Chapter 5 Modulation of the fear response in sheep *via* the GABAergic and serotonergic pathways

5.1 Introduction

Temperament of farm animals has been defined earlier, as the inherent fearfulness of an animal in response to humans or to strange, novel or threatening situations. Consequently, animals that are calm or less fearful are favored over those with a more fearful or nervous temperament. Excessive or over-expression of fearfulness can be an undesirable trait in farm animals contributing to decreases in welfare, growth and reproduction (Boissy 1995). However, fearfulness plays an important role in the ability of the animal to adapt to challenging situations and is therefore inextricably linked with temperament. Temperament in livestock is largely based on behavioural fear responses. The neurophysiological basis for individual differences in temperament in ruminants is not known. Boissy *et al.* (2005) stated that the behavioural patterns relating to fear can be variable dependent on the situation. Furthermore, they assert that this variability in behaviour is dependent on both genetic and epigenetic factors. The evidence in cattle clearly shows that behavioural temperament traits are moderately heritable (Burrow 1997) and that breed variation in fearfulness is apparent even when the breeds are reared under the same conditions (Romeyer and Bouissou 1992). Given this potential to identify and select farm animals with a reduced expression of fear when presented with challenging or stressful situations could bring about genetic improvements not only in the trait but yield benefits in terms of animal welfare and productivity. Ultimately then, animals with calm temperament and low fearfulness would be hypothesized to be more capable of adapting to particular environments and more suitable for selection for production situations.

The importance of temperament in livestock production is generally recognized, however, our basic understanding of the neuro-regulatory mechanisms that underpin differences in temperament in livestock is relatively weak. What is generally known is that when animals are challenged with a fear-eliciting situation, various reactions occur in the central nervous system (CNS), including changes in many neuro-regulatory systems. In mammals, the serotonergic and GABAergic systems have been shown to be centrally involved in the expression of fear and anxiety. Support for the association between these neurotransmitter systems and the

expression of fear and anxiety arises from several research areas such as molecular and gene knock-out studies, receptor binding studies and from pharmacological challenge studies (Bond 2001; Carrasco and Van de Kar 2003).

GABA receptors are found throughout the brain, particularly in regions associated with the regulation of fear, such as the amygdala (Fuchs and Flügge 2003) and the frontal cortex (Swanson and Petrovich 1998). Biggio *et al.* (1990) and Drugan *et al.* (1994) assert that the GABAergic system plays a key role in the modulation of stress mediated changes in behaviour and physiology. This assertion stems from the considerable weight of evidence from both pharmacological and neurological studies. For example, it is well known that benzodiazepines (BZPs) enhance GABA neurotransmission and are effective in relieving anxiety disorders. Furthermore, decreases in GABA neurotransmission have been associated in the aetiology of some neurologic disorders (see review by Shiah and Yatham 1998). GABA receptor dysfunction has also been implicated in the expression of fear and anxiety, for example, $GABA_A$ receptor knock-out mice exhibit more fear related behaviours (e.g. inhibition and hyper-responsiveness) (Crestini et al, 1999). Furthermore, Frolund *et al.* (2002) and Primus and Kellogg (1991) reported changes in the $GABA_A$ receptor complex when rodents were exposed to a variety of different stressors. Martijena *et al.* (1997) also proposed an association between reduced GABAergic neurotransmission and enhanced fearfulness following exposure to a stressor. Such evidence presents a compelling case for the role that the GABAergic system plays in the expression and regulation of fear and anxiety.

The other neurotransmitter system of interest is the serotonin system. Serotonin (5-HT) is a neurotransmitter in the brain that is involved in the regulation of a variety of normal physiological functions. However, it is also involved in the aetiology of several psychiatric disorders (Sternbach 1991) and has been associated with fearful and anxious behaviours in mink (Malmkvist *et al.* 2003), rodents (Toth 2003), monkeys (Barr *et al.* 2004) and humans (Strobel *et al.* 2003). There is good evidence to show an association between serotonergic system dysfunction and the development of anxiety, depression, panic and CNS disorders, especially when aggravated by stress (Bohus *et al.* 1990). Low levels of the $5\text{-}HT_{1A}$ receptor in the brain have been repeatedly associated with mood and anxiety disorders (Heisler *et al.* 1998; Mayorga *et al.* 2001; Overstreet *et al.* 2003), whilst chronic stress (e.g. long-term isolation) has been shown to reduce $5\text{-}HT_{1A}$ hippocampal receptor levels in the rat (Popova

and Petkov 1990). Additional evidence is provided by various 5-HT receptor knock-out studies in rodents. Mice without 5-HT_{1A} receptors exhibit increased fearfulness in the elevated plus maze (EPM), the open field test and novelty test (Parks *et al.* 1998; Heisler *et al.* 1998; Ramboz *et al.* 1998). Furthermore, dysfunction of the serotonergic system can occur through slow developing changes in the monoamine oxidase A genes (responsible for 5-HT degradation) and 5-HT transporter genes which increase in expression in response to chronic stress in mice (Filipenko *et al.* 2002). Additionally, mice with the 5-HT transporter gene knock-out exhibit increased ACTH levels when exposed to an acute stressor and increased corticosterone levels in response to chronic stressors (Lanfumey *et al.* 2000; Li *et al.* 1999). Although evidence of the relationship between increased expression of fear and anxiety and dysfunction of the serotonergic system is evident in rodents, there is limited data available for farm animals. Nevertheless, this association is potentially important in the context of understanding the inter-animal variability in the expression of temperament of livestock.

The study of neurotransmitter systems has been greatly enhanced through the use of pharmacological models, such as the administration of specific ligands to activate particular receptor and block or active the responses (Cloninger *et al.* 1993). This approach allows investigation into the relationship between fearfulness and the reactivity of the targeted neurotransmitter system (Weijers *et al.* 2001). We chose to use this type of approach to study the GABA and 5-HT neurotransmitter systems which we hypothesised are involved in the expression of temperament in livestock. Diazepam was selected because of its well characterized effects of increasing GABA transmission resulting in attenuation of fear and anxiety (Korte *et al.* 1990). The serotonin agonist, 1-*m*-chlorophenylpiperazine (*m*-CPP) was chosen, as its administration has been found to reduce the exploratory behaviour of rats when placed in a light-dark box, a typical anxiety challenge for rodents (Bilkei-Gorzó 1998). Additionally, when rats were administered *m*-CPP, the number of escape attempts tended to increase when the animals were exposed to the EPM (Jones *et al.* 2002). Research on the administration of these treatments to livestock, particularly sheep, is limited and therefore this represents a novel approach to investigate their effects on temperament. The work presented here follows on from that investigated in Chapter 4 assessing pharmacological treatments to block or activate the GABA and 5-HT receptors. However, although the hypotheses being tested are based on responses in other species, it was recognized that species may vary in their responses to these pharmacological agents.

The evidence presented suggests the GABAergic and serotonergic systems may play a role in the expression of temperament in livestock. In the following experiments, the role of these systems in the expression of temperament of sheep was investigated using sheep divergent for temperament (calm and nervous) on exposure to the fear potentiation model developed in chapter 4. The sheep were selected on the basis of their response to the original Isolation box test context (agitation measured over a minute). Consequently the expectation was that these animals will have a different response, behaviourally and physiologically, which can be manipulated to allow a better understanding of the role that these neurophysiological pathways play in livestock temperament.

5.1.1 Hypotheses

Experiment 1: Administration of the 5-HT receptor agonist, *m*-CPP would increase the fear response more in the animals from the nervous selection line compared to the calm selection line.

Experiment 2: Administration of the GABA receptor agonist, DZP would decrease the fear response more in the animals from the calm selection line compared to the nervous selection line.

5.2 Materials and methods

Experiments were conducted at The University of Western Australia's (UWA) Allandale research farm. The use of the sheep selection lines and the procedures for the experiments in this chapter were approved by the University's Animal Ethics Committee (AEC Number: RA/3/100/289).

5.2.1 Animals

Animals were sourced from the Allandale research flock, a Merino flock managed and maintained by UWA, which comprises two temperament lines, identified as calm or nervous (Murphy 1999) (see chapter 2 for more details). For experiment 1, 56 Merino ewes, aged 12 months (liveweight range 38 - 55 kg) were used and in experiment 2, 56 Merino ewes aged 10 months (liveweight range 28 - 35 kg) were used. Additional animals were used in each experiment as companions to prevent the last and the first test animals from being isolated prior to and immediately after the challenge, respectively. Animals were randomly allocated

to treatment groups from within the temperament selection line after stratification by liveweight.

5.2.2 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.

5.2.3 Manipulation of fear *via* the serotonergic pathway

A factorial design comprising the two temperament selection lines (calm and nervous), two challenge contexts (**Iso** and **Iso+Dog**) and two pharmacological treatments (*m*-CPP and the control) were used. The serotonergic treatment, *m*-CPP, is a serotonin agonist which acts as an anxiogenic, altering serotonergic neurotransmission to elicit an increase in the fear response of the animal (Bagdy *et al.* 2001).

The sheep received the treatments 20 minutes prior to the commencement of the third step of the model. The treatments were 2 mg/kg *m*-CPP, the serotonergic agonist and the control (physiological saline). The dose chosen was based on the results from the earlier dose response experiment (see chapter 3). The *m*-CPP (Sigma-Aldrich Australia Pty Ltd.) was made up in 70% ethanol (ETOH) and saline to make a standard stock solution (20 mg/ml). Both *m*-CPP and saline were administered *via* intramuscular injection into the hind leg.

5.2.4 Manipulation of fear *via* the GABAergic pathway

A second experiment was undertaken to test the GABA agonist, diazepam (DZP). The same experimental design used above was applied on a different set of animals from the selection line flock. Also, each step was conducted on one rather than two days. The treatments administered during the third exposure included DZP (0.4 mg/kg) and the control (physiological saline). Diazepam is a GABAergic agonist which works by enhancing the inhibitory effects of GABA, thereby eliciting anxiolytic responses, or a decrease in the fear response of the animals (Wilson *et al.* 2004).

5.2.5 Experimental procedure

On the morning of each test day, the sheep were bought into the yards and drafted into their two challenge groups and moved to separate areas. The sheep were fitted with a heart rate monitor 20 min prior to the challenge, and then left in their groups until the challenges commenced. They were held in a race with other sheep until the challenge commenced. Blood samples were collected at 0, 15, 30, and 60 minutes relative to start of the challenge for each animal. After the challenge, the animals were moved to a nearby pen. The pharmacological treatments were administered 20 min prior to the commencement of the third step. Based on the literature and previous experiments in Chapters 3 and 4, 20 min was considered to be an effective time period to elicit measurable responses (See chapter 3; Haleem 1993; Korte *et al.* 1990; Siemiatkowski *et al.* 2000; Wallis and Lal 1998; Wongwitdecha and Marsden 1996).

5.2.6 Sampling measures

5.2.6.1 Blood sampling

For both experiments, four blood samples were collected by jugular venepuncture (6 ml serum separator vacutainers, (Becton Dickinson Ltd)) at 0, 15, 30 and 60 min relative to the commencement of the challenge. The samples were then processed according to the procedures described in chapter 2.

5.2.6.2 Heart rate monitoring

Polar heart rate monitors were used for both experiments. The monitors recorded the average heart rate every 1 min over a 2 h episode (see chapter 2).

The heart rate profile was recorded over 2 hours. Three periods were selected from the entire dataset to analyse which consisted of comparing the amount of time sheep spent in the box, with the same amount of time prior to- and post-challenge. Therefore we assessed three periods of 8; pre-challenge, during the challenge, and post-challenge. The mean heart rate and standard deviation was determined for each of the three periods.

In preliminary analysis we looked at the variability of the heart rate as a measure of stress and found very little differences, therefore we decided not to present these results within the thesis.

5.2.7 Cortisol assay

Serum concentrations of cortisol were measured using a commercially available cortisol radioimmunoassay kit (Spectria Cortisol RIA, Orion Diagnostica) as described in chapter 2. For experiment 1, the intra- and inter-assay coefficients of variation were 4.83 % and 13.65 % for high, 7.4 % and 4.73 % for medium and 7.72 % and 6.77 % for low control samples. For experiment 2, the intra- and inter-assay coefficients of variation were 9.73 % and 9.17 % for high, 10.0 % and 8.95 % for medium and 3.88 % and 9.27 % for low control samples.

The total integrated cortisol response over time (0 – 60 min) (AUC) was calculated using the area under the curve technique based on the trapezoidal rule (refer chapter 2).

5.2.8 Statistical analysis

All statistical tests were performed using SAS Version 8.2 (SAS Institute Inc., 1999). The homogeneity of variance was tested for all variables and either logarithmic or square root transformations were used to normalize the data prior to analysis. The dependent variables were first minute in the isolation box (IBT min1), the 2-9 minute period in the box when the door was opened to present either the presence or absence of the dog (IBT min2-9), the last minute when the door was closed (IBT min10), number of vocalizations (Vocs), heart rate (HR) mean and standard deviation for each period and the integrated serum cortisol response (AUC). Least square means (LSM) = standard errors are presented and where data was transformed, the back-transformed LSMs are presented.

For both experiments (5-HT and GABA), the GLM procedure in SAS was used for the analysis. For each of the first two steps, the model contained the main effects of selection line

(nervous and calm), challenge (**Iso+Dog** and **Iso**), day of testing (1 and 2) plus the first and second order interactions. The model for the GABA experiment data, did not include the fixed effect of day as each step was conducted over 1 day. The model for the final step 3 (expression of the fear potentiated response) contained the same main effects, plus pharmacological treatment (control and *m*-CPP or DZP) and the various interactions. Non-significant interactions were sequentially removed until the simplest significant ($P<0.05$) models were obtained.

5.3 Results

5.3.1 Manipulation of fear *via* the serotonergic pathway

*5.3.1.1 Step 1: Initial response to the **Iso+Dog** challenge*

A significant interaction between selection line x challenge for the IBT min2-9 agitation score, number of vocalizations, AUC and mean HR during the post-challenge period was observed (Table 5.1).

Table 5.1: Effect of temperament selection line, fear challenge, day of testing and significance of the interactions on the agitation scores (IBT min1, IBT min2-9, IBT min10 and number of vocalizations), total integrated cortisol response (AUC), mean heart rate and heart rate variability (Step 1)

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	61.9	105.4	15.2	2.2	572.4	70.8	73.3	80.5	5.5	6.3	6.6
Nervous	140.9	341.5	52.5	8.9	550.0	74.3	75.4	93.6	5.2	6.2	8.0
Sed	8	38	9	2.8	49	1.81	2.59	2.18	0.7	1.7	1.1
Significance	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*	ns	*P=0.05*	ns	*P<0.001*	ns	ns	ns
Challenge											
Isolation	111.5	504.9	41.7	8.7	614.0	72.0	73.5	92.0	6.2	6.7	8.3
Isolation + Dog	84.4	39.1	22.0	2.3	512.8	73.1	75.1	82.1	4.6	5.8	6.4
Sed	8	38	9	2.8	49	1.81	2.59	2.14	0.6	1.6	1.1
Significance	*P<0.01*	*P<0.001*	*P<0.01*	*P<0.001*	*P<0.05*	ns	ns	*P<0.001*	*P<0.01*	ns	ns
Day											
1	95.8	199.6	28.0	4.2	584.0	73.9	75.6	86.0	4.9	5.5	6.7
2	99.2	212.8	34.3	5.9	539.1	71.2	73.0	88.1	5.8	7.1	8.0
Sed	8	38	9	2.8	49	1.8	2.5	2.1	0.7	1.7	1.06
Significance	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Interactions											
Selection line x Challenge	Ns	*P<0.001*	ns	*P<0.05*	*P<0.01*	ns	ns	*P<0.05*	ns	ns	ns
Selection line x Day	Ns	*P<0.01*	*P<0.01*	ns	ns	ns	ns	ns	ns	ns	ns
Challenge x Day	Ns	ns	*P<0.01*	ns	ns	ns	ns	*P<0.01*	ns	ns	ns
Selection line x Challenge x Day	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

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

For IBT min2-9, no differences were observed between the selection lines when presented with the **Iso+Dog** challenge, however there was a marked difference between the selection lines (nervous > calm) when placed in the **Iso** challenge (Fig 5.1). The IBT min2-9 agitation scores were significantly higher for the **Iso** compared to the **Iso+Dog** challenge.

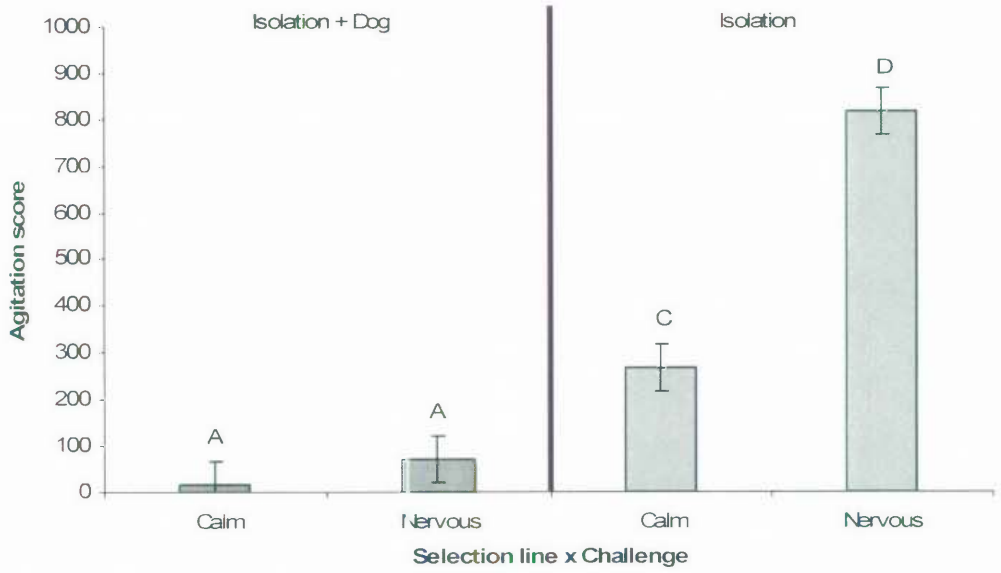


Figure 5.1: Effect of selection line x challenge (±se) for the IBT min2-9 agitation score (Step 1)

The nervous line vocalized significantly more during the **Iso** challenge compared with the calm line during either challenges or the nervous line presented with the **Iso+Dog** challenge (Figure 5.2).

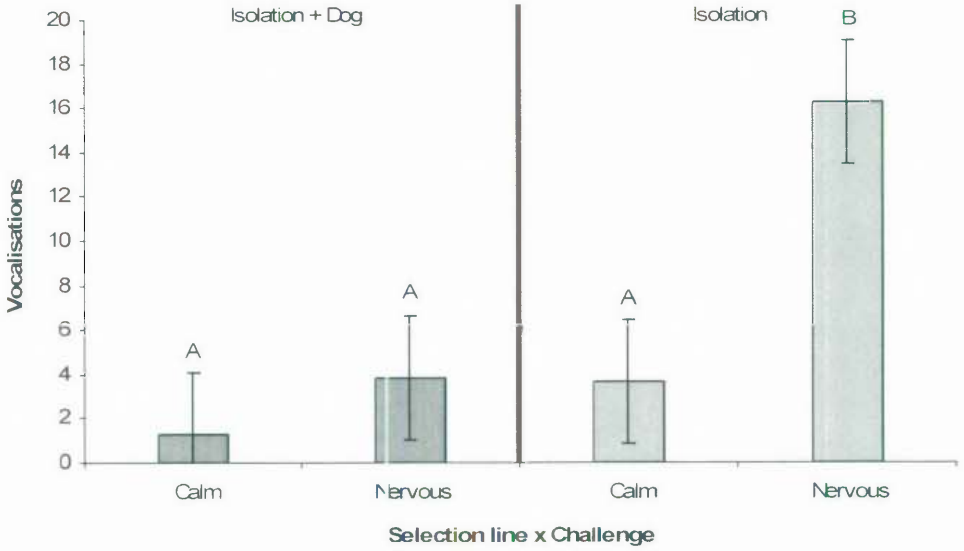


Figure 5.2: Effect of selection line x challenge (±se) for the number of vocalisations (Step 1)

The selection line x challenge interaction ($P<0.01$) for AUC showed that the calm line presented with the **Iso** challenge had a higher cortisol response compared with the other three selection line x challenge groups (Figure 5.3).

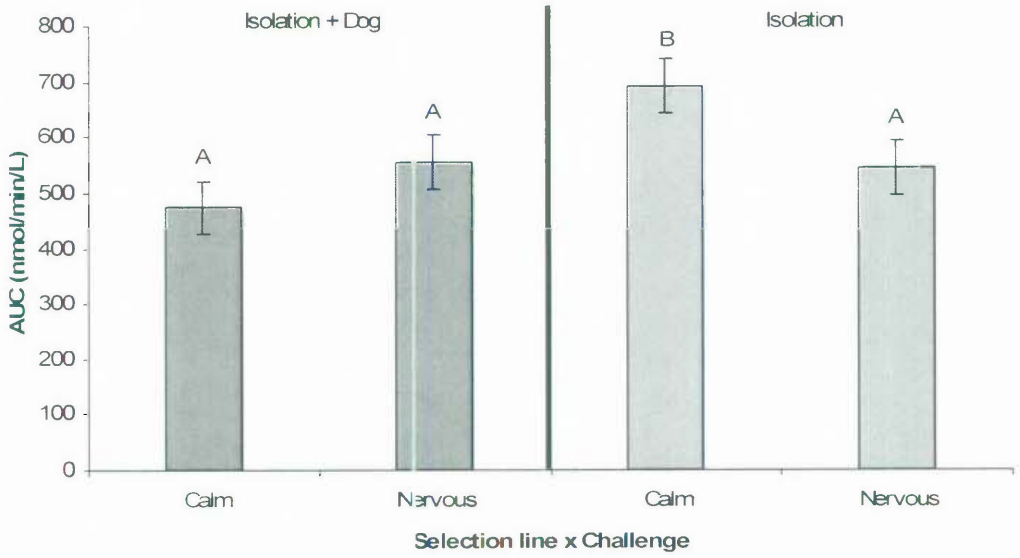


Figure 5.3: Effect of selection line x challenge (±se) for the total integrated cortisol response (AUC) (Step 1)

The interaction between selection line x challenge was only significant for mean heart rate during the post-challenge period. The results indicated the nervous line on presentation of the **Iso** challenge had higher mean heart rates compared with the other three groups (Figure 5.4). Additionally, the calm line presented with the **Iso+Dog** challenge had a lower mean heart rate compared with both the nervous line groups.

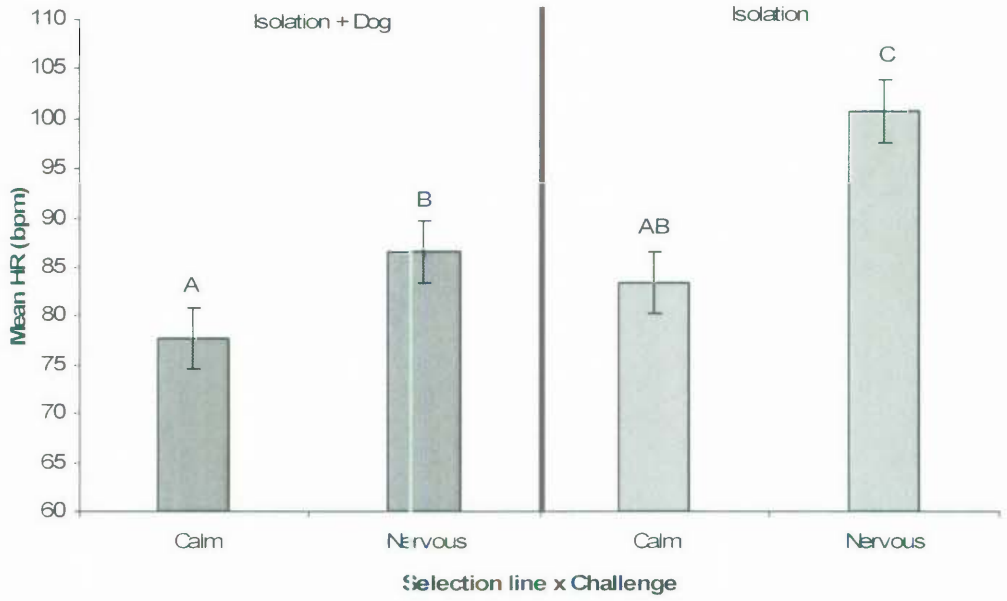


Figure 5.4: Effect of selection line x challenge (±se) for the mean heart rate during the post challenge period (Step 1)

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

The results for exposure 2 were similar to those from exposure 1 (Table 5.2). However, the main difference was that the interaction between selection line x challenge was only significant (P=0.05) for mean HR during the challenge. The interaction between selection line x day was significant for all the agitations scores. The nervous line presented with the **Iso** challenge had a significantly higher heart rate during the challenge period compared with the other groups (Figure 5.5).

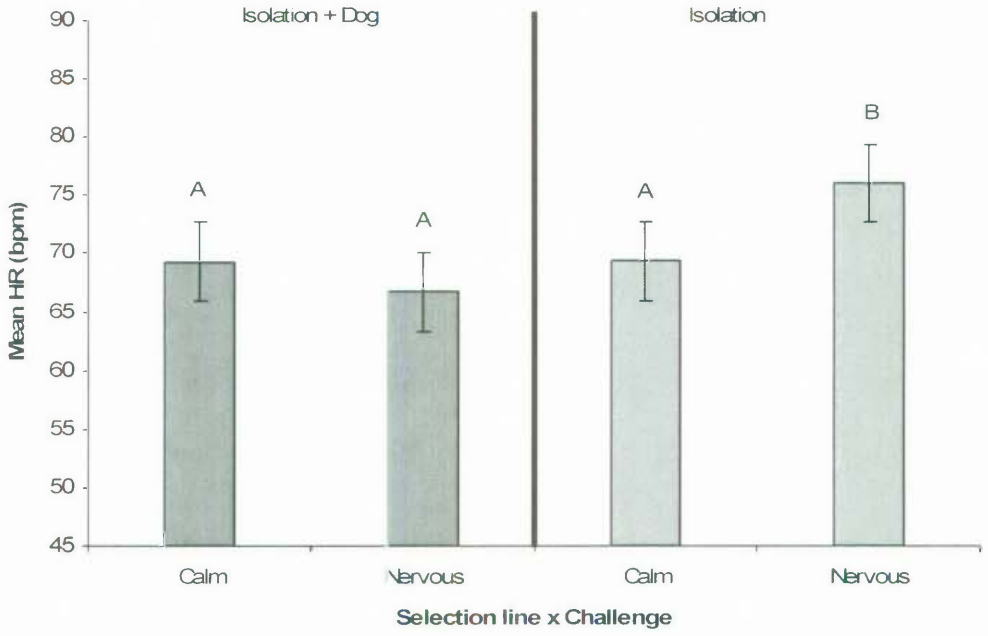


Figure 5.5: Effect of selection line x treatment (±se) for the mean heart rate during the challenge period (Step 2)

Table 5.2: Effect of temperament selection line, fear potentiation challenge and day of testing and significance of the interactions on the agitation scores (IBT min1, IBT min2-9, IBT min10, and number of vocalizations), total integrated cortisol response (AUC), mean heart rate and heart rate variability (Step 2)

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	58.87	132.25	22	1.93	415.72	66.43	67.98	81.86	4.10	4.85	9.49
Nervous	132.25	431.39	66.75	7.39	482.99	69.80	72.69	94.18	5.10	6.36	9.39
Sed	9.5	70.2	8.8	3.7	51	2.05	2.4	2.7	0.6	1.7	2.3
Significance	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*	ns	ns	*P=0.05*	*P<0.001*	*P=0.05*	ns	ns
Challenge											
Isolation	97.61	529.00	38.94	5.26	464.05	68.52	71.37	91.66	5.05	5.87	10.08
Isolation + Dog	80.28	85.93	43.96	2.72	432.68	67.71	69.30	84.37	4.14	5.26	8.25
Sed	9.5	71	8.9	3.7	51	2.05	2.4	2.7	0.7	1.7	2.3
Significance	Ns	*P<0.001*	ns	*P<0.001*	ns	ns	ns	*P=0.01*	ns	ns	ns
Day											
1	85.19	218.45	36.76	3.06	368.71	69.93	72.13	86.80	4.44	5.64	9.39
2	92.35	305.55	47.33	4.66	544.57	66.31	68.55	89.23	4.71	5.53	9.49
Sed	9.5	70.2	8.8	3.7	51	2.05	2.4	2.7	0.7	1.7	2.3
Significance	Ns	Ns	ns	ns	*P<0.001*	ns	ns	ns	ns	ns	ns
Interactions											
Selection line x Challenge	Ns	Ns	ns	ns	ns	ns	*P=0.05*	ns	ns	ns	ns
Selection line x Day	*P<0.05*	*P<0.05*	*P<0.05*	ns	ns	ns	ns	ns	ns	ns	ns
Challenge x Day	Ns	Ns	ns	ns	*P<0.05*	ns	ns	ns	ns	ns	ns
Selection line x Challenge x Day	Ns	Ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

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

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

The serotonergic agonist, *m*-CPP, was administered during this step and the animals were presented with the **Iso** challenge. The results are presented in Table 5.3. There were very few significant interactions between the main effects. The notable exceptions were a significant effect of selection line x treatment for mean heart rate pre-challenge and post-challenge. Several interactions were also found for HR standard deviation (Table 5.3).

Table 5.3: Effect of temperament selection line, fear potentiation challenge, pharmacological treatment and day of testing and significance of the interactions on the agitation scores (IBT min1, IBT min2-9 and number of vocalisations (Step 3)

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	27.39	144.72	13.19	4.16	1114.3	69.57	71.20	78.86	8.41	5.75	5.93
Nervous	86.88	307.68	34.12	6.91	1043.1	70.02	73.74	82.90	8.25	7.61	5.47
Sed	12.1	61.4	8.7	3.5	46.1	2.2	3.1	3.1	1.1	1.2	1.0
Significance	*P<0.001*	*P<0.01*	*P<0.01*	ns	ns	ns	ns	ns	ns	ns	ns
Challenge											
Isolation	59.52	273.27	21.75	7.20	1037.9	69.65	66.76	83.41	7.85	5.47	4.57
Isolation + Dog	46.77	170.04	20.90	3.99	1091.1	69.93	76.17	78.34	8.85	8.08	7.10
Sed	12.1	61.4	8.7	3.5	46.1	2.2	3.1	3.1	1.1	1.2	1.0
Significance	ns	ns	ns	ns	ns	ns	*P<0.05*	ns	ns	*P<0.01*	*P<0.01*
Treatment											
m-CPP	28.46	143.25	13.87	3.50	1137.9	68.98	71.87	77.90	8.94	6.96	4.81
Control	84.98	309.79	32.78	8.20	365.04	70.60	73.07	83.86	7.69	6.30	6.75
Sed	12.1	61.4	8.7	3.5	46.1	2.2	3.1	3.1	1.0	1.2	1.0
Significance	*P<0.001*	*P<0.01*	*P<0.01*	*ns*	*P<0.001*	ns	ns	ns	ns	ns	*P<0.05*
Day											
1	64.80	256.57	18.17	5.91	1137.9	71.48	72.08	80.59	7.24	6.30	6.17
2	42.41	183.68	24.77	4.86	996.2	68.11	72.85	81.17	9.58	6.96	5.26
Sed	12.1	61.4	8.7	3.5	46.1	2.2	3.1	3.1	1.1	1.2	1.0
Significance	*P<0.05*	ns	ns	ns	ns	ns	ns	ns	*P<0.01*	ns	ns
Interactions											
Selection line x Challenge	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Treatment	ns	ns	ns	ns	ns	*P<0.01*	ns	*P<0.05*	ns	ns	ns
Selection line x Day	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Challenge x Treatment	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Challenge x Day	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Treatment x Day	ns	ns	ns	ns	ns	ns	ns	ns	*P<0.001*	ns	*P<0.01*
Selection line x Challenge x Treatment	ns	ns	ns	ns	ns	ns	ns	ns	*P<0.01*	ns	ns
Selection line x Challenge 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
Challenge x Treatment x Day	ns	ns	ns	ns	ns	ns	ns	ns	*P<0.01*	ns	ns

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

The nervous line administered *m*-CPP had a lower mean rate compared with the calm line administered *m*-CPP and the nervous line control group, whilst calm controls had a lower heart rate compared with the nervous control ($P<0.01$; Figure 5.6).

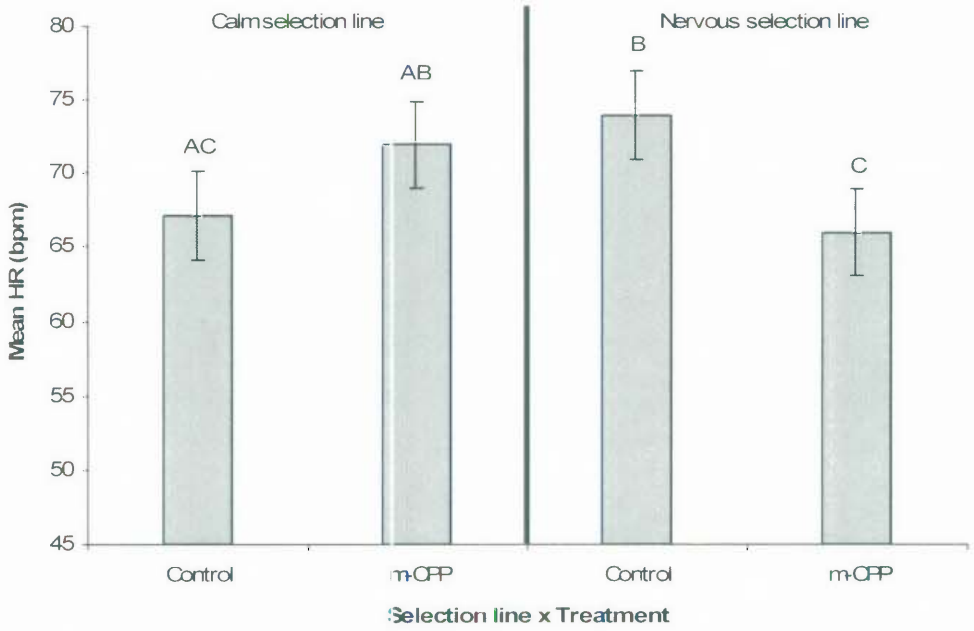


Figure 5.6: Effect of selection line x treatment (±se) for the mean heart rate during the pre-challenge period (Step 3)

During the post-challenge period the nervous control group had a significantly higher mean heart rate compared with the other three groups ($P < 0.05$, Figure 5.7). An interaction between temperament selection line x treatment x and challenge was observed during the third exposure prior to the challenge for HR standard deviation. In the nervous selection line, administration of the treatment, m-CPP decreased heart rate variability compared with the nervous control group, whilst the treated group and control were similar in the calm selection line.

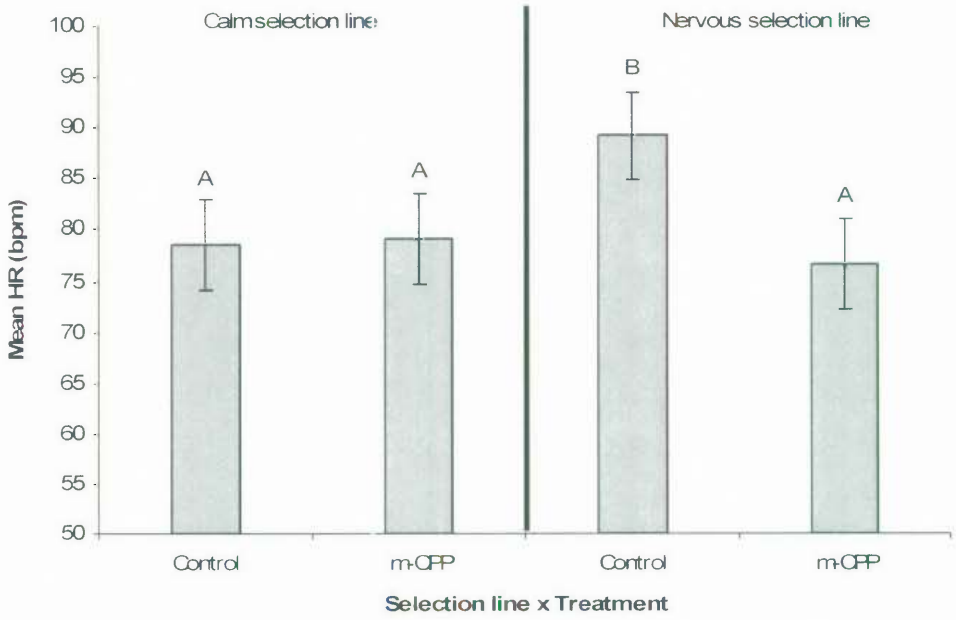


Figure 5.7: Effect of selection line x treatment (±se) for the mean heart rate during the post challenge period (Step 3)

5.3.2 Manipulation of fear *via* the GABAergic pathway

*5.3.2.1 Step 1: Initial fear response to the **Iso+Dog** challenge*

Results presented in Table 5.4 below indicate a selection line by challenge for the first minute in the IBT and the following 2-9 min agitation scores, as well as an effect on heart rate during the actual challenge period.

Table 5.4: Effect of temperament selection line and fear potentiation challenge for the agitation scores on the IBT min1, IBT min2-9, IBT min10 and vocalisations (Step 1)

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	37.21	131.1	19.62	2.75	860.25	89.80	103.20	91.94	5.0	7.6	5.4
Nervous	108.16	299.3	43.56	5.7	718.93	86.29	102.49	91.25	5.9	10.5	4.6
sed	8.3	52	6.4	5.2	98.06	2.39	3.28	2.7	0.8	1.6	1.0
Significance	*P<0.001*	*P<0.001*	*P<0.01*	*P<0.05*	ns	ns	ns	ns	ns	*P<0.05*	ns
Challenge											
Isolation	77.96	493.3	43.03	2.33	829.61	85.19	104.59	87.89	5.5	8.6	4.0
Isolation + Dog	58.82	42.9	19.98	0.4	749.57	90.91	101.09	95.30	5.3	9.3	5.9
sed	8.3	2.4	6.4	5.2	98.06	2.39	3.28	2.7	0.8	1.6	1.0
Significance	ns	*P<0.001*	*P<0.01*	*P<0.001*	ns	*P<0.05*	ns	*P<0.01*	ns	ns	*P<0.05*
Interactions											
Selection line x Challenge	*P<0.05*	*P<0.01*	ns	ns	ns	ns	*P=0.05*	ns	ns	ns	ns

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

The interaction between challenge x selection line was found to be significant for IBT min1, IBT min2-9 and mean HR during the challenge period. For IBT min1, there was no difference between the challenges for the calm selection line whereas the agitation score was significantly higher for the nervous particularly during the Iso challenge (Figure 5.8).

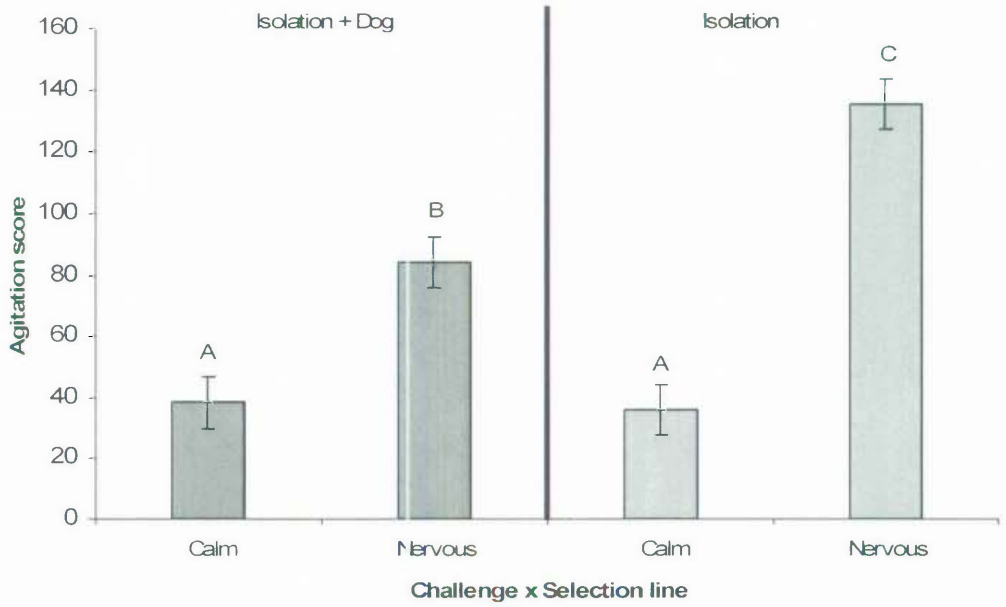


Figure 5.8: Effect of challenge x selection line (±se) for the IBT min1 agitation score (Step 1)

For IBT min2-9, there was a significant selection line difference in the **Iso** challenge and no difference for the **Iso+Dog** challenge (Fig. 5.9). Also, the agitation scores were significantly lower for the **Iso+Dog** challenge.

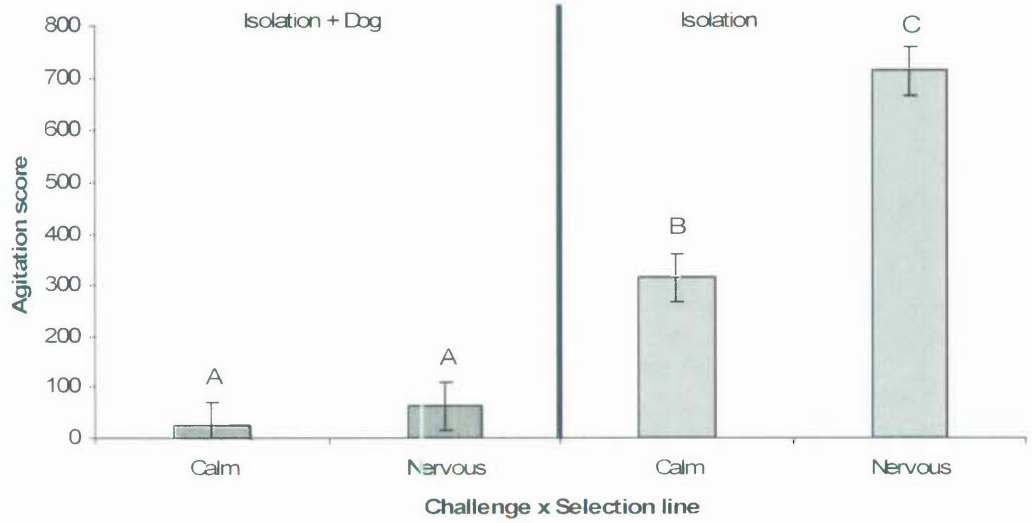


Figure 5.9: Effect of challenge x selection line (±se) for the IBT min2-9 agitation score (Step 1)

For mean heart rate during the challenge, the challenge x selection line interaction revealed a difference for the nervous selection line where the HR was higher during the **Iso** challenge ($P < 0.05$; Figure 5.10).

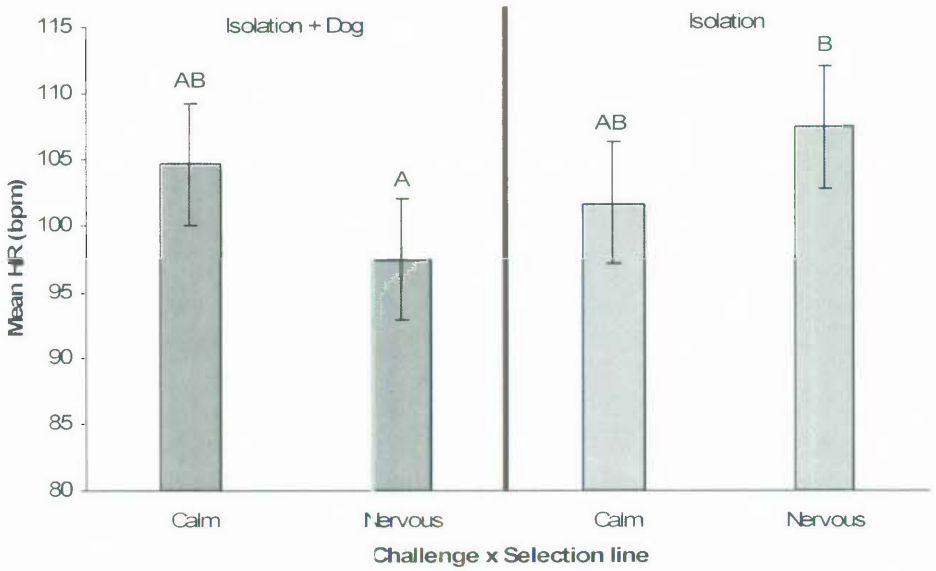


Figure 5.10: Effect of challenge x selection line (±se) for mean heart rate during the challenge period (Step 1)

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

The results presented in Table 5.5 reveal that the interaction between selection line x challenge was only significant for the agitation score IBT min2-9.

Table 5.5: Effect of temperament selection line and fear potentiation challenge for the agitation scores of IBT min1, IBT min2-9, IBT min10 and vocalisations

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	35.88	123.65	20.70	3.10	694.50	110.19	126.88	114.96	6.1	9.7	5.0
Nervous	108.58	392.44	54.32	5.47	617.71	103.55	125.30	110.60	5.2	10.4	7.9
Sed	11.6	52.4	10.6	5.1	80.24	4.65	5.15	4.7	1.3	1.5	1.3
Significance	*P<0.001*	*P<0.001*	*P<0.05*	ns	ns	ns	ns	ns	ns	ns	*P<0.05*
Challenge											
Isolation	83.72	527.16	46.51	9.49	774.71	99.20	123.83	106.37	5.1	9.3	4.9
Isolation + Dog	52.71	63.52	36.01	1.77	537.50	114.53	128.35	119.19	6.3	10.9	8.0
Sed	11.6	52.4	10.6	5.1	80.24	4.65	5.15	4.7	1.3	1.5	1.3
Significance	*P<0.01*	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.01*	*P<0.01*	ns	*P<0.01*	ns	ns	ns
Interactions											
Selection line x Challenge	ns	*P<0.05*	ns	ns	ns	ns	ns	ns	ns	ns	ns

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

The trends were similar to those observed in step 1 where the nervous selection had significantly higher agitation scores compared to the calm line and this difference was much more apparent during the **Iso** challenge ($P<0.05$; Figure 5.11).

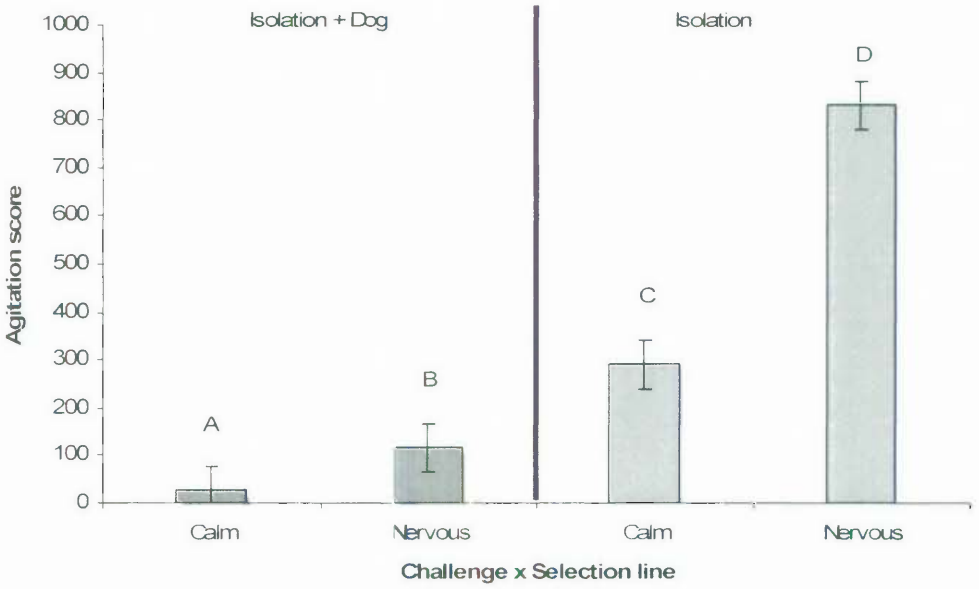


Figure 5.11: Effect of challenge x selection line (±se) for the IBT min2-9 agitation score (Step 2)

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

During step 3, the GABA agonist, DZP and the control were administered, and animals were presented with the **Iso** challenge only. There were very few significant interactions between the main experiment effect and most of these were found for the heart rate parameters (Table 5.6). The same trend between the selection lines observed during steps 1 and 2 was evident during step 3 (fear potentiation).

Table 5.6: Effect of temperament selection line, fear potentiation challenge and pharmacological treatments on the agitation scores (IBT min1, IBT min2-9, IBT min10 and vocalisations), total integrated cortisol response, mean heart rate and variability (Step 3)

Main effects	***Agitation score**				**Cortisol**	**HR mean (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	34.57	158.26	24.90	1.21	464.05	90.95	108.83	87.91	4.9	9.3	3.8
Nervous	100.06	497.74	53.58	4.80	468.72	86.12	118.28	86.29	5.5	12.8	11.9
Sed	12.1	61.4	2.4	2.5	35.4	3.9	5.4	3.4	1.2	1.4	1.4
Significance	*P<0.001*	*P<0.001*	*P<0.01*	*P<0.05*	ns	ns	ns	ns	ns	*P<0.01*	ns
Challenge											
Isolation	67.40	362.90	45.70	4.71	528.48	92.60	114.95	88.53	4.9	11.3	4.4
Isolation + Dog	59.44	251.22	30.80	1.25	411.58	84.48	112.16	85.68	5.4	10.4	4.2
Sed	12.1	61.4	2.4	2.5	35.4	3.9	5.4	3.4	1.2	1.4	1.4
Significance	ns	*P=0.05*	ns	*P<0.05*	ns	*P<0.05*	ns	ns	ns	ns	ns
Treatment											
Control	49.98	264.06	35.16	2.56	595.86	86.09	110.75	85.36	5.4	10.3	4.2
DZP	78.15	347.45	40.70	2.86	365.04	90.98	116.36	88.35	4.9	11.4	4.4
Sed	12.1	61.4	2.4	2.5	35.4	3.9	5.4	3.4	1.2	1.4	1.4
Significance	*P<0.05*	ns	ns	ns	*P<0.01*	ns	ns	ns	ns	ns	ns
Interactions											
Selection line x Challenge	ns	ns	ns	ns	ns	ns	*P*=0.01	*P=0.05*	ns	ns	ns
Selection line x Treatment	ns	ns	ns	ns	ns	ns	ns	*P<0.05*	ns	*P<0.01*	ns
Challenge x Treatment	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Selection line x Challenge x Treatment	ns	ns	ns	ns	ns	ns	ns	*P<0.05*	ns	ns	ns

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

During the actual challenge period, a selection line x challenge interaction was found, indicating a challenge difference between the calm selection line where the mean heart rate was higher in the group presented with the **Iso+Dog** challenge (P=0.01; Figure 5.12).

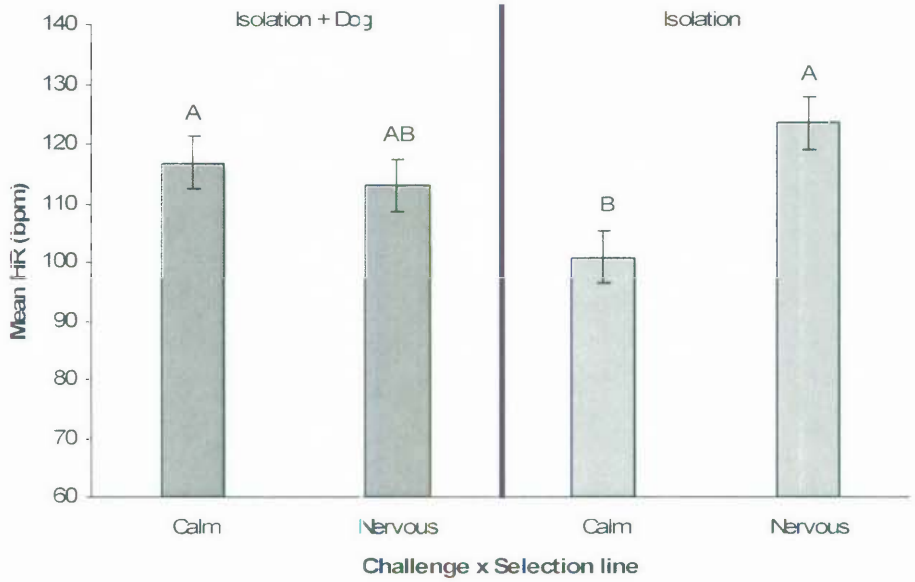


Figure 5.12: Effect of challenge x selection line (±se) for heart rate during the actual challenge period (Step 3)

During the post-challenge period the calm line presented with the **Iso** challenge had a significantly higher mean heart rate compared with the calm line presented with the **Iso+Dog** challenge ($P<0.05$; Figure 5.13).

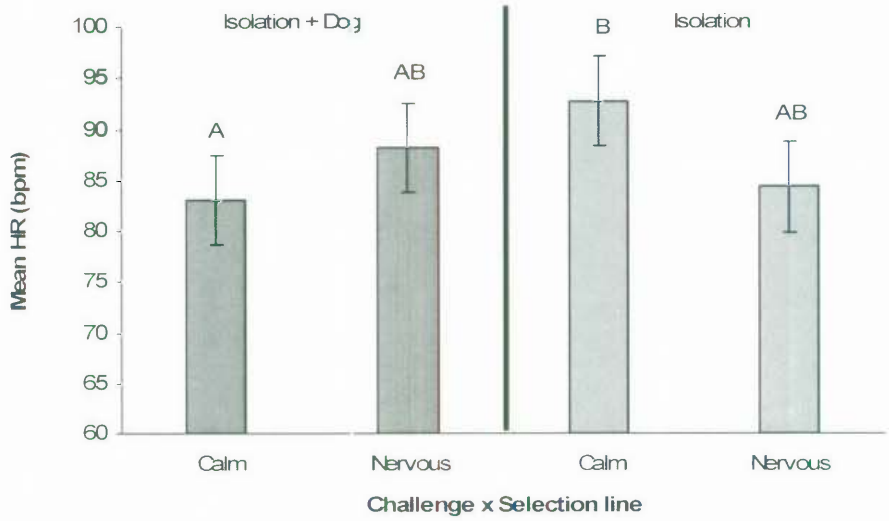


Figure 5.13: Effect of challenge x selection line (±se) for heart rate during the post-challenge period (Step 3)

Before the challenges were presented, there was a selection line x treatment interaction that showed that the control nervous line had a lower mean heart rate compared to the control calm selection line ($P<0.05$; Figure 5.14).

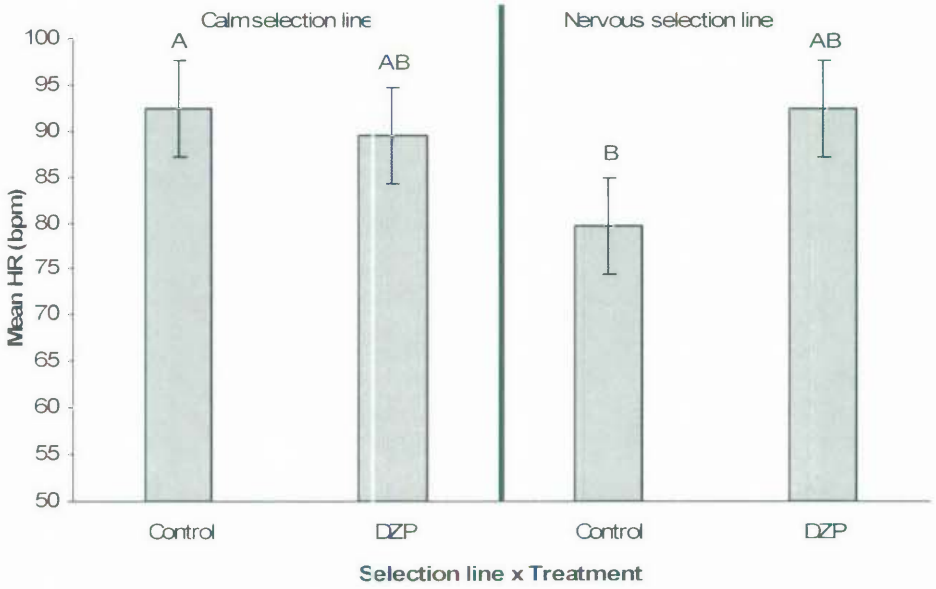


Figure 5.14: Effect of selection line x treatment (±se) for heart rate during the pre-challenge period (Step 3)

There was a significant (P<0.01) interaction between selection line x treatment where the heart rate of DZP nervous line group was significantly more variable compared with the other groups during the challenge period (Figure 5.15).

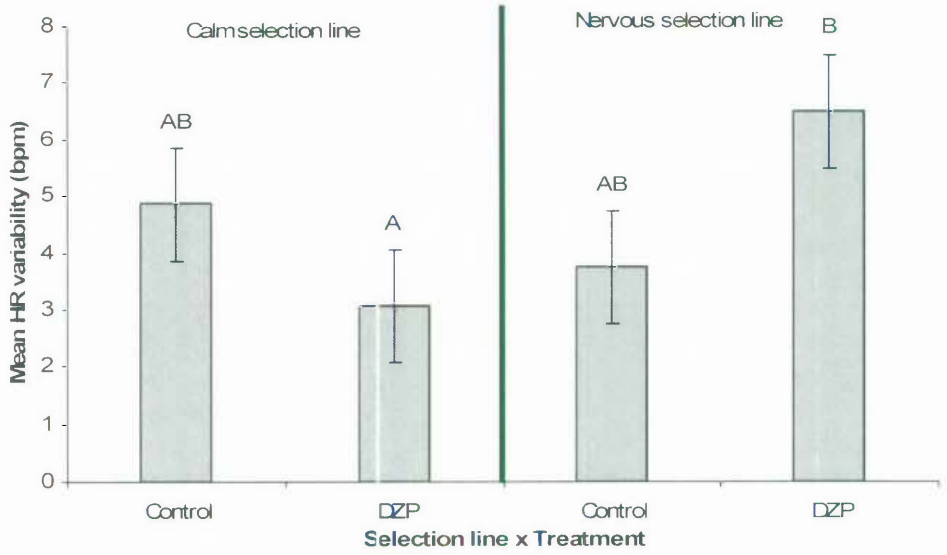
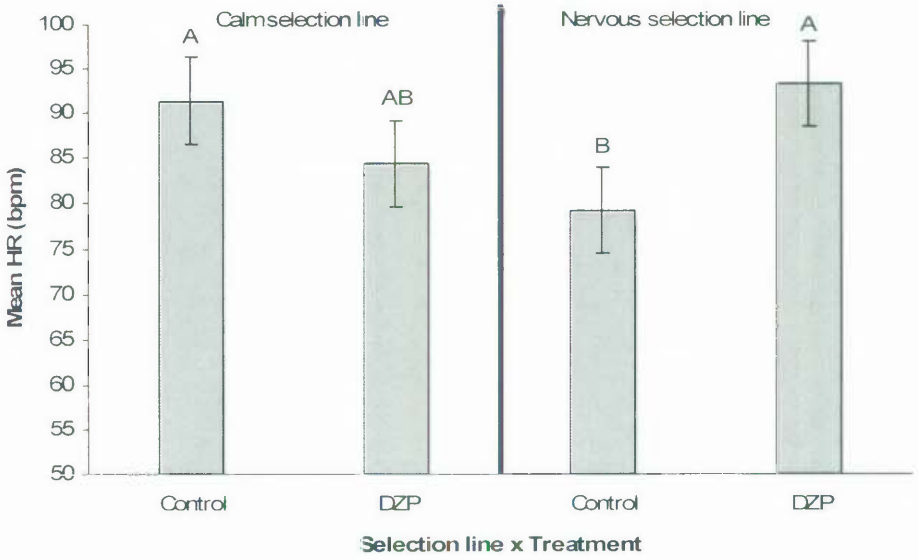


Figure 5.15: Effect of selection line x treatment (±se) for HR variability during the actual challenge period (Step 3)

A similar pattern to that in the pre-challenge period was observed during the post-challenge period, however the control calm line group had a higher mean rates compared with the control nervous line group ($P<0.05$; Figure 5.16).



Figures 5.16: Effect of selection line x treatment (±se) for heart rate for the post-challenge period (Step 3)

5.4 Discussion

The administration of *m*-CPP was hypothesized to cause increased fearfulness on exposure to the challenge, and it was anticipated that this would be higher in the nervous selection line. The administration of the 5-HT agonist, *m*-CPP was highly effective in eliciting large increases in plasma cortisol, with sustained elevation over time compared with the control treatment. However, there were no selection line interactions with *m*-CPP. This possibly indicates that this agonist was not effective in altering the fear responses between the calm and nervous lines using the fear potentiation model. There may also be other factors or reasons for this result such as the efficacy of the model. That rise in the total cortisol response following *m*-CPP administration aligns with other research (Bagdy *et al.* 2001; Fiorella *et al.* 1995; Takamatsu *et al.* 2003) showing it is highly effective in altering the HPA axis. *m*-CPP has been shown to increase stress parameters in animals and humans, such as blood cortisol and ACTH concentrations, blood pressure, heart rate, and in humans, it causes subjective feelings of anxiety (Broocks *et al.* 1997).

In rats, *m*-CPP administration also causes reduction in a variety of behaviours, including decreased exploration in the open arm and light-dark tests, and decreased activity of mice in the light compartment test (Griebel 1995). These behaviours are indicative of fear-like behaviour. In the present experiments, relative to the control group, decreases in agitation were observed across all the variables measured following treatment with *m*-CPP, which could be interpreted as increased fearfulness given the rodent findings. However, the Isolation box measures the amount of movement and noise within the box and this can be translated in several different ways, as many other tests show. For example a low agitation score was observed when the sheep were presented with the **Iso+Dog** challenge, because sheep possibly perceived the dog as a threat, and research shows sheep can respond to presentation of a dog by freezing in certain situations. However, a low agitation score in the Isolation box also reflects a calm temperament, as work by Blache and Ferguson (2005) show in Merino sheep when investigating lamb mortality from calm and nervous ewes. Therefore agitation scores from the IBT can be context dependent in these situations (e.g. **Iso+Dog**). The arena test used for a variety of different livestock (e.g. sheep, cattle and pigs), measures several different variables, including locomotion. In this test, increased locomotion can be regarded as fearful behaviour as shown by Kilgour and Szantar-Coddington (1997), along with increases in urination and defecation in single versus group arena tests. Alternately, increased exploration,

is not considered a behavioural trait associated with fear (Erhard 2003; Romeyer and Bouissou 1992), therefore ambivalence exists regarding the relationship between fear and exploration (cited in Roussel *et al.* 2004).

Diazepam is a GABA agonist, developed initially for treatment of anxiety disorders, and at low doses can cause sedation and muscle relaxation (Goldberg and Finnerty 1979). Diazepam facilitates an increase of the effect of GABA on the $GABA_A$ receptor, which is associated with a reduction in fearfulness and anxiety (Ferreira *et al.*; Korte and De Boer 2003; Sandem *et al.* 2006). For example, Korte *et al.* (1999) showed that administration of DZP to rats before placement in the EPM decreased anxiety-like behaviours. The administration of DZP in this experiment caused an increase in the amount of movement (agitation) within the isolation box, as well as a decrease in the cortisol response, and a reduction in the mean heart rate. In general, it was observed that the calm selection line had a lower heart rate compared with the nervous selection line, and there was further evidence to suggest that the DZP-mediated reduction in HR was greater in the calm selection line. Diazepam typically reduces fear associated with a novel environment to release suppressed behaviour (Marin *et al.* 1997) and if this is also the case for sheep, it could explain the increase in activity measured in the isolation box test. Further evidence to support this argument stems from the observed attenuation in the cortisol response in the DZP animals which has also been observed in rodents (Stock *et al.* 2000).

Sheep selected for calm temperament consistently responded to both challenges with lower levels of agitation compared to those from the nervous selection line. As the lines were initially selected based on their behavioural response to isolation, this was not unexpected. No interaction between the temperament selection line, challenge and the treatment were evident for the cortisol response or agitation scores. However, heart rate mean and the standard deviation were influenced by the treatment interacting with the selection line. The nervous group administered DZP responded with higher and more variable heart rates before and after the challenge compared with the other groups. Although we did see some interaction with heart rate, selection line and treatment, the lack of a similar interaction for the other parameters perhaps infers that differences in the GABAergic system may not account for the fundamental differences in temperament between the lines.

The line difference (calm<nervous) was reflected in differences of heart rate for the group administered the serotonergic anxiogenic, *m*-CPP, with an increase in the nervous group during the challenge. The divergence in behaviour and heart rate was not paralleled by the cortisol response and it seems that the selection of the animals based on temperament, either calm or nervous, has impacted largely on their behavioural response and possibly only very transient or small physiological response to fear-eliciting situations. The temperament selection process has altered behavioural reactivity to isolation, but it would appear it has not necessarily altered the HPA response. The results also suggest that fear potentiation to the **Iso+Dog** context was largely driven by altered behaviour with concomitant increases in heart rate, and not necessarily by activation of the HPA axis. This could suggest that sheep have an optimal behavioural profile for particular challenges, not a typical generalized stress response of an increase in heart rate and cortisol, therefore individual responses to the specific challenges making for a more efficient adaptive response.

Hessing *et al.* (1994) found consistent individual differences in cardiac responses of pigs with different coping strategies, categorised as active or passive, when tested in an open field and back test. The active pigs had a higher mean heart rate compared with the pigs considered to be passive. Additionally, when a novel object was introduced, all pigs with the active strategy responded with a tachycardic response, whilst a third postulated to be passive, responded with a bradycardic heart rate. The results reported by Hessing *et al.* (1994) were thought to occur mainly *via* increased sympathetic nervous activity as a strategy used by the active pigs when responding to stress, whilst the passive strategist pigs responded parasympathetically. There may be similarities here to the sheep selection lines where the nervous sheep primarily respond more sympathetically, whilst the calm selection line responds with more parasympathetic activation.

When the sheep were exposed to the context involving the dog (**Iso+Dog**), movement in the box almost ceased, which was indicative of freezing behaviour. An additional behaviour observed (results not presented), during exposure to the dog and absent in the **Iso** challenge, was an aggressive footstamp. This aligns with the results of Beausoleil *et al.* (2005) who found that sheep foot-stamped exclusively in the presence of a dog but not when they were confronted with a human or unfamiliar animal. This behaviour was postulated to have evolved directly in response to predators (Dwyer *et al.* 2004) and the increase in foot-stamping

reflects an aggressive response to a threat. An interesting aside for future studies would be to investigate whether sheep that utilized this behaviour (footstamp) in response to the **Iso+Dog** challenge had lower stress responses compared with those that did not footstamp, as this may suggest behaviour by the sheep related to their perceived control over the situation. The ability to control a stressful situation has been found to attenuate the stress response, physiologically and behaviourally (Mormede *et al.* 1988). Additionally, sheep vocalized more when exposed to the **Iso** challenge compared with the **Iso+Dog** challenge and vocalisations can be considered as an attempt to identify others in the vicinity, play a role in communication, attract conspecifics or used as an alarm (Manteuffel *et al.* 2004).

The presentation of a dog to sheep has been found to cause increases in cortisol, ACTH, adrenaline, noradrenaline and heart rate (MacArthur 1979; Beausoleil *et al.* 2005b; Torres-Hernandez and Hohenboken 1979). However, in this study, differences in the total cortisol response between the challenges was only evident in step 1 (initial exposure) and step 2 (reinforcement) in the serotonergic pathway and GABAergic pathway experiments, respectively. In both these cases, the cortisol response was higher in animals exposed to the **Iso** challenge than for the challenge when the dog was present. This was unexpected as Komesaroff *et al.* (1998) found acute increases in cortisol, ACTH, adrenaline and noradrenaline in sheep on exposure to a barking dog and Cook (1996) similarly found large stress responses in sheep when exposed to a dog stressor. In this case it appears that the sheep may have largely accommodated the stress of the challenge by their particular behavioural defense mechanism. Some researchers have reported behavioural stress reactions to be independent of the HPA axis (Courvoisier *et al.* 1996). Liebsch *et al.* (1998) suggested that although selection for particular behavioural traits, such as high levels of anxiety in rats when exposed to the plus-maze, or in this case, temperament selection based on behavioural response, there may not necessarily be concomitant change in neuroendocrine activation. In this case, there was little evidence to suggest a strong relationship between behavioural measures of fear and a concomitant increase in the cortisol response, in relation to the **Iso+Dog** challenge. A dissociation between emotionality and the HPA axis of Syracuse and Maudsley rats has also been suggested by Abel (1991) and Brush (1991). Schrader and Ladewig (1999) provide further evidence when they exposed pigs to repeated isolation from conspecifics, a reduced pituitary adrenocortical response, with no behavioural or cardiac effects observed. This was postulated to be indicative of a dissociation of the pituitary adrenal

response from behaviour and other sympathetic activities, a pattern for which there is some evidence here. Such dissociation indicates the possibility that neuro-endocrinological and behavioural responses may be regulated independently from the adreno-pituitary responses to stressors. However, Bickell and Blache (in preparation) found distinct cortisol responses correlated with the temperament selection when exposed to a novel object during the same isolation context used in the current experiments. The nervous selection line displayed higher cortisol responses. The challenge used by Bickell and Blache (in preparation) was regarded as a more neutral stimulus with no biological significance, being that of a long, white bag tube attached to a fan to make it move and be noisy. Alternatively, Beausoleil *et al.* (2005) showed that sheep from the calm selection line had a higher cortisol response compared with a nervous selection line during three different challenges (e.g. isolation box, human and dog). Differing coping strategies were suggested to be the reasoning behind the differences. We found very little evidence of cortisol differences between the calm and nervous animals on presentation to either challenge during the three steps. This suggests that the level of fear potentiation achieved was weak at best which contrasts the behavioural results observed in chapter 4.

Behavioural patterns related to fear and anxiety are often highly variable and context specific (Boissy *et al.* 2005a). Tallet *et al.* (2006) stated that behavioural and physiological responses are not always consistent and represent particular aspects of emotion. The actual level reached may consequently affect whether behavioural or physiological, or both types of responses are activated. Moberg (1985) stressed the point that there is large inter-animal variability in the stress response, as well as a large diversity of situations that would additionally affect the response. This lends itself to the idea that once the CNS has perceived what is considered a stressful situation, it responds with a defense that can vary from all or part of a behavioural, autonomic, neuroendocrinological or immunological response, of which the behavioural response would be likely to be activated first because it is the most cost effective biologically (Moberg 2000). This may also point to the existence of an optimal behavioural profile being reached first, before the activation of latter responses, for example physiological responses.

5.5 Conclusion

Sheep that were selected for calm temperament were less agitated in the isolation box test, and are therefore suggested to be more adaptable and able to cope with isolation stress, than those with a nervous temperament. The divergence between temperament lines was consistent only for behaviour, whilst physiological differences were inconsistent suggesting that temperament selection based on behavioural responses may not necessarily convey a correlated neuroendocrine response. Results of the pharmacological treatments suggest that both the GABA and 5-HT systems are involved in the behavioural expression of fear in the sheep tested. However, the evidence presented here does not support the notion that altered GABAergic or serotonergic function accounts for the temperament differences in the selection lines. Having said that, this is by no means conclusive as the pharmacological approach and fear model used may not have been sensitive or reliable enough to confirm the association.

The following chapter investigates if the response to cortisol is one of the primary mechanisms which could contribute to differences in temperament between selected lines of Merino sheep using the fear potentiation model.