undernutrition

401 when tested at an appropriate age. In response to stressors imposed on the ewe, Roussel *et al*

402 (2004) and Roussel-Huchette *et al* (2008) did not find a consistent pattern of change. As

403 pointed out by Coulon *et al* (2015) the stress protocols imposed allowed the possibility of

404 habituation by the ewe and this should be taken into account in interpreting these studies.

405 Further, in all the above studies, males had not been castrated and therefore the relevance of

406 the results can be questioned as many males destined for slaughter will be castrated. Thus

407 overall, current evidence does not suggest increased fearfulness in young sheep destined for

408 slaughter as a result of maternal stress imposition, although this conclusion must be heavily

409 qualified because of the small number of studies carried out at the relevant age and the

410 inherent limitations of the studies.

411 For breeding ewes, especially when grazing, increased emotional reactivity may not be an

412 adverse consequence of prenatal stress as it may enhance the ability of the ewe to deal with

413 challenges such as predators in the grazing environment, although as discussed above for the

414 young sheep increased reactivity may not be an advantage when the ewe is exposed to novel

415 environments and challenges such as housing and transport. Considering responses measured

416 at the relevant age (more than 12 months in age; Erhard *et al* 2004; Corner *et al* 2005; Chadio

417 *et al* 2007; Hernandez *et al* 2009, 2010) and only responses for female, there were few

418 significant responses when the dam was under-nourished. Thus, there is little evidence for

419 adverse effects of undernutrition on emotional reactivity. Only Roussel *et al* (2004),

420 measured responses of offspring at a relevant age in response to maternal stress challenges

421 and recorded an increase in emotional reactivity at 8 months of age. Overall therefore,

422 undernourishment of the ewe appeared to have little adverse consequences for young sheep

423 or breeding females but there was insufficient evidence to make any conclusions in respect of

424 stress challenges imposed on the ewe as only offspring of betamethasone-challenged ewes

425 were tested at the relevant age (Sloboda *et al* 2007; Long *et al* 2013). One factor that future

426 research should consider, is the inherent stress responsiveness of the ewe subjected to

427 pregnancy challenges as Coulon *et al* (2015) found that reactivity of young sheep was

428 increased when the dam was classified as having high stress responsiveness. It is possible

429 also that since Long *et al* (2013) found that basal cortisol concentrations were increased in

430 the grand-daughters of ewes exposed to dexamethasone, that high stress responsive ewes

431 (Coulon *et al* 2015) may themselves have been exposed to stress-related events during

432 pregnancy.

433 **Suggestions for future research work and strategies**

434 Overall it is clear that prenatal challenges, either in the form of sub-optimal maternal

435 nutrition or maternal stress have the potential to alter, after weaning, aspects of biology that

436 could have implications for welfare. A similar conclusion was drawn following a review of

437 the same literature in relation to pre-weaning lamb outcomes (Rooke *et al* 2015). However, in

438 many studies it is difficult to draw clear conclusions in relation to the relative welfare status

439 of prenatally challenged animals versus controls. As it stands now, the research literature in

440 this area does not form a solid basis on which advice to farmers could be based. Partly this is

441 of course because the field is relatively young and direct practical relevance is often not the

442 primary motivation of those conducting the study. Based on these reviews, along with others

443 recently conducted in cattle (Arnott *et al* 2012), poultry (Dixon *et al* 2016) and pigs (Otten *et*

444 *al* 2015), where similar issues exist, it is possible to provide some suggestions for a way

445 forward. These relate to: i) the choice of treatments, ii) the choice of outcome measures and

446 iii) possible factors that may modify the effects of prenatal challenge on welfare outcomes.

Choice of treatments

448 Work in this area is conducted for two reasons. Firstly, many studies are used to examine

449 basic biology or to inform human relevance. Secondly, studies are conducted to inform

450 considerations of sheep health, welfare and production in commercial practice. The former is

451 much more common, whilst the latter is more useful from an applied perspective. Basing

452 treatments on practically relevant factors runs the risk that the differences between treated

453 and control animals will be too small for statistical significance to be achieved. However,

454 such a finding adds valuable information about animal management and welfare outcomes.

455 The reality is that such 'negative findings' may be harder to publish and do little for the

456 career progression of the researchers involved. However, a good example from recent work

457 with applied relevance (not included in the current review because neonatal outcomes were

458 measured) is that of Averós *et al* (2015) who kept ewes at three different stocking densities

459 (1, 2 or 3m^2 per ewe) during gestation and examined the impact of this housing on progeny.

460 Although main effects of stocking density were not significant there were interactions

461 between maternal stocking density and post-natal stress (early separation from dam) such that

462 negative effects of post-natal stress on the offspring were exacerbated by reduced stocking

463 density.

20

464 Beyond the specific choice of treatment, the general lack of attempted replication is an

465 important problem. Demonstration of the repeatability of a finding is the cornerstone of

466 science, yet prenatal stress studies are rarely conducted in the same way twice, and this

467 greatly limits the robustness of conclusions about the reproducibility or generalizability of

468 findings. As with other areas of research (Ioannidis 2005) many of the findings will likely be

469 false. Indeed, Ioannidis (2005) identified characteristics of a research field that increase the

470 likelihood of individual findings being false, and several of these are potential problems in

471 the field of prenatal stress and animal welfare, including: small studies, small effect sizes, a

472 large number of tested relationships and a high level of flexibility in study design, choice of

473 outcome and methods of analysis. There are likely to be various structural and institutional

474 factors which limit attempts at replicating key findings. Funding bodies and journals are not

475 keen on studies which repeat other work, indeed a replication study can even be criticised on

476 ethical grounds, and career progression is similarly not rewarded by studies that confirm

477 previous findings. This means that conclusions are often drawn on the findings of single

478 studies. These conclusions are particularly precarious in light of the fact that prenatal stress

479 studies often measure multiple outcomes, with statistical accounting for this being rare.

480 Despite the fact that the ewe is likely to encounter multiple stressors during pregnancy, for

481 example undernutrition and handling, none of the reviewed studies investigated interactions

482 between nutrition and stressors. Further while Rooke *et al* (2015) found no disease-related

483 studies, the one study reported here (Fisher *et al* 2010) did report changes in lamb physiology

484 following maternal exposure to endotoxins. Both these areas (interactions between stressors

485 and responses to disease challenge) are worthy of further experimentation.

486 *Choice of Outcome measures*

21

487   Prenatal stress studies often involve measurement of many different outcomes, across various

488   areas of biology. Across the literature as a whole there is a lack of consistency in choice and

489   application of outcome measures. Even where the same parameter is assessed individual

490   studies often vary in the exact approach taken. This is particularly notable in relation to tests

491   of emotionality, where unlike the situation in rodents where tests are generally highly

492   standardised (e.g. open field, elevated-plus maze) there is still substantial variability in test

493   parameters, including arena size and design, and also the nature of outcome measures

494   recorded. Furthermore, there is also a limited degree of prior validation work which allows

495   for variable interpretation of these outcome measures. Another common issue is that many

496   outcome measures are not measured at multiple time points either in the same or different

497   cohorts of animals. This means that the time course of biological changes induced by prenatal

498   challenge is uncertain. A broader issue, which hampers clear animal welfare conclusions, is

499   that individual measures are rarely integrated into some clear understanding of whether the

500   welfare state of the animals involved is overall better or worse as a consequence of the

501   experiences of their mother. Even if it is believed that a physiological or behavioural measure

502   assessed does indeed represent altered emotionality, it is often not clear what the implications

503   of such alterations are for the lifetime welfare of affected animals.

504   ***Experimental design factors which may modify offspring outcomes from ewe treatments***

505   *Variable postnatal environments*

506   Whilst discussing experimental design issues in developmental plasticity studies Groothuis

507   and Taborsky (2015) noted that most theoretical frameworks for understanding prenatal

508   effects rely on a comparison of outcomes under different postnatal conditions. Yet such a

509   comparison is almost never made in research studies involving captive species and animal

510 welfare issues. In interpreting the studies reviewed one must consider that the welfare

511 relevance of any change in responsiveness may be situation-specific. Thus increased

512 responsiveness in an environment that induces fear and changed HPA axis responsiveness

513 may not have adverse consequences for welfare where the sheep may be exposed to

514 predation, e.g. in a hill environment, but will be relevant in situations such as transport,

515 lairage or slaughter of the animal. Similarly it may be necessary to distinguish between

516 responses which arise from permanent programming of the HPA axis from those which are

517 expressions of developmental flexibility. Whilst for some species, such as pigs, where the

518 range of environments encountered under production conditions is relatively narrow, sheep

519 are managed in different systems, varying from a semi-wild extensive existence to a more

520 intensive lowland system. Changes that are seen in a research context in housed animals may

521 actually be neutral or even beneficial in an extensive setting.

522 *Maternal effects*

523 In risk assessment terms, it is important to consider whether different ewe breeds or ages can

524 be considered as the same or different target populations for the purposes of drawing

525 conclusions about animal welfare impact. Over 100 different breeds of sheep are used in the

526 UK alone (EBLEX 2014) and this range would be extended by consideration of different

527 countries. These breeds vary widely in their productivity and reproductive characteristics, as

528 well as in key aspects of their health, overall robustness and behaviour. Yet it is rare (with

529 some exceptions: Burt *et al* 2007; Rooke *et al* 2010) that studies attempt to expose different

530 breeds to the same treatment. A very tentative conclusion from these studies is that animals

531 selected for lean tissue growth are more sensitive to prenatal effects as the birth-weight of

532 lambs born to the selected breed in each study was reduced to a greater extent by under-

533   nutrition of the ewe. However, more direct testing of this in future studies would be welcome

534   and an important contribution to elucidating the true industry relevance of this area.

535   Within breeds, differences between ewes in reactivity may also be important determinants of

536   response although evidence is not consistent. While Roussel et al. (2004) found no effect of

537   maternal reactivity on offspring response, Coulon et al. (2014) reported that pre-natally

538   stressed offspring of high emotional reactivity ewes were more affected. These differences in

539   response may be related to either breeds used or the methods used to characterize the ewes.

540   From a practical perspective another factor which may alter the effects of a standard

541   challenge is maternal parity. Parity effects could occur in two different ways. Firstly it is

542   possible that previous experience has a mediating effect on how ewes respond to

543   environmental factors. For instance, younger ewes may find handling and housing more

544   stressful during their first pregnancy compared to later. Secondly, body reserves may differ

545   over several breeding seasons altering the impact of a standard level of nutrition. Finally,

546   there are well known effects of parity on maternal care (Dwyer and Smith 2008; Munoz *et al*

547   2009). As noted above (in relation to variable ewe temperament: Coulon *et al* 2015) and

548   previously (e.g. body reserves: Rooke *et al* 2015), other types of variation in ewe biology will

549   likely modulate the impact on fetal lambs.

550   **Animal welfare implications and conclusion**

551   The data gathered together here and in a related review (Rooke *et al* 2015) suggest, as

552   expected from other species, that the nutrition and stress state of pregnant ewes can effect

553   many aspects of their progeny's biology, at birth and throughout their life. In some cases

554   these changes may even carry-over into subsequent generations. Furthermore, some of the

555   identified changes clearly have implications for animal welfare. However, detailed analysis

556 of the literature shows several deficiencies in the field as a whole which greatly limit the

557 ability to i) draw conclusions about how welfare may be affected by prenatal challenges in

558 commercial sheep, or ii) suggest ways that these effects could be avoided, or even how

559 maternal treatment during gestation might contribute to improving the welfare of farmed

560 sheep. Suggestions have been made relating to how experimental designs could be improved

561 to aid translation to applied relevance. Particularly in respect of both behavioural and

562 physiological outcomes, there is a need for measures that can be integrated to give a global

563 picture of offspring welfare (i.e. a stated conclusion that progeny welfare is overall better or

564 worse for the animals concerned).

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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
Under (UN) or Over (ON) Nutrition					
N1	Bloomfield *et al* 2003	UN: 0.02 or 0.04 requirement	105-115 or 105-125	30	F
N2	Erhard *et al* 2004	UN: 0.5 requirement	0 – 95	18	F, M
N3	Corner *et al* 2005	UN: 0.6 requirement	64 – 132	12	F
N4	Gardner *et al* 2006	UN: 0.5 requirement	1-30	12	F, M
N5	Chadio *et al* 2007; Simitzis *et al* 2009	UN: 0.5 requirement	0 – 30; 30 - 100	2, 3, 4, 5, 6, 10	F, M
N6	Hernandez *et al* 2009	UN: 0.5 - 0.8 requirement	-60 - 30	4, 18	F, M
N7	Hernandez *et al* 2010	UN: 0.5 - 0.8 requirement	-60 – 30; -60 – 0; 0 - 30	4, 18	F, M
N8	Long *et al* 2010	UN: 0.5 requirement	28-147	12	F
N9	Wallace *et al* 2011	UN: 0.75 requirement; ON: 2.2 requirement	7-147	9, 18, 24	F, M
N10	Long *et al* 2012	ON: 1.5 requirement	-60 - 147	20	F, M
N11	Oliver *et al* 2012	UN: 0.5 - 0.8 requirement	-60 - 30	4, 10, 18	F, M
N12	Donovan *et al* 2013	UN: 0.5 - 0.8 requirement	-60 - 30	18	F, M
Stress					

S1	Sloboda *et al* 2002, 2007	Betamethasone	104; 104-125	6, 12, 24, 36	F, MC
S2	Roussel *et al* 2004	Isolation (1h ; 2 x weekly)	112–147	1, 8	F, M
S3	Roussel-Huchette *et al* 2008	Isolation (1h; 2 x weekly)	115-147	4	F, M
		Isolation and transport			
S4	Coulon *et al* 2011	Aversive handling	115-147	1	F, M
S5	Long *et al* 2013	Dexamethasone	103-104	16, 28, (F2) 6	F
S6	Coulon *et al* 2014, 2015	Isolation; mixing; transport; dog handling; sham shearing; delayed feeding (total n=16 / ewe)	94 - 103	1	F, M
Other					
O1	Erhard and Rhind 2004	Sewage sludge containing parachlorobenzoates	0-147	5	F, M
O2	Fisher et al 2010	Lipopolysaccharide	135; 135-137	5, 18	F, M
					(5 mo only)
O3	Gutleb *et al* 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.

Table 2 Changes in basal concentrations of cortisol and ACTH in young sheep whose dams were 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	Undernutrition: no difference
		Overnutrition: 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	

Table 3 Responses of young sheep whose dams had been exposed to gestation treatments to physiological or behavioural challenges. Responses are given as differences in the increase in plasma adrenocorticotropic 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).

	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	

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

	Novel environment		
Study	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 prenatal stress. Stress reactivity of ewe influenced response in absence of prenatal stress.		
O1		Increase in time spent exploring arena in males only	Increased number of vocalizations 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 whose dams were exposed to gestation challenges (responses are changes with reference to controls) (Identity of studies given in Table 1).

	Novel stimulus		
	Object	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 metres of object	
S4		Tendency for increase in flight distance from stimulus	Reduced vocalization
S6		Prenatal stress treatment lambs spent more time distant from object. Prenatal 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	Prenatal stress effects only in offspring of high stress reactive ewes: presence of human reduced locomotor activity, vocalization, 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 whose dams had been exposed to gestation challenges (responses are changes with reference to controls) (Identity of studies given in Table 1).

Study and test	Response	Comment
T Maze		
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
Blind Maze		
S4	Latency to solve	No differences
	Re-test response	No differences
S6	Latency to solve	Prenatal stress offspring slower to complete test
	Re-test response	Prenatal stress offspring slower to complete test