Chapter 6 The role of glucocorticoids in the expression of temperament in sheep

6.1 Introduction

When animals fail to adequately obviate or react appropriately to environmental or situational stressors, it can adversely affect the animal, and particular traits, such as reproduction, fertility, growth, immunity, animal welfare and may also lead to the development of chronic stress (Boissy *et al.* 2005). Therefore, the activation of an appropriate response to a stressor is of prime importance for both adaptation to the situation and the subsequent welfare of the animal. The inherent fearfulness of the animal will influence the magnitude and duration of this response to a challenge. In livestock this is assessed *via* behavioural measurements and observations which can be used to define the temperament of the animal. The neurophysiological differences that underpin divergences in livestock temperament are not known, but it is hypothesized that the mechanisms involved in the control of stress reactivity and fear are involved. The hypothalamic pituitary adrenal (HPA) axis is clearly a candidate given its central role to the expression of fearfulness in animals (Archer 1979).

When an animal is exposed to a stressor, be it physiological or psychological in nature, a number of behavioural and physiological changes are initiated that are generally adaptive (Boissy 1995), helping to improve the animal's probability of restoring homeostasis. Strategies based on a combination of the behavioural response, the autonomic nervous system response, the neuroendocrine response and the immune response are then initiated in the body (Moberg 2000). Depending on the nature of the challenge, behavioural adaptations can include increased alertness, vigilance, improved cognition, arousal (Price 1999), freezing and flight (Chrousos 1998). The physiological interactions are myriad during a stress response, consisting of a cascade of events, leading to, among others, redirection of energy, movement of oxygen and nutrients to the central nervous system (CNS), muscles and other sites where most needed, resulting in an increase in cardiovascular tone and respiratory rate. Specific functions can also be inhibited, such as hunger, metabolism and reproduction. Additionally, animals will respond to different stressors with different responses. For example, studies by Djordjevic *et al.* (2003) investigated the response by rats to different stressors, ranging from fasting, cold and heat stress and heat stress of different durations (20 and 60 min). They found

heat stress caused the largest increases in ACTH compared to the other stressors, suggesting each stressor has a particular stress response.

Stress exposure in a normal vertebrate leads to activation of both the sympatho-adrenal medullary (SAM) system and the HPA axis. In general, the SAM system is associated with release of catecholamines, increased cardiac output and redistribution of blood flow to the brain and skeletal muscle. In general, the HPA axis responds to a stressor by secretion of corticotrophin releasing hormone (CRH) and vasopressin from the hypothalamus, which in turn causes the release of adrenocorticotrophine (ACTH) from the anterior pituitary, triggering secretion of glucocorticoids (GC), such as cortisol from the adrenal cortex (Minton 1994). Subsequent regulation of the HPA axis depends on the GC negative feedback mechanism, whereby circulating cortisol inhibits the release and synthesis of CRH and vasopressin, leading to a decreased concentration of ACTH, inducing a decline in the level of cortisol. Cortisol inhibition is mediated by two corticosteroid receptor subtypes; the mineralocorticoid (MR) and the glucocorticoid (GR) receptor. These receptors are located in areas that have been identified as important in the expression of fear and anxiety such as the hippocampus and amygdala. Additionally, Henry and Stephens (1977) hypothesized that the amygdala and hippocampus were involved in different coping processes enabling an animal to respond to threatening and frustrating situations. However, the ability to adjust to some stressors seems to be under the control of the amygdala through activation of the sympathetic nervous system (von Borell 1995). Research indicates that the MR mediates cortisol inhibition of the HPA axis during resting, basal and non-stress periods, whilst the GR mediates cortisol regulation during stress induced changes (De Kloet *et al.* 1998). However, it has been recently suggested that regulation of the HPA axis during stress may require both receptors to mediate the negative cortisol feedback effect, which would be dependent on the level of stress, particularly mild stressors where a strong corticosterone response would not be expected (Pace and Spenser 2005). The two receptor subtypes differ in their affinity for, and binding capacity of corticosteroids. Mineralocorticoid receptors have a higher affinity for corticosterone and are mostly saturated under normal resting conditions, whilst GRs have a lower affinity and so only become activated with higher levels of circulating cortisol, such as those present when activated by stress (Spencer *et al.* 1998). This also influences activation of the receptors in regards to the diurnal rhythmicity of corticosteroid secretion, so that normal basal corticosteroids prepare the animal for the day *via* MR action, whilst the suppressive action

regarding the negative feedback of corticosteroids is coordinated by GRs, to ensure a balance between the two to achieve homeostasis (De Kloet *et al.* 1998).

Corticosteroid hormones modulate both behavioural adaptation and cognitive functions (Sapolsky *et al.* 2000). Behavioural activities in rodents have been shown to be affected by high amounts of circulating corticosterone, such as swim patterns, and processing of information for learning and memory (Erickson *et al.* 2003). The increase in cortisol during a stress response can initiate a sequence of events that can mediate an animal's response. This results in a variety of different changes within the body, but individuals will be affected differently depending on particular neurotransmitters and brain areas. For example, elevated levels of cortisol generally indicate a heightened level of stress, but Zorrilla *et al.* (1994) found that unstressed people can also exhibit elevated basal cortisol levels. People with depressive illnesses have been found to have high levels of cortisol in their blood and a novel treatment for such conditions would be the administration of treatments that reduce the elevated levels (Healy *et al.* 1983). Cook (2002) also showed that cortisol can increase the sensitivity of the limbic system in response to stress. This suggests that cortisol could possibly modulate temperament due to its potential ability to sensitize the CRH response to subsequent, same or differing stressors. CRH has been shown to mediate a variety of fear-related behaviours (Davis 1992; Erickson *et al.* 2003), and elevated cortisol promotes the facilitation of CRH gene expression in the amygdala, which consequently can enhance the perception of fear and fear related behaviours (Erickson *et al.* 2003). Additionally, cortisol has been shown to affect individuals differently shown by the large variation in behavioural responses following glucocorticoid infusions into the brain. Gomez *et al.* (1998) administered a synthetic GC (dexamethasone) to five different rat strains selected for differing behavioural responses to the forced swim test. Rats were then exposed to electric tail shocks and restraint. Results indicated inter-strain differences, such as ACTH and corticosterone hypo-responsiveness in one strain compared with the others during acute testing, whilst dexamethasone administration was far less effective in one other strain as compared to the others. It was hypothesized that differences were related to the regulation of the HPA axis, particularly related to the negative feedback mechanisms. The evidence indicates that individual differences in HPA function affect how an animal reacts, copes or adapts to a physiological or psychological stressor.

The possibility exists, that selection for temperament differences may have altered the HPA axis regulatory mechanisms so that there is a divergence in HPA function particularly during exposure to stress. If this is the case then blockage of the GR, with the GC antagonist should alter the expression of fearfulness and/or cortisol in lines selected divergently for temperament. Mifepristone (RU-486) is a glucocorticoid and progesterone receptor antagonist (Healy *et al.* 1983) and can prevent some of the actions of cortisol that are receptor mediated *via* binding to the GR (Bertagna 1997; Bertagna *et al.* 1984; Weidemann *et al.* 1992). By binding to the GR, RU-486 reduces the transmission of the receptors and reduces the negative feedback mechanism resulting in a rise in circulating cortisol (Born *et al.* 1991; Weidemann *et al.* 1992).

The expression of fear is dependent on a large number of neural pathways, including learning and memory (Fuchs and Flügge 2003; McEwen 2000). In addition to their regulatory role *via* negative feedback of the activity of the HPA axis, the glucocorticoid receptors are involved in many of those functions (see review by Erickson *et al.* 2003). In the fear potentiation challenge model, the sheep are exposed to the stimulus (dog) in a particular setting (isolation box) and then re-exposed to the same setting in absence of the stimulus. Therefore, during the first 2 steps (initial exposure and re-exposure), learning and memory functions will be activated to initiate the fear response during step 3. The lack of cortisol response observed previously (Chapters 4 and 5) illustrates the possibility that the HPA axis might not be directly involved in the building of the fear potentiated response (step 3). However, it is evident that the activation of the glucocorticoids receptors are involved in the building of the actual fear response (steps 1 and 2). To test this hypothesis, we have used the glucocorticoid receptor antgonist RU-486 in an experiment involving the fear potentiation model. Furthermore, we have used sheep from the two temperament selection lines because they exhibit differences in their behavioural responses during all steps. Therefore, if the glucocorticoid receptors are involved in the building of the actual fear response, administration of RU-486 will differentially affect the fear response between the selection lines particularly during the initial two steps.

6.2 Hypothesis

The following hypothesis was basis of the experiment, which aimed to determine if a response to cortisol was one of the primary mechanisms' contributing to differences in the temperament selection lines of Merino wethers.

(i) Administration of RU-486 would differentially affect the fear response between the temperament selection lines.

6.3 Materials and methods

6.3.1 Animals

Animals were sourced from the Allandale research flock, a temperament resource flock managed and maintained by the University of Western Australia (UWA), School of Animal Biology (refer chapter 2). The use of the selection line animals and procedures for this experiment were approved by The UWA Animal Ethics Committee (AEC RA/3/100/289). Fifty-six Merino wethers aged 10 mo. (liveweight 30 - 40 kg) were used. Fifty-two sheep were used in the actual experiment, and additional sheep were used pre- and post-challenge to prevent the first and last sheep from being isolated outside of the challenge period. Animals were allocated at random after stratification for liveweight to the challenge and pharmacological treatments.

6.3.2 Fear potentiation model

The fear potentiation model comprised two challenges consisting of 10 min of isolation, in either the absence (**Iso.**) or presence (**Iso+Dog**) of a dog (refer to Chapter 4). The model was applied in three steps (each step was conducted over a two day period) with two days separating each step.

Step 1: Initial response to exposure of **Iso+Dog**

Step 2: Reinforcement of exposure to **Iso+Dog**

Step 3: Expression of the fear potentiated response to **Iso+Dog** context in the absence of the dog

Each animal was placed in the isolation box (described in chapter 2) for 10 min and the degree of agitation was objectively measured. Agitation scores were recorded at 1 min intervals across the 10 min period. The number of vocalisations was recorded during the entire time the animal was within the box.

6.3.3 Experimental design and procedure

A factorial design was utilized for this study and included: treatment (control and RU-486), selection line (nervous and calm) and day of testing (1 and 2). All sheep were presented with the **Iso+Dog** challenge on two occasions (steps 1 and 2) with two days between each challenge. The treatments (RU-486 and control) were administered during the first two steps before exposure to the challenge. During the third and final step, the pharmacological treatments were not administered and all animals received only the **Iso** challenge (Table 6.1).

Each morning animals were bought into the yards and drafted into their two treatment groups approximately 1 hour prior to the start of the challenges. The pharmacological treatments were administered and heart rate monitors attached after which the animals were then left to settle. Blood samples were collected immediately prior to the animals entering the isolation box and again at 15 (after the challenge), 30, 60 and 90 min relative to the commencement of the challenge. After the 90 min blood sample was taken, the heart rate monitors were removed.

6.3.4 Treatment

Animals were administered either the treatment drug, Mifepristone (RU-486) or the control treatment (treatment vehicle, saline and 70 % ethanol). Animals were administered RU-486 and the control vehicle intravenously 3 mg/kg 20 min prior to the **Iso+Dog** challenge in steps 1 and 2. RU-486 (supplied by Sapphire Bioscience Pty. Ltd., Redfern, NSW 2016, Australia) was dissolved at 100 mg/ml in 70% ethanol, vortexed and sonnicated for approximately 1 hr.

6.3.5 Sampling

6.3.5.1 Blood sampling

Blood samples (6 ml) were collected by jugular venepuncture into serum separator vacutainers (Becton Dickinson Ltd). The samples were then processed according to the procedures described in chapter 2.

6.3.5.2 Heart rate monitoring

Polar heart rate monitors (S810i, Polar Electro, 2000) were attached to each animal 30 min prior to the **Iso+Dog** challenge. Monitors were set to record at 1 min intervals and data were recorded over a 2 hr period (see chapter 2 for further details).

The heart rate profiles were first divided into three periods for analysis, consisting of 8 minutes prior to the challenge (pre-challenge), 8 minutes during the challenge (chall) and 8 minutes post-challenge (post-chall) for analysis. Heart rate was averaged every 1 minute, therefore each period per animal consisted of eight points, of which no handling or bleed points were included. The mean heart rate and standard deviation was determined for each of the three periods.

6.3.5.3 Cortisol assay

Serum concentrations of cortisol were measured using a commercially available cortisol radioimmunoassay kit (Spectria Cortisol RIA, Orion Diagnostica). All samples were run in duplicate and the lower detection limit was 5ug/mL. Intra- and inter-assay coefficients of variation were 6.26% and 5.31% for high, 3.43% and 6.17% for medium and 9.38% and 4.02% for low control samples.

6.4 Statistical analysis

All statistical tests were performed using SAS Release 8.2 (SAS Institute Inc., 1999). Data distribution and homogeneity of variance were analysed for all variables and transformations were used to normalize the data when required using logarithmic or square root transformation prior to analysis. Values that were transformed were back-transformed for presentation.

The dependent variables included the agitation score after 1 min (IBT min1), cumulative score between 2 – 9 min (IBT min2-9) and the score during the final minute (IBT min10) during the **Iso+Dog** challenge and **Iso** challenge. The total number of vocalisations (Vocs) during the challenge, mean heart rate (HR) and integrated cortisol response (AUC) were also analysed.

The responses for each of the three steps were each analysed separately and a general linear model (Proc GLM) was used. The model contained the main effects of selection line (nervous and calm), treatment (RU-486 and control), day (1 and 2) and the various interactions. Non-significant interactions were sequentially removed until the simplest significant ($P<0.05$) models were obtained. Data are expressed as least squares mean (LSM) ± the standard error.

6.5 Results

6.5.1 Step 1: Initial response to the Iso+Dog challenge

The results are presented in Table 6.1. There were very few significant interactions apart from selection line x day for IBT min10 and treatment x day for AUC. These were mainly due to differences in the response between each day of testing.

The nervous selection line had significantly higher agitation scores (IBT min1 $P<0.001$, IBT min2-9 $P<0.001$ and IBT min10 $P<0.01$) and increased vocalisations ($P<0.001$). There were no significant differences in the integrated cortisol response between the temperament selection lines, although the AUC response tended to be higher for the nervous line. The administration of RU-486 resulted in a higher AUC compared to the control group ($P<0.01$). Mean heart rate pre- and post-challenge and HR variability in the post-challenge period were influenced by day of testing (Table 6.1). No other significant effects on the HR parameters were observed.

Table 6.1: Effect of temperament selection line, treatment, day of testing and significance of the interactions for the agitation scores (IBT min1, IBT min2-9, IBT min10) number of vocalisations, total integrated cortisol response, mean heart rate and variability during the first step of the **Iso+Dog** challenge (Step 1)

Main effects	***Isolation box test**				***Cortisol**	**Mean HR (bpm)**			**HR standard deviation (bpm)**		
Selection line	**Min 1**	**Min 2-9**	**Min 10**	**Vocs**	****AUC**	**Pre-chall**	**Chall**	**Post-chall**	**Pre-chall**	**Chall**	**Post-chall**
Calm	29.9	22.2	3.3	1.3	992.2	85	87	85	8.6	8.0	4.9
Nervous	99.4	148.1	9.0	4.9	1339.4	85	89	83	10.3	9.8	4.9
Sed	11.6	58	7.9	3.8	153.0	3.3	3.4	3.3	1.6	1.1	0.6
Significance	*P<0.001*	*P<0.001*	*P<0.01*	*P<0.001*	ns	ns	ns	ns	ns	ns	ns
Treatment											
Control	54.6	73.7	15.4	4.8	998.2	84	89	84	8.1	8.7	5.1
RU-486	59.6	40.4	12.0	2.6	1438.9	86	86	84	10.8	9.1	4.8
Sed	11.6	58	7.9	3.8	153	3.3	3.4	3.3	1.6	1.1	0.6
Significance	Ns	Ns	ns	ns	*P<0.01*	ns	ns	ns	ns	ns	ns
Day											
1	49.4	49.4	24.5	2.4	1096.6	89	90	88	10.4	9.5	5.7
2	60.3	66.6	5.2	3	1211.9	81	85	80	8.5	8.3	4.2
Sed	11.6	22.2	7.9	1.0	153.0	3.3	3.4	3.3	1.6	1.1	0.6
Significance	Ns	Ns	*P<0.001*	ns	ns	*P<0.01*	ns	*P<0.01*	ns	ns	*P<0.01*
Interactions											
Selection line x Treatment	Ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Day	Ns	Ns	*P<0.01*	ns	ns	ns	ns	ns	ns	ns	ns
Treatment x Day	Ns	Ns	ns	ns	*P<0.05*	ns	ns	ns	ns	ns	ns
Selection line x Treatment x Day	Ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Backtransformed means shown **Units nmol/L/min

6.5.2 Step 2: Reinforcement of exposure to the Iso+Dog challenge

The results (Table 6.2) revealed a significant interaction between selection line x treatment for IBT min2-9 ($P<0.01$; Figure 6.1) and IBT min 10 ($P<0.05$; Figure 6.2). The nervous selection line, regardless of treatment, was more agitated than the calm selection line. Additionally, the administration of RU-486 caused a divergent effect in the agitation scores between the selection lines, reducing agitation scores for the calm line and increasing it in the nervous line. This was significant in the case of the IBT min10 score.

During the challenge period, there was a selection line x day effect ($P<0.05$) for mean HR indicating the calm selection line on day 1 had lower mean heart rate compared with the nervous group on day 1 and the calm group on day 2. A significant treatment x day effect on agitation score for the IBT min10 ($P<0.05$) was also observed with animals treated with RU-486 on day 1 having higher scores than those treated on day 2.

Notwithstanding the above interactions, the nervous selection line was generally more agitated than the calm selection line for all agitation measures (IBT min1, IBT min2-9, IBT min10) and vocalized more during isolation ($P<0.001$) (Table 6.2). Administration of RU-486 resulted in a significant decrease in agitation the IBT min2-9 score ($P<0.05$). A difference between the selection lines for AUC was not evident, however, treatment with RU-486 resulted in an increase in the total integrated cortisol response compared with the control group ($P<0.05$). The calm selection line had a lower mean heart rate pre- ($P<0.01$) and during the challenge ($P=0.05$) compared with the nervous selection line, whilst the opposite occurred during the post-challenge period ($P=0.05$). Furthermore, heart rate variability was higher in the nervous line during the post-challenge period.

Table 6.2: Effect of temperament selection line, treatment, day of testing and significance of the interactions on the agitation scores (IBT min1, IBT min2-9, IBT min10), number of vocalizations, total integrated cortisol response (AUC), mean heart rate and variability during the second step of the **Iso+Dog** challenge (Step 2)

Main effects	***Isolation box test**				**Cortisol**	**Mean HR (bpm)**			**HR standard deviation (bpm)**		
Selection line	**Min 1**	**Min 2-9**	**Min 10**	**Vocs**	****AUC**	**Pre-chall**	**Chall**	**Post-chall**	**Pre-chall**	**Chall**	**Post-chall**
Calm	33.1	33.1	16.4	1.6	1286.3	80	83	79	6.9	7.6	3.4
Nervous	121.5	165.6	60.0	8.1	1191.7	83	90	75	7.1	10.5	5.9
Sed	12.1	52.6	10.7	0.9	116.1	2.5	3	2.2	1.2	1.6	1.2
Significance	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*	ns	*P<0.01*	*P=0.05*	*P=0.05*	ns	ns	*P=0.05*
Treatment											
Control	60.3	165.6	40.4	3.3	1110.15	77	88	78	7.7	8.5	4.5
RU-486	66.6	68.0	33.1	3.6	1367.39	75	85	76	6.3	9.6	4.8
Sed	12.1	52.6	10.7	0.9	116.1	2.5	3	2.2	1.2	1.6	1.2
Significance	ns	*P<0.05*	ns	ns	*P<0.05*	ns	ns	ns	ns	ns	ns
Day											
1	66.6	99.4	44.7	3.6	1144.0	74	86	73	7.4	10.2	5.2
2	60.3	102.0	29.9	3.3	1333.8	78	87	80	6.6	7.9	4.1
Sed	12.1	52.6	10.7	0.9	116.1	2.5	3	2.2	1.2	1.6	1.2
Significance	ns	Ns	ns	ns	ns	ns	ns	*P<0.01*	ns	ns	ns
Interactions											
Selection line x Treatment	ns	*P<0.01*	*P<0.05*	ns	ns	ns	*P<0.05*	*P<0.05*	ns	ns	ns
Selection line x Day	ns	Ns	ns	ns	ns	ns	*P<0.05*	ns	ns	ns	ns
Treatment x Day	ns	Ns	*P<0.05*	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Treatment x Day	ns	Ns	ns	ns	ns	ns	*P<0.05*	*P<0.05*	ns	ns	ns

*Backtransformed means shown **Units nmol/L/min

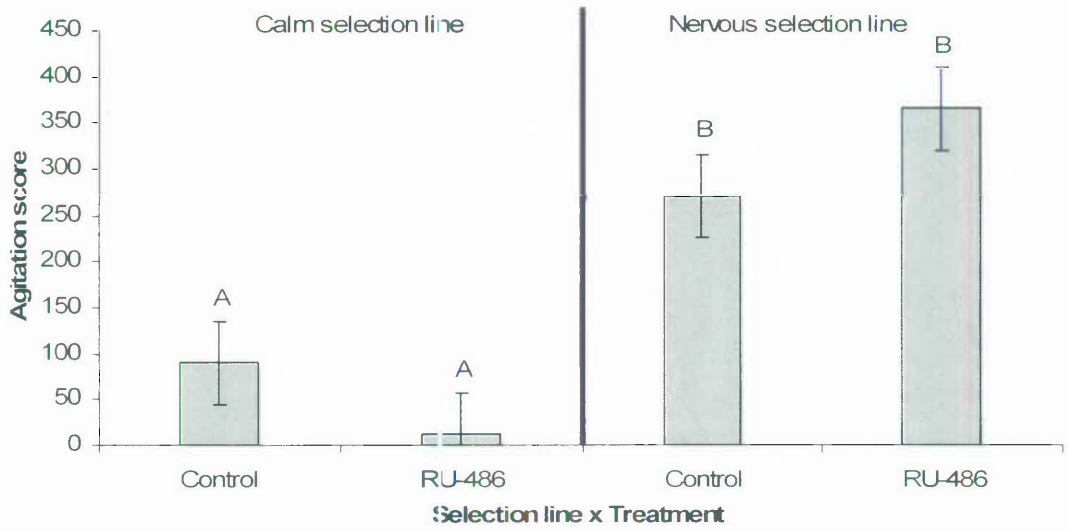

Figure 6.1: Effect of selection line x treatment for IBT min2-9 (±se) to exposure to the **Iso+Dog** challenge during exposure 2

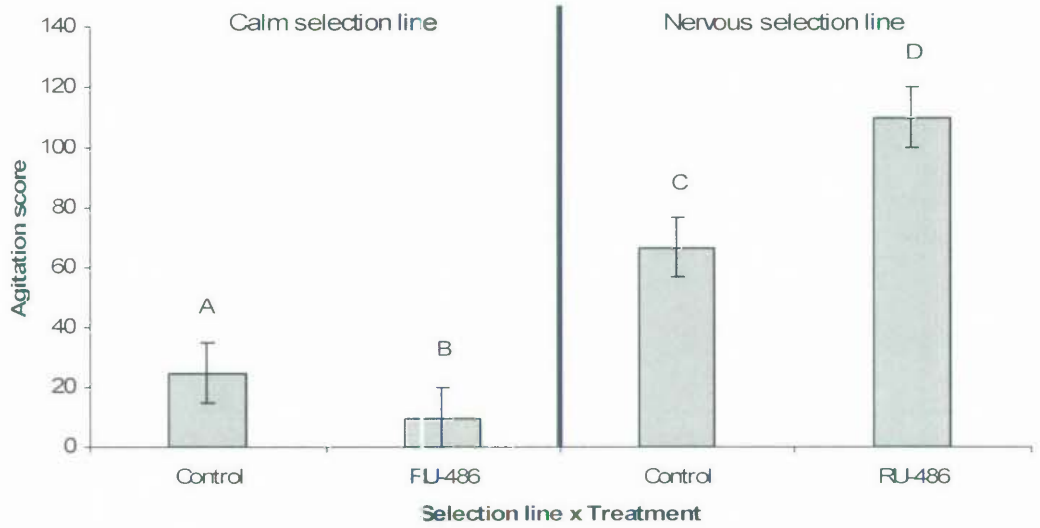

Figure 6.2: Effect of selection line x treatment for IBT min10 (±se) during the second exposure to the **Iso+Dog** challenge

The same interaction also significantly influenced mean heart rate during the challenge ($P<0.05$; Figure 6.3) and post challenge ($P<0.05$; Figure 6.4) periods. The calm selection line administered RU-486 had lower heart rates compared to the other line x treatment groups during the challenge period. Similar to the trend observed for agitation score, the effect of RU-486 on mean heart rate during the challenge was different between the selections lines.

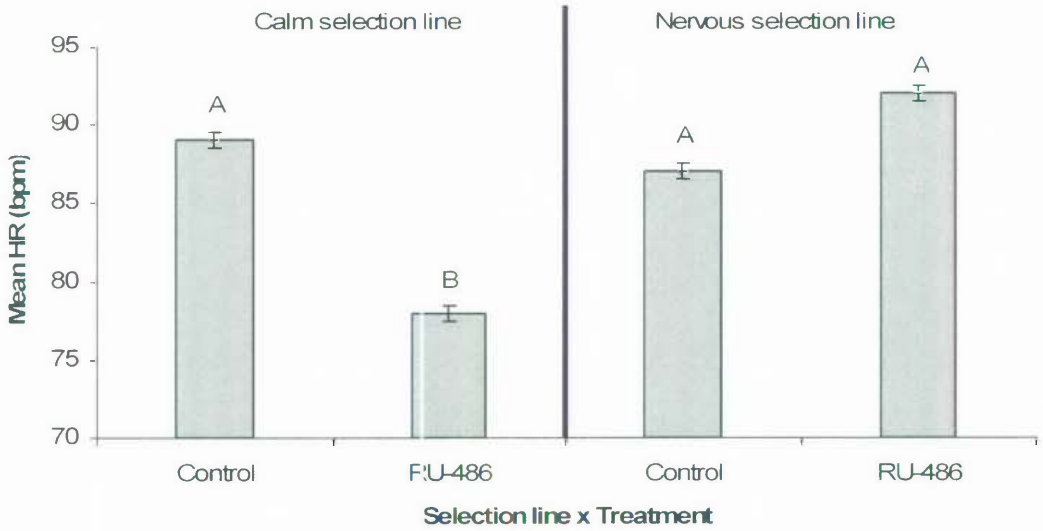

Figure 6.3: Effect of selection line x treatment for heart rate (±se) during the **Iso+Dog** challenge period

The effect of the interaction during the post-challenge period indicated a difference between the lines for control treatment (Figure 6.4).

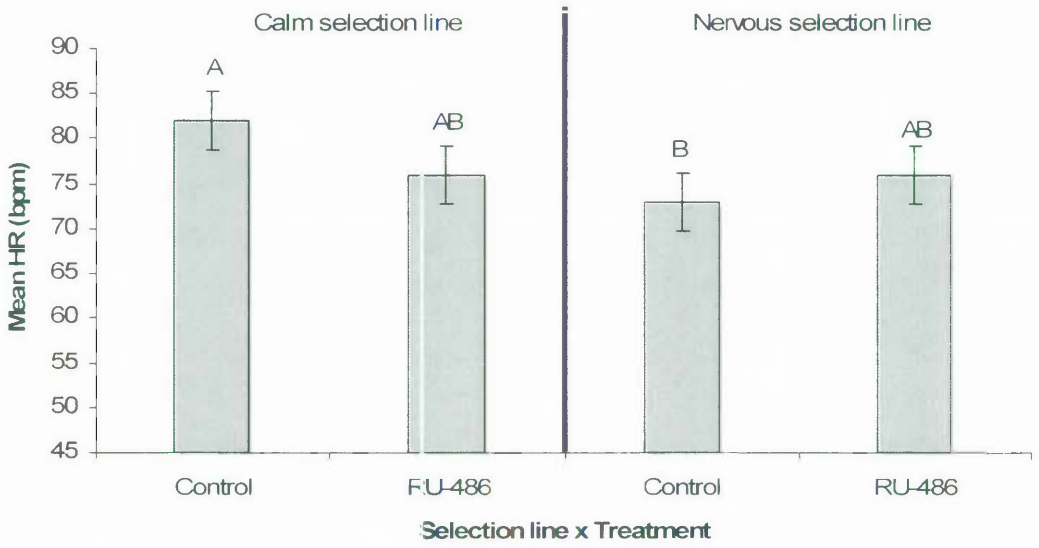

Figure 6.4: Effect of selection line x treatment on heart rate (±se) during the post-challenge period presenting the **Iso+Dog** challenge

6.5.3 Step 3: Expression of the fear potentiated response to the Iso challenge

During the final challenge period, the dog was removed and the sheep exposed to the **Iso** challenge with no treatment administered.

The results are presented in Table 6.3. No interactions between the main effects were evident during this period. The nervous selection line was significantly more agitated (IBT min1, IBT min2-9) and vocalizations were significantly greater ($P<0.001$). The trend was similar for the IBT min10 score but not significant. Prior administration of RU-486 did not affect the agitation scores. A day of testing effect was observed for IBT min2-9 and AUC. No difference in AUC between the treated and control groups was observed, however, animals previously administered RU-486 tended to have slightly higher AUC responses than the control group. During the challenge period, the nervous selection line had a higher and greater variability compared with the calm selection line ($P<0.01$) during the period.

Table 6.3: Effect of the temperament selection line, treatment, day of testing and significance of the interactions for the agitation scores (IBT min1, IBT min2-9, IBT min10), number of vocalisations, total integrated cortisol response, mean heart rate and variability on presentation of the **Iso** challenge (step 3)

Main effects	***Isolation box tests**				**Cortisol**	**Mean HR (bpm)**			**HR standard deviation (bpm)**		
Selection line	**Min 1**	**Min 2-9**	**Min 10**	**Vocs**	****AUC**	**Pre-chall**	**Chall**	**Post-chall**	**Pre-chall**	**Chall**	**Post-chall**
Calm	20.9	99.4	27.1	11.0	1183.9	87	100	86	4.8	8.6	4.0
Nervous	90.0	492.7	36.6	4.0	1137.7	82	113	84	3.8	12.5	4.7
Sed	10.7	64.3	20.9	8.4	160.1	2.8	4.5	2.1	0.6	1.5	0.4
Significance	*P<0.001*	*P<0.001*	ns	*P<0.001*	ns	ns	*P<0.01*	ns	ns	*P<0.05*	ns
Treatment											
Control	49.4	221.0	27.1	4.9	1063.7	85	107	85	4.6	9.8	4.3
RU-486	36.6	225.0	36.6	4.0	1257.9	84	106	85	4.1	11.3	4.5
Sed	10.7	64.3	20.9	2.2	160.1	2.8	4.5	2.1	0.6	1.5	0.4
Significance	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Day											
1	49.4	330.3	33.1	4.9	966.6	84	109	84	4.3	10.5	4.4
2	36.6	148.4	29.9	4.4	1355.0	85	104	87	4.3	10.6	4.3
sed	10.7	64.3	20.9	0.9	160.1	2.8	4.5	2.1	0.6	1.5	0.4
Significance	ns	*P<0.01*	ns	ns	*P<0.05*	ns	ns	ns	ns	ns	ns
Interactions											
Selection line x Treatment	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Day	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Treatment x Day	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Treatment x Day	ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

*Backtransformed means shown **Units nmol/L/min

6.6 Discussion

Administration of the cortisol antagonist, RU-486, resulted in a sustained cortisol response in sheep during the first two stages of the challenge. During the third fear potentiated stage, there was a similar trend, albeit not significant, that suggested that previous administration of RU-486 had influenced the HPA response. This type of response is consistent with results of Healy *et al.* (1983) and Weidenfeld and Feldman (1993) in rodents. Rats which received chronic intra-cerebral treatment with RU-486 displayed an enhance ACTH and corticosteroid response to stress (van Haarst *et al.* 1996). RU-486 however, did not elicit any differences in the cortisol response between the selection lines. This perhaps suggests that the genetic selection for temperament has had a small, at best or negligible effect on HPA responsiveness. This is further reinforced by the fact that the AUC response did not significantly differ between the lines during any of the three steps of the fear potentiation model in the current experiment and similar observations were found in the experiments detailed in chapter 5.

The lack of a cortisol response between the selection line, with and without the glucocorticoid antagonist administration, indicates that in this case there was very little difference in the HPA response between the two selection lines. This is opposite to the marked differences observed in behaviour between the two lines when presented with the challenge. Moberg (2000) suggests that one of the 'first lines of defense' when an animal responds to a stressor is that of an economic strategy to employ a behavioural response. This appears to be the case for this experimental situation that the animals, both calm and nervous, were able to effectively cope with the challenge by responding behaviourally, with divergent responses. However, the possibility should not be discounted that there are glucocorticoid differences between the calm and nervous sheep, as the divergence in behavioural responses between the two selection lines is very apparent, and what causes these differences still remains. Gomez *et al.* (1998) investigated different rat strains which exhibited different levels of activity in the forced swim test and found those that had lower activity in the test displayed a defect in the negative feedback mechanism of the HPA axis. It would be interesting to have utilized a different pharmacological method to assess this pathway, with administration of the dexamethasone suppression test, used to assess the negative feedback mechanism of glucocorticoids in the HPA axis.

There was evidence to suggest that there was also a slight activation, at times, of the autonomic nervous system, in regards to fluctuations of heart rate when RU-486 was administered, however this was a little inconsistent. Previous research in humans shows generally that RU-486 does not affect heart rate (Bertagna 1997). In the present experiment, given the trend for differences between the selection lines, a more detailed analysis of heart rate may be of value, but it must be remembered it can be a very transient measure. Differential heart rates can demonstrate the response to different types of stressors such as handling practices in sheep and isolation in cattle (Hargreaves and Hutson 1990; Boissy and Le Neindre 1997), characterizing the ability for an animal to adapt and cope with an adverse situation. Variability in heart rate between individuals is well documented (Palestrini *et al.* 1998). Desire *et al.* (2004) found lambs responded to the suddenness of a novel event with a marked transient (10 secs only) increase in heart rate following the appearance of a fearful object. The equipment used in this experiment only recorded heart rate at every one minute interval, which does not give perfect accuracy For example, Boissy and Le Neindre (1997) recorded the heart rate of cattle in 15-sec intervals to short term isolation and reunion, and found this to be sufficient in detecting differences between Friesian and Aubrac heifers.

The neuroendocrinal responses to stress in different lines or strains of animals selected for or against differences in temperament or emotional reactivity can be difficult to interpret (Abel 1991; Brush 1991; Courvoisier *et al.* 1996). Liebsch *et al.* (1998) suggested the possibility that the activity of the HPA axis was not necessarily as tightly linked to behavioural reactivity in stressful conditions. This was postulated in their experimental research as rats selected for high and low levels of plus-maze anxiety did not show any correlation between behavioural anxiety measures from the plus maze test and increases in ACTH and corticosterone concentrations in either lines, therefore there may not necessarily be co-selection for neuroendocrine reactivity when selecting on particular behavioural responses. Additionally, Pich *et al.* (1993) showed that anxiety related behaviour in the EPM was independent of the HPA axis response after rats had been presented with a social stressor.

It has been stated that the overall reaction to a challenge is specific to the stressors involved. Response depends on the type of stressor(s), duration of exposure or unpredictability of the situation. As stated succinctly by Moberg (2000) there is no non-specific stress response that applies to all stressors. Consequently, the activation of the primary biological response

pathways (behavioural, autonomic, neuroendocrine and immunological) will vary depending on the type, intensity and duration of the stimulus (Moberg 2000). Komesaroff and Funder (1994) showed that sheep had divergent responses in ACTH depending on whether they were exposed to a metabolic challenge (insulin-induced hypoglycemia) or an audiovisual stressor (barking dog). The results presented in Chapter 5 showed no significant differences in the cortisol response between the calm and nervous lines whilst exposed to the **Iso+Dog** challenge. This tends to support earlier observations by Beausoleil *et al.* (2005). However, Bickell and Blache (in preparation), using the same lines, did find a selection line difference in the cortisol response, when the isolation challenge was coupled with the presence of white, noisy, moving tube. Clearly, the challenge context has influenced the neuroendocrinal response. In addition to the fact that sheep will respond differently depending on the nature of the challenge, it must also be remembered that there are other intrinsic factors that can further affect the response. For example, Tilbrook and Clark (2006) found that the sex of the animal affected the cortisol response to isolation and restraint stress where it was higher in female sheep than males, whilst the opposite was observed by (Turner *et al.* 2002) during the challenge of insulin-induced hypoglycemia.

As reported by Schrader and Ladewig (1999), it may be that in this set of experiments the experience was only perceived by the animals as a mild stressor, causing behavioural changes and little differential activation of the HPA axis between the selection lines at this level. Pigs subjected to a variety of different stressors were also found to react more consistently with changes in behaviour, as opposed to changes in physiology (Hicks *et al.* 1998). For acute mild stressors, behavioural indicators shown by the temperament selected sheep may be more important as indicators of how the animal can cope with changes in the environment.

During the reinforcement step (step 2), RU-486 appeared to differentially affect behaviour between the selection lines after the first minute. The RU-486 appeared to cause a decrease in agitation in the calm group, whilst the nervous group had an increased agitation score. Such a differential effect may suggest that although cortisol differences were not significant the treatment did affect behaviour: the agitation score of the nervous group being more sensitive to the increase in cortisol associated with the RU-486. This provides some limited evidence that selection for agitation differences may have been linked to sensitivity to cortisol changes. It is notable that heart rates changes appear to occur for the calm rather than the nervous

animals, affirming findings of earlier chapters that physiological and behavioural responses are not as tightly linked. During the fear potentiation step (step 3), agitation increased, similar to that observed in earlier experiments (Chapters 4 and 5). One major contrast between the results here and those reported in chapter 5 was that the agitation scores were generally much lower in the present experiment. This could further indicate an effect of RU-486 on behaviour. However, this was not tested, so cannot be stated with certainty, additionally the effect could also be attributed to a range of other factors such as age of the sheep and sex differences.

6.7 Conclusion

Short term administration of the glucocorticoid receptor antagonist, RU-486, resulted in a sustained cortisol response during an acute stress challenge. Furthermore, there was evidence of a carry-over effect of prior treatment with RU-486 on the cortisol response. Administration of RU-486 also caused differential effects on agitation score and heart rate (however, these were not consistent), but not the integrated cortisol response, between the selection lines. The differential effect on the agitation behaviour of the selection line when exposed to the RU-486 treatment suggests that the nervous group maybe more sensitive to the increase in cortisol in acutely stressful situations. It provides some evidence that selection for agitation may be linked to sensitivity to cortisol changes as hypothesized. However the findings were not consistent at all stages and further studies will be needed for confirm the associations identified.