Chapter 3 Dose response studies of GABAergic and serotonergic agonists and antagonists

3.1 Introduction

Temperament is defined here as the inherent fearfulness of an animal in response to a variety of situations, including the response to humans or to strange, novel or threatening situations. Kilgour (1975) defined animal temperament as the behavioural characteristics that result from an individual's physical, hormonal and nervous relationship, encompassing the animal, as well as environmental influences. Factors influencing measures of temperament include weight, age, sex, health status, breed, maternal effects, previous experience, as well as previous physical, social, environmental and genetic factors (see review by (Burrow 1997)). For example, experience plays a major role as evidenced by findings that sheep inverted during one adverse handling situation were far more difficult to handle one year later in the same yards (Hutson 1985). Variation in temperament is also evident in a variety of species, including mice (Gershenfeld and Paul 1998), mink (Malmkvist *et al.* 2003), foxes (Harri *et al.* 2003) and monkeys (Fairbanks *et al.* 2004), as well as livestock species, including sheep (Gelez *et al.* 2003), pigs (Forkman *et al.* 1995), cattle (Burrow 1998; Sato 1981), horses (Le Scolan *et al.* 1997) and poultry (Cheng *et al.* 2003). This type of variation may explain some of the differences that exist between animals in their individual responses to challenges and therefore, in their ability to cope.

Genetic factors play a large role in influencing temperament; therefore temperament varies widely between genotype, strain, and even between individuals of the same strain. Genetic factors influence how an animal reacts to a fearful situation (Murphey *et al.* 1981; Boissy and Bouissou 1995). Therefore, animal temperament equates to the behavioural fear response, where animals with calmer or better temperament scores are generally considered less fearful. Given this, the ability to select for less fearful or less anxious animals could potentially yield benefits in terms of animal welfare during typical farming practices. It could then be argued that animals with a less fearful temperament are potentially better suited for livestock production given the above-mentioned. Animals which express lower levels of fear and are more adaptable in changing circumstances, such as those involved in the farming industry, would obviously be advantaged in regards to welfare. If this is true, additional knowledge of

the factors regulating temperament would increase our ability to select livestock that are better-adapted to cope with challenges related to particular production systems. Livestock temperament is generally assessed using tests that measure behaviour, specifically focusing on those measuring 'escape and avoidance' behaviour in response to specific challenges. Although these types of tests are useful and informative, a better understanding of the underlying neurophysiological mechanisms that influence temperament is needed.

Specific neurological pathways have been implicated in differences associated with animal responses to stressful challenges. Therefore some of these, such as gamma-amino butyric acid (GABA), serotonin (5-HT) pathways may play a role in the observed variation of livestock temperament and stress responsiveness. There is a plethora of evidence showing that GABA receptor dysfunction is inter-connected with fear, anxiety and stress responsiveness. For example, many human neurological pathologies have been linked to GABA receptor dysfunction, including anxiety (Frolund *et al.* 2002), whilst in mice, stress-induced helplessness caused decreased GABA release, which can then be reversed by GABA injections into the frontal cortex (Petty and Sherman 1981). Down-regulation of $GABA_B$ receptors is also seen in the rat helplessness model (Martin *et al.* 1989), whilst knockout mice with a $GABA_{B(1)}$ subunit deletion were found to be more anxious in a light-dark anxiety model than those without the deletion (e.g. a reduced number of moves from the light to dark areas indicates increased anxiety) (Mombereau *et al.* 2004). Major changes in $GABA_A$ receptor binding affinities have also been shown after severe stress exposure (Chadda and Devaud 2005) and further studies attest to changes in $GABA_A$ receptor levels in different brain regions when animals are subjected to acute or repeated stressors (Martijena *et al.* 2002; Montpied *et al.* 1993; Clément 1996; Korpi *et al.* 2002; Krogsgaard-Larsen *et al.* 1994; Primus and Kellogg 1991). Finally, some of the most compelling evidence emerges from GABA ligand studies. Therefore an association between reduced GABAergic transmission and enhanced stress responsiveness, leading to increased fearfulness and anxiety has been clearly shown.

Serotonin (5-HT) dysfunction has also been associated with disorders, including anxiety, depression and panic in humans and animals, especially when associated with stress. Prozac © (Fluoxetine) up to 2003 was one of the leading treatments used in the United States for treatment of depression in humans and animals (Business Insights 1999). This treatment works by blocking the uptake of 5-HT into platelets, thereby increasing 5-HT levels in the

brain. The particular localisation of 5-HT receptor subtypes, especially in the cortex and amygdala, lends itself to the theory that the 5-HT neurotransmitter system is involved with the expression of fear and anxiety (Bhatnagar *et al.* 2004). Enhanced serotonergic transmission has been shown to increase anxiety related behaviour (Chilmonczyk 2002; Díaz-Véliz *et al.* 1997) and mice lacking the 5-HT_{1A} receptor are found to actively avoid novel and challenging situations, indicating increased fearfulness and anxiety (Parks *et al.* 1998). The administration of particular serotonergic antagonists can consistently reduce behavioural measures of anxiety (Kilfoil *et al.* 1989) and Van den Buuse and Wegener (2005) found that administration of 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) and Buspirone lowered the effects of stress on cardiovascular responses in rats.

It has been suggested that assessing the effect of ligands known to have specific neuro-physiological pathways, in addition to more traditional measures of physiological and behavioural indicators of stress, would be of value for investigation of stress responses. The aim of these experiments was to investigate the effects of two GABAergic ligands and two serotonergic ligands on the stress responsiveness of sheep during exposure to acute stressful challenges involving social isolation, the arena test and the isolation box test. It was hoped that the results would help in the interpretation of the role of these specific neurophysiological pathways in the expression of temperament and stress in livestock, and show how these specific pathways were implicated in the expression of temperament was also investigated. Another aim was to identify the appropriate dose to use in subsequent studies as this was not known.

3.2 Hypotheses

The hypotheses of the experiments were that:

i. The GABA antagonist, Pentylenetetrazol (PTZ) would reduce the inhibitory effect of GABA, and the GABA agonist Diazepam (DZP) would enhance the inhibitory effect of GABA, resulting in behavioural and physiological changes that were indicative of higher and lower fear responses in sheep, respectively.
ii. The 5-HT agonists, 1-(*m*-chlorophenyl)piperazine (*m*-CPP) and 8-hydroxy-2-(di-n-propyl-amino) tetralin (8-OH-DPAT) would alter serotonergic neurotransmission causing behavioural and physiological changes that were indicative of higher and lower fear responses in sheep, respectively.

3.3 Materials and methods

Experiments were conducted at the FD McMaster Laboratories, 'Chiswick' (Armidale) and the FD McMaster Laboratories Animal Ethics Committee approved the use of all animals and procedures used in these experiments (AEC Numbers: 02/34 and 03/19).

3.3.1 Animals

Animals were sourced from the McMaster research flock and forty Merino wethers aged 18 months were used for each of the two studies (GABA and 5-HT) (liveweight range 32 – 45kg). Sheep were randomly assigned to each experimental group after stratification by liveweight. Additional animals were used in the testing areas to avoid the experimental animals being isolated prior to testing.

3.3.2 Experimental design

Sheep were presented with two behavioural challenges, the arena test and the isolation box test (IBT) (See Chapter 2 for further description of the IBT) on three different occasions (exposures), with seven days separating each exposure.

- Exposure 1: sheep were assessed during the first presentation to the IBT and arena test
- Exposure 2: sheep were assessed during the second presentation to the tests after administration of the pharmacological treatments
- Exposure 3: sheep were assessed following the third presentation to the tests.

3.3.3 Treatments

Three dose rates of Diazepam (DZP), a GABA receptor agonist (0.3, 0.6 and 0.9 mg.kg) and Pentylenetetrazol (PTZ), a GABA receptor antagonist (1, 5 and 10 mg/kg) were selected. The pharmacological treatments and dose rates chosen to effect the 5-HT pathway were 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) (2, 5 and 7 mg/kg) and 1-(m-chlorophenyl)piperazine (m-CPP) (0.5, 1 and 2 mg/kg). Both are reported as receptor agonists, but with different physiological actions (anxiolytic and anxiogenic, respectively) (Table 3.1). All treatments were chosen based on the evidence that they elicit anxiolytic and anxiogenic responses via interaction with the targeted receptors.

The highest dose for each drug was based on the literature where levels and behavioural responses were stated with negligible side-effects. In a majority of cases, there was no

evidence of the use of these in sheep, therefore treatments and doses were extrapolated from mainly rodent based studies. The three dose rates were extrapolated from research based on (Ferreira et al. ; Andersen et al. 2000b; Hill et al. 1992; Rodgers et al. 1995; Siemiatkowski et al. 2000; Ahlenius et al. 1991; Baldwin and De La Riva 1995; Bagdy et al. 2001; Broocks et al. 1997). All chemicals were supplied by Sigma Aldritch Pty Ltd, (PO Box 970, Castle Hill, NSW 1765, AU), except DZP which was supplied by Cenvet Australia Pty Ltd (PO Box 4365, Maryong, NSW 2148, AU). Physiological saline was used as the control and was injected in a corresponding amount to the maximum dose rate of the specific pharmacological treatments. The treatments were administered intramuscularly (i.m.) into the hind leg. DZP was administered directly from the storage container (initial concentration 5mg/ml), and the maximum of any treatment injected at any one time was 7ml. The other treatments, PTZ, m-CPP and 8-OH-DPAT were dissolved in physiological saline to achieve similar injection volumes to the DZP treatments for each animal (Table 3.1).

Table 3.1: Pharmacological treatment, dose rates (mg/kg liveweight) and number of animals in each group

Receptor	Ligand	Action	Dose	Expected effect	Animals
GABA	DZP	Agonist	0.3	Anxiolytic	5
GABA	DZP	Agonist	0.6	Anxiolytic	5
GABA	DZP	Agonist	0.9	Anxiolytic	5
GABA	PTZ	Antagonist	1	Anxiogenic	5
GABA	PTZ	Antagonist	5	Anxiogenic	5
GABA	PTZ	Antagonist	10	Anxiogenic	5
5-HT	m-CPP	Agonist	0.5	Anxiogenic	5
5-HT	m-CPP	Agonist	1	Anxiogenic	5
5-HT	m-CPP	Agonist	2	Anxiogenic	5
5-HT	8-OH-DPAT	Agonist	0.2	Anxiolytic	5
5-HT	8-OH-DPAT	Agonist	0.5	Anxiolytic	5
5-HT	8-OH-DPAT	Agonist	0.7	Anxiolytic	5
		Control		minimal	10

3.3.4 Procedure

Seven animals per day (one sheep from each of the receptor treatment/dose rate groups and 1 control animal) over five days were exposed to the arena test and the IBT on three occasions (exposures). Each exposure was separated by a period of seven days. On each day of testing, sheep were yarded (0700 h), drafted into groups and left to settle for 20 minutes. Sheep were then sequentially moved into the arena test, whilst an observer recorded their behaviour for

three minutes (refer 3.3.5.1). On completion of the arena test, animals were moved into a transit pen for five minutes before placement into the IBT for one minute. After one minute, sheep were then released into a pen containing conspecifics. Throughout the experiment, five blood samples were collected at different times relative to the start of the arena test and once the final blood sample was taken (90 min) from the last animal, sheep were released into their home paddock. The same procedure was used for all three exposures, the only difference was that on the second exposure, the pharmacological treatments were administered 20 minutes before the start of the arena test (Table 3.2).

Table 3.2: Number of exposures, days and animals

Exposure	Days	No. of animals
1	1 – 5	7 sheep/day (35 total)
2	6 – 10	7 sheep/day (35 total)
3	11 - 15	7 sheep/day (35 total)

3.3.4.1 Blood sampling measures

Five blood samples were taken by jugular venepuncture at 0, 20, 40, 60 and 90 minutes relative to commencement of the arena test for analysis of cortisol. All samples were collected in 10ml serum vacutainers (Becton Dickinson Ltd) and stored on ice until removal to the lab where the samples were centrifuged at 3000g for 10 minutes and duplicate 3 - 4ml aliquots of serum were stored at -20°C until analysis.

Due to missing data the first blood sample was not analysed within the results section. However, the original inclusion of the first blood sample was to obtain an indication of the cortisol concentration before any experimental procedures had occurred on the animals, with the possibility that the first blood sample could be used as a potential control for the animal itself, as a baseline before stress testing the sheep.

3.3.4.2 Cortisol assay

Serum concentrations of cortisol were determined according to the method described in Chapter 2. All samples were run in duplicate and the lower detection limit was five ug/mL. The intra- and inter-assay coefficients of variation were 14.93 and 10.24 for high, 9.55 and 3.25 for medium and 13.68 and 13.74 for low control samples. The integrated cortisol response (AUC) was measured according to the method described in Chapter 2.

3.3.5 Challenges

3.3.5.1 Arena test

The original arena test was developed by Fell and Shutt (1989) as a means to evaluate how repelled sheep were to humans after mulesing surgery. Since then, the test has been adapted for use in quantifying livestock behaviour to various stressors, including assessment of parasite load in sheep (Fell *et al.* 1991) and ewe maternal traits (Kilgour and Szantar-Coddington 1997). The arena test involves a motivational choice for the sheep, (Kilgour 1998) by creating a conflict situation between the social attraction of the test animal to its flockmates at the end of the arena, versus the fear of the observer stationed in front of the pen of flockmates.

Sheep were placed into a 7 x 3 m arena for three minutes. The walls of the arena were 1.2 m high covered with shade cloth. The floor of the arena was marked in 1 m increments with painted lines. At one end of the arena was a pen containing a number of flockmates, whilst an observer stood between the animal and flockmates. During the 3 minute interval, a number of behavioural parameters were recorded (see below). A handler quietly moved individual sheep into the arena, where the observer (already in position before the test animal entered) stood in front of the holding pen. The test began when the animal entered the arena test. Sheep were considered to have entered a zone when both forefeet were over the painted line. Once the test was completed the animal was quietly moved toward the exit (Figure 3.1).

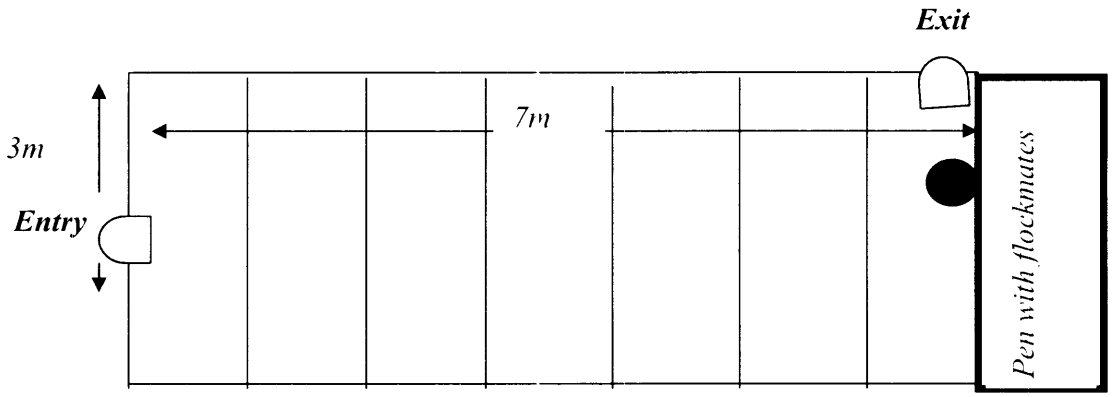

Figure 3.1: The arena test layout (black circle marks observer)

3.3.5.2 Behavioural measures

A number of behaviours were recorded during testing these included:

- Vocalisation (Vocs): number of bleats
- Zones crossed (ZX): the number of zones the animal entered

- Zone in which most time spent (ZT): the zone in which an animal spent the most time
- Closest approach (CLOSE): the zone in which the animal got closest to the observer/flockmates
- Total time in marked zone (TIME): the total time spent in each zone multiplied by the number of the zone. The higher the number, the more time the animal spent further away from the observer. This indicates a measure of the time spent in each zone and proximity to zone 1 (where observer was situated).

Elimination (faeces and urine) and jumps were recorded initially, but the incidence was very low and therefore these were not used in the analysis.

3.3.5.3 Isolation box test (IBT)

The IBT involves objectively measuring the degree of agitation during one minute of isolation in the 1.5 m^3 box (Chapter 2). The agitation score and number of vocalisations during the test were recorded.

3.4 Statistical analysis

All statistical analyses were performed using SAS, Version 8.2 (SAS Institute Inc., USA, 1999-2001). The homogeneity of variance was assessed for all variables, and where appropriate logarithmic or square root transformations were used to normalize the data. The dependent variables were: the integrated cortisol response over 90 minutes (AUC), IBT agitation score and IBT vocalizations (IBT Vocs). For the arena test, the dependent variables included: vocalisations (Vocs), zones crossed (ZX), zone most time spent (ZT), total time spent in each of the marked zones of the arena (TIME) and closest approach to observer (CLOSE). All data were expressed as least squared means (LSM). Where data were transformed, the back-transformed LSMs are presented.

For exposure 2 (when the pharmacological treatments were administered), a general linear model (GLM) in SAS was used to analyse the data. The full model contained the main effects of testing day (1 - 5), treatment group (pharmacological treatment and dose) (control, DZP-low, DZP-med, PTZ-low, PTZ-medium, PTZ-high, m-CPP-low, m-CPP-medium, m-CPP-high, 8-OH-DPAT-low, 8-OH-DPAT-medium, 8-OH-DPAT-high)) and the interaction term.

For exposures 1 and 3, a MIXED model in SAS was used to analyse the data. The full model contained the main effects of testing day (1-5), exposure (1 and 3), treatment group and the interaction terms. Animal was used as the random term. The contrast between exposure 1 and 3 enabled some assessment of whether there were any carry-over effects, however, differences in the response between exposure 1 and 3 could simply be due to either habituation or attenuation to repeated exposure to the challenge and handling context.

3.5 Results

3.5.1 GABA receptor treatments (Exposure 2)

The highest dose of DZP (0.9mg/kg) resulted in some ataxia and reduced locomotion in some sheep during the arena test, therefore a decision was made to exclude this treatment group as it could unduly bias the analysis of the data.

There was a significant effect ($P<0.05$) of treatment on the total integrated cortisol response (AUC), with a lower response in the group administered the low dose DZP treatment (0.3 mg/kg) compared with the control (Table 3.2). However, PTZ did not affect the AUC (Table 3.2 and Fig. 3.3). No significant treatment effect was evident for measures from the arena test (TIME, number of zones crossed, time spent in each zone, closest zone to the observer or vocalisations) or when tested in the isolation box test (IBT score and Vocs). There was a slight trend for PTZ to cause a higher latency to approach the observer/flockmates as evidenced by the higher TIME variables compared with the DZP treatment and control. There was a DZP dose dependent reduction in the IBT scores and vocalisations. Additionally, DZP reduced the TIME variable (willingness to approach the observer) relative to the control. The reduction was significant at the low DZP dose. At no time was day of testing or the interaction between treatment and day significant for any of the variables (Table 3.3).

Table 3.3: Least square means for the integrated cortisol response (AUC), arena test variables (TIME, ZX, ZT, CLOSE and Vocs) and for the isolation box score and vocalisations for the GABA treatments (DZP, PTZ and control)

Main effects	Cortisol	Arena test					IBT	
Treatment	**AUC	TIME	*ZX	*ZT	*CLOSE	*Vocs	Score	Vocs
Control	2692.5^{A}	714.5	4	3.96	2	3	23.0	2.2
DZP (low: 0.3 mg/kg)	1460.7^{B}	574.2	2	2.52	1	3	20.3	1.4
DZP (med: 0.6 mg/kg)	2231.6^{AB}	670.8	4	2.85	1	5	14.2	1.0
PTZ (low: 1 mg/kg)	1899.2^{AB}	843.8	5	4.79	1	5	30.1	1.4
PTZ (med: 5 mg/kg)	2458.1^{AB}	744.4	3	3.72	1	3	20.3	1.7
PTZ (high: 10 mg/kg)	1982.0^{AB}	815.1	4	3.57	1	4	18.0	2.3
Sed	152.3	156.92	0.7	0.7	0.4	1.1	9.8	0.7
Significance	*P<0.05*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*

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

Figure 3.2 and 3.3 illustrate the unadjusted means of the cortisol responses for different dose of DZP and PTZ treatments respectively, over the 90 minute sampling period. The low DZP dose rate shows a continuously low response over time, including the period of testing (at approximately 20 min), whilst the medium dose shows an increase at 40 minutes, and both the control and medium DZP groups show decreases at 60 minutes.

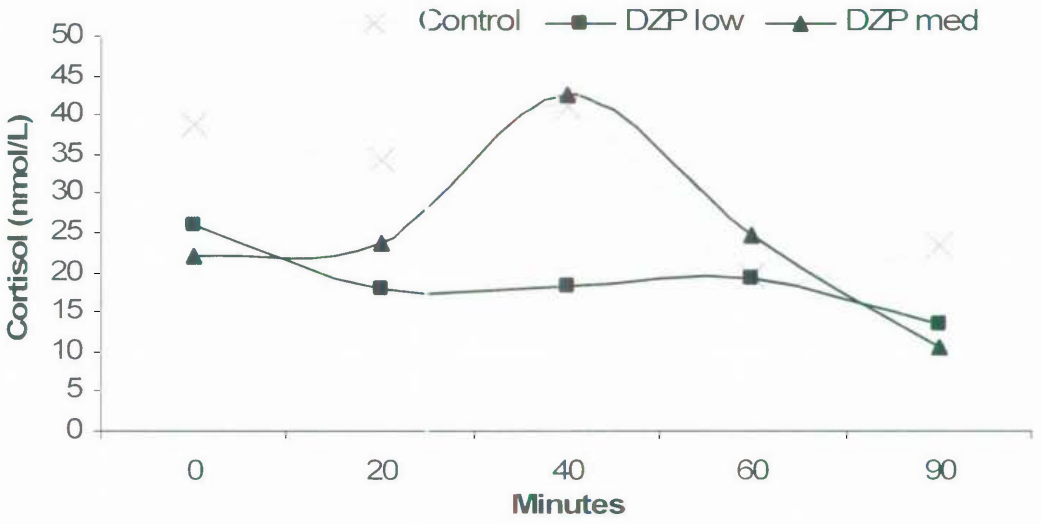

Figure 3.2: Mean cortisol concentration (nmol/L) for the five blood sampling times for the two Diazepam treatment dose groups (low - 0.3 mg/kg and med – 0.6 mg/kg)

The raw means of the cortisol responses for the PTZ treatment groups show little differences between the treatment groups and the control.

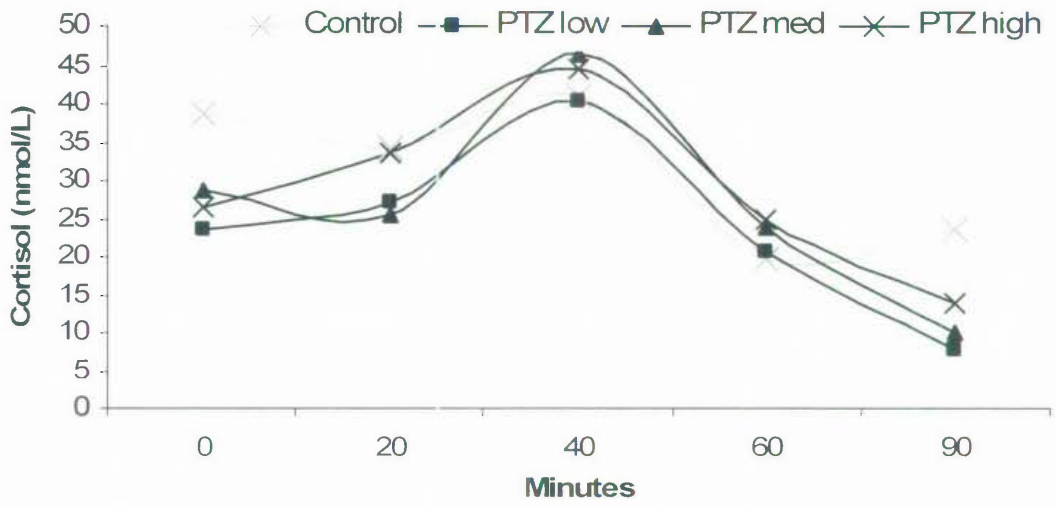

Figure 3.3: Mean cortisol concentration (nmol/L) for the five blood sampling times for the PTZ treatment dose groups (low - 1 mg/kg, medium - 5 mg/kg and high - 10 mg/kg)

3.5.2 GABA treatment groups (Exposures 1 and 3)

There was a significant exposure by day effect for the IBT score ($P<0.05$) (Table 3.3). Additionally, treatment by day was significant for the number of zones crossed in the arena test, agitation score and vocalisations in the isolation box. The interaction between exposure and treatment group was not significant for any of the variables measured.

The IBT score ($P<0.001$) and IBT vocalizations ($P<0.01$) were approximately 50% lower during exposure 3 compared with exposure 1. Animals vocalized significantly less in the arena during exposure 3, compared with the first exposure ($P<0.01$). Day was significant in both the arena (number of zones crossed, closest zone and zone in which the most time was spent) and the IBT agitation score (Table 3.4).

Table 3.4: Least square means of the arena test (vocalisations, TIME, number of zones crossed, closest zone to observer and amount of time spent in zone), IBT score and vocalisations during the first and third exposures

	*Arena test					*IBT	
Main effects	**Vocs**	**TIME**	**ZX**	**CLOSE**	**ZT**	**IBT score**	**IBT Vocs.**
Exposure							
1	5.7	629.9	7	2.0	3.4	46.53	4
3	1.5	618.5	7	1.9	3.8	26.84	2
sed	1.4	90	0.5	0.3	0.4	2.3	0.1
Significance	*P<0.01*	*ns*	*ns*	*ns*	*ns*	*P<0.001*	*P<0.01*
Treatment (Exp 2)							
Control	4	552.3	7	3.2	4.5	39.6	4
DZP (low: 0.3 mg/kg)	4	521.1	6	2.6	3.5	43.3	4
DZP (med: 0.6 mg/kg)	3	478.5	8	2.	4.4	28.2	3
PTZ (low: 1 mg/kg)	5	568.8	10	3.2	4.3	35.8	3
PTZ (med: 5 mg/kg)	3	562.1	7	2.4	4.4	32.5	2
PTZ (high: 10 mg/kg)	4	592.2	9	2.4	3.7	34.2	3
sed	3	97.3	1.9	0.5	0.9	10.8	1.4
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Day							
1	1.0	496.2	10.1^{A}	1.9^{A}	2.8^{A}	29^{AB}	3
2	5.1	667.4	5.2^{B}	2.7^{BC}	5.1^{B}	32^{ABC}	3
3	6.1	643.8	7.3^{AB}	2.5^{AB}	4.1^{AB}	46^{A}	4
4	3.2	641.9	9.3^{A}	2.9^{BC}	4.4^{B}	25^{B}	3
5	2.5	692.4	7.4^{AB}	3.6^{C}	4.6^{B}	48^{C}	2
sed	2.3	95.1	1.8	0.3	0.8	9.8	1.2
Significance	*ns*	*ns*	*P<0.05*	*P<0.05*	*P<0.05*	*P<0.05*	*ns*
Interactions							
Exposure x Treatment	ns	ns	ns	ns	ns	ns	ns
Exposure x Day	ns	ns	ns	ns	ns	*P<0.05*	ns
Treatment x Day	ns	ns	*P<0.05*	ns	ns	*P<0.01*	*P<0.05*

*Backtransformed LSM

3.5.3 5-HT receptor treatments (Exposure 2)

There was a significant treatment effect on the integrated cortisol response (AUC) where both 5-HT treatments, 8-OH-DPAT and *m*-CPP, caused an elevated cortisol response compared to the control group ($P<0.001$; Table 3.4 and Figs. 3.4 and 3.5). Additionally, there was a dose dependent increase in AUC for the *m*-CPP treatment. .

The medium dose (1 mg/kg) of *m*-CPP caused sheep to remain further away from the observer (TIME) for longer periods compared to the control ($P<0.05$). The medium dose (0.5 mg/kg) 8-OH-DPAT caused the sheep to remain closer to the observer/flockmates compared with the control group ($P<0.05$) based on the results for ZT (zone in which the sheep spent the most time). Both serotonin treatments at all dose rates caused a reduction in the number of vocalisations made within the arena, compared with the control group ($P<0.01$; Table 3.5).

Neither of the serotonin treatments significantly altered the IBT score or vocalisations during the test compared with the control. However, there was a trend that all of the treatment groups (except the high *m*-CPP dose) had higher IBT scores and in some circumstances, this bordered on significance compared with the control group.

Table 3.5: Least square means of the integrated cortisol response (AUC), arena test measures and IBT score and vocalisations at low, medium and high dose rates for the serotonin anxiogenic (*m*-CPP) and anxiolytic (8-OH-DPAT) treatments

Main effects	*Cortisol	Arena test					Isolation box test	
Treatment	**AUC**	**TIME**	***ZX**	***ZT**	**Close**	***Voc**	**Score**	**Vocs**
Control	2692.5^{A}	817.6^{AC}	7	6^{AC}	4	6.8^{A}	31.0	2.7
8-OH-DPAT low (2 mg/kg)	5225.8^{BE}	882.9^{AD}	7	5^{AC}	4	1^{B}	42.5	1.4
8-OH-DPAT med (5 mg/kg)	7377.0^{C}	685.4^{AC}	8	3^{B}	3	1^{B}	50.9	1.6
8-OH-DPAT high (7 mg/kg)	6475.4^{BCE}	625.1^{BC}	7	4^{A}	3	1^{B}	38.5	1.4
m-CPP low (0.5 mg/kg)	5584.5^{BCE}	919.7^{AD}	5	6^{AC}	5	1^{B}	37.7	1.8
m-CPP med (1 mg/kg)	6192.1^{BCE}	1057^{D}	6	7^{C}	4	1.5^{B}	34.2	1.8
m-CPP high (2 mg/kg)	10545.2^{D}	853.4^{ABCD}	6	6^{AC}	3	1^{B}	28.5	1.4
sed	358.5	119.9	1	1	1	1.3	9.8	0.7
Significance	*P<0.001*	*P<0.05*	*ns*	*P<0.05*	*ns*	*P<0.01*	*ns*	*ns*

*Backtransformed LSM

The cortisol concentration at each of the five sampling times following administration of the serotonergic treatment 8-OH-DPAT are shown in figure 3.4. Regardless of dose, the 8-OH-DPAT treatment resulted in a higher cortisol response from 20 minutes relative to the control.

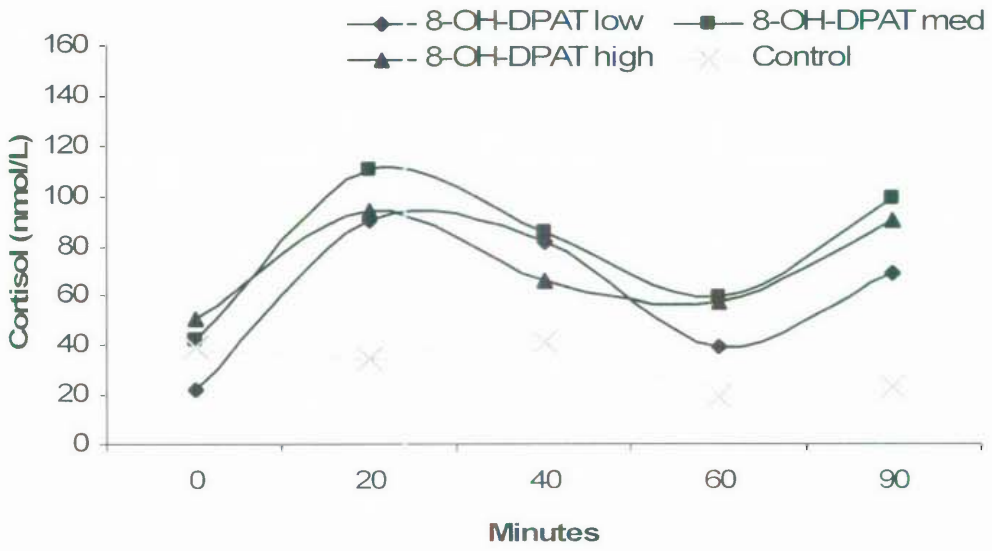

Figure 3.4: Mean of the cortisol concentration for the 5-HT agonist 8-OH-DPAT at low (0.2 mg/kg), medium (0.5 mg/kg) and high (0.7 mg/kg) dose rates for each of the five sample times

The raw mean cortisol concentration at each of the five sampling times for the serotonergic *m*-CPP treatment doses are shown in figure 3.5, showing the cortisol concentration was higher from the 20 minute sample time onwards compared with the control; additionally the high dose (2 mg/kg) of *m*-CPP caused an elevated increase in the cortisol concentration over time.

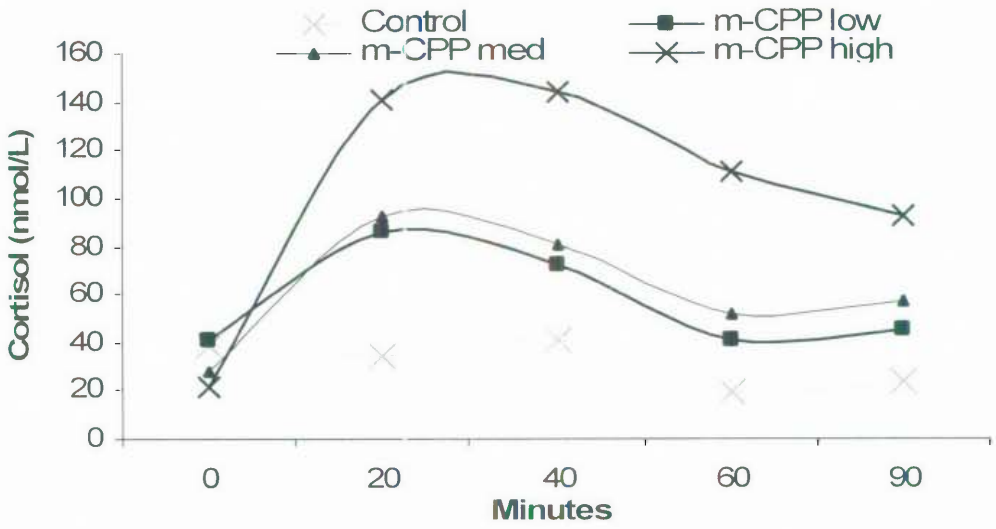

Figure 3.5: Mean for the cortisol concentration for the 5-HT agonist *m*-CPP at low (0.5 mg/kg), medium (1 mg/kg) and high (2 mg/kg) dose rates for each of the sample times

3.5.4 5-HT treatment groups (Exposures 1 and 3)

There was an interaction between exposure and day ($P<0.05$) indicating a decrease in the IBT score during the third exposure on days 1, 2 and 3 compared with all days during exposure 1 (Table 3.6). The interaction between exposure and treatment group was not significant for any of the behavioural or physiological measures.

Animals were more agitated in the IBT during the first exposure compared with the last exposure ($P<0.001$). Additionally, animals vocalized less during exposure 3 compared with exposure 1 ($P<0.05$). During the arena test, there was a day effect with the animals vocalizing more on days 2 and 5 compared with day 1, and vocalisations were lower on day 3 compared with day 5 ($P<0.05$) (Table 3.6).

Table 3.6: Least square means from the arena test (vocalisations, TIME, number of zones crossed, closest zone to observer and amount of time spent in each zone) and score and vocalisations during the IBT for the first and third exposures

Main effects	***Arena test**					***Isolation box test**	
Exposure	**Vocs**	**TIME**	**ZX**	**CLOSE**	**ZT**	**IBT score**	**IBT Vocs**
1	3	719.1	5.3	3.4	4.8	30.4	3
3	2	751.3	5.1	3.7	4.8	13.8	1
Sed	1.3	72.1	0.5	0.5	0.2	2.2	0.08
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*P<0.001*	*P<0.05*
Treatment (Exp 2)							
Control	3	680	4.3	2	3.7	19	2
8-OH-DPAT low (2 mg/kg)	1	948	3.8	1.5	5.5	14	1
8-OH-DPAT med (5 mg/kg)	1	760	4.3	3.2	4.1	16	2
8-OH-DPAT high (7 mg/kg)	1	655	5.2	2	3.4	21	1
m-CPP low (0.5 mg/kg)	3	762	3.5	2.2	4.2	21	3
m-CPP med (1 mg/kg)	4	811	4.7	2	4.2	23	1
m-CPP high (2 mg/kg)	0	665	5.2	3.2	3.3	13	1
Sed	1.3	111.2	0.6	0.5	1.7	6.6	0.4
Significance	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Day							
1	1^{A}	750.5	4.9	4.0	5.0	24.3	1
2	3^{B}	693.4	5.6	3.3	4.3	19.9	2
3	2^{A}	729.8	4.5	3.6	5.1	24.6	1
4	2^{A}	722.6	6.1	3.0	4.7	17.7	2
5	4^{B}	780.5	5.1	3.9	5.1	20.3	1
Sed	0.9	52.8	0.4	1.3	0.9	1.6	0.1
Significance	*P<0.05*	*ns*	*ns*	*ns*	*ns*	*ns*	*ns*
Interactions							
Exposure x Treatment	ns	ns	ns	ns	ns	ns	ns
Exposure x Day	ns	ns	ns	ns	ns	*P<0.05*	ns
Treatment x Day	ns	ns	ns	ns	ns	ns	ns

*Backtransformed LSM

3.6 Discussion

3.6.1 GABA treatments

The GABAergic pathway was a logical choice to target given its well documented role in the expression of fear and anxiety, which in most instances has evolved through pharmacological studies. Diazepam is a classical benzodiazepine (BZP), which has been used in a large number of animal models for investigation of fear and anxiety (Wilson *et al.* 2004). It is a prime candidate for assessment of its effects in sheep when exposed to acute fear-eliciting challenges. Our hypothesis was that DZP would reduce the effects of fear on the sheep when exposed to the challenges. In this experiment, the low dose of DZP caused a decrease in the cortisol response compared with the control, which would tend to indicate an anxiolytic effect, or reduction of fear. However, when the sheep were exposed to the arena test and isolation box test, there was no significant change in their behavioural responses, relative to the control group. Although not significant, there were trends for decreased movement between the zones, reduced latency to approach the observer/flockmates and reduced distance between the sheep and the observer. These could be interpreted as indicative behaviours of sheep that were generally less fearful during the arena test. This tends to reinforce the physiological data that DZP had an anxiolytic effect.

The lack of significant behavioural differences contrasts somewhat with studies in rodents investigating the use of DZP and its relationship to fear and anxiety. However, in this type of context there was limited information available on sheep. Stock *et al.* (2000) found both long and short term administration of DZP in rats at 1, 2 and 3 mg/kg caused a decrease in corticosterone during the plus maze test, similar to that seen in the swim stress test (Wilson *et al.* 2004)) and similar to that observed here. In contrast, corticosterone release in rats has also been shown to increase when tested in other types of stress tests after administration of DZP (e.g. elevated plus maze (EPM) and the prod-burying test) (Wilson *et al.* 2004), and it was suggested that the anxiolytic properties of DZP may be dependent on the initial state of anxiety when the animals are first tested. Another possible reason for the somewhat equivocal results is that perhaps the effects of DZP (and other pharmacological treatments) differ between different types of challenges. Andersen *et al.* (2000) tested pigs in three anxiety tests (elevated plus maze, light/dark test and the open-field test) after treatment with DZP, and observed that the anxiolytic responses were only evident in response to one of the tests. The interpretation as to why no additional anxiolytic effects of DZP were observed was unclear,

but the authors speculated that it may have been linked to the innate behaviours of pigs on exposure to anxiety contexts. Given these results, the lack of dose response, especially for the behavioural parameters, may therefore be linked to the type of challenge and context under which it was applied. Perhaps these tests did not elicit a sufficiently large or sustained fear response to detect behavioural differences between the treatments.

If true, then it is possible that the isolation box test may have not been the right context in which to evaluate the effects of these pharmacological treatments on the fear responses in sheep. The sheep were placed into the box for very short duration (1 minute), which may not have been a sufficient enough amount of time to allow for discrimination between the control animals and the animals treated with DZP in regards to the behavioural differences, particularly the agitation score. Perhaps a longer period of isolation in the box would be more applicable to allow assessment of the differences in the expression of fear. In summary, anxiolytic-like effects of the low dose of DZP were found in relation to cortisol, with similar trends in the behavioural responses measured during the arena test and isolation box test.

There is also potentially a risk of bias of the responses; particularly to the second test (IBT) due to stress induced by the previous exposure to the arena test. Potentially because of the short duration between the two tests, the arena test and the IBT (5 mins), there is a possibility that stress shown in the second test could have been potentiated by the animals experience in the first test, thereby influencing the results. However, we know from previous work with the IBT {Blache & Ferguson 2006 #12120}, that it is a stressful test, in that it affects the sociability of the animal, as sheep are highly gregarious and do not like being isolated from conspecifics. A potential way to remove this bias, if it existed, would be to increase the amount of time between tests, however, it also needs to be considered that the other types of stress associated with testing could also affect the results, for example, handling of the sheep to get them into the experiment in the first place. Additionally, different tests assess different aspects of fear and temperament, and we were perhaps assessing for the different types of reactions to the fear tests, and regardless of the test and in what sequence we the test needed to be stressful to the sheep. Another way to assess which test was the most stressful, would have been to run the arena test first and then the IBT and have also assessed the IBT first and then the arena test and tested for any sequence effect in the analysis. However, due to time considerations this could not be done.

The pharmacokinetics of a drug is also an important consideration and it refers to the increase and decrease of a drug within the body, which determines the course of action. This also relates to the half-life of the drug, which is the time taken for the plasma concentration of the drug to fall to 50% of the initial administered amount and influences the longevity of the drug treatment effects. Diazepam, when used at low or moderate doses is a powerful anxiogenic, however it does have sedative side-effects at high doses. It is also a quick acting drug on the brain, and initial effects have been shown to dissipate rapidly as it is distributed into fatty tissues of an animal {Mandelli, Tognoni, et al. 1978 #13300}. During development of our experimental protocol, we initially used three different doses of DZP; 0.3, 0.6 and 0.9 mg/kg, and our pilot data indicated that the higher dose (0.9 mg/kg) adversely affected ambulation during the arena test. Due to the observable effects on ambulation at the higher dose, a decision was made restrict dosage to 0.3 and 0.6 as restricted ambulation would confound measurement.

When DZP has been used in experimental protocols it has been commonly given between 10 and 30 minutes prior to testing. Stock et al. (2000) {Stock, Foradori, et al. 2000 #11340} administered DZP at both acute and chronic doses in rats and observed behaviourally and physiological effects. Similar effects have been noted by Wilson et al. (2004) {Wilson, Burghardt, et al. 2004 #7540}and Andersen et al. (2000) {Andersen, Gaerevik, et al. 2000 #6690}.

Pentylenetetrazol has anxiogenic effects in humans and rodents where it acts to reduce GABA mediated inhibition in the central nervous system (CNS), reducing chloride ion influx and enhancing CNS excitability (Wallis and Lal 1998). It produces intense anxiety at sub-convulsive doses and panic attacks at convulsive doses in both animal and human subjects (Jung et al. 2002). Given this, we expected PTZ to increase the cortisol response and increase the expression of fearful behaviours measured in the arena and isolation box test. However this was not the case as there was no increase in cortisol or fear-related behaviours during the two tests relative to the control group. Indeed, there were slight non-significant trends of a reduced cortisol response at all doses of PTZ. There were slight trends that could be interpreted as heightened fear, such as reduced locomotion and latency to approach flockmates observed in the arena test. PTZ is considered to be a classical anxiogenic ligand (Jung et al. 2002), however, it is evident from the literature that equivocal effect have been reported. PTZ

is reported to produce mostly anxiogenic behavioural responses in animal anxiety models (Rodgers et al. 1995; Carey and Fry 1993). However, some studies have also reported anxiolytic and non-specific responses. De Vry (1995) found anxiolytic-like behaviours of rats in the defensive burying tests. Biphasic responses have also been observed, with low doses (< 4 mg/kg) causing anxiolytic effects and high doses (> 15 mg/kg) causing anxiogenic effects in mice when exposed to the elevated plus maze (Rodgers et al. 1995). Non-specific effects have also been identified in the punished responding test (Hill et al. 1992).

Once again pharmacokinetics need to be considered and Palit et al. (1998) {Palit, Kumar, et al. 1998 #2550} conducted research on rhesus monkeys to assess anxiety in the social colony and administered PTZ as the anxiogenic drug of choice. They found effects, both behaviourally and physiologically, with responses within 10 minutes after administration, with the peak reached between 50-60mins and dissipate after 2-4 hours. They also considered that the use of this anxiogenic drug was advantageous, as it was easy to administer, had dose-dependent responses, and had behavioural effects that were easy to observe and quantify. The use of the PTZ discrimination test has also been used extensively in animal models {Lal & Emmett-Oglesby 1983 #2730} {Lal, Mann, et al. 1981 #13440}, whilst a review by Jung et al. (2002) {Jung, Harbans, et al. 2002 #2530} discusses further work on PTZ as an anxiogenic.

The duration and intensity of the tests once again may have contributed to the lack of significant effects on administration of PTZ, and it is possible that peak behavioural effects may have been missed, whilst the cortisol response could also have possibly been washed-out due to that of extended handling and testing throughout the day. One study by Palit et al. (1998) examined the effects of 30 mg/kg PTZ on the social behaviour of monkeys, and found effects to start within 10 minutes of administration, with peak effects between 50 and 60 minutes, whilst all effects had disappeared within 2 hours of administration. In summary, the use of PTZ at these dose rates and in combination with the isolation box test and arena test are not robust enough to warrant continued investigation within this thesis.

3.6.2 5-HT treatments

The 8-OH-DPAT treatment was expected to induce an anxiolytic effect through a reduction in 5-HT transmission following the activation of 5-HT cell body autoreceptors which in turn caused a decrease in 5-HT release in areas of the limbic system (File et al. 1996). As a result,

a lower cortisol response and changes in behaviour, relative to the control group were expected. However, this did not occur and in fact, a higher cortisol response ($P<0.05$) was observed for all three dose rates compared with the control group. Additionally, no apparent effect in the behavioural responses during the arena test was observed with the exception of vocalisations, where the treatment elicited a significant reduction relative to the control group. These responses were not clear or consistent; therefore the outcomes cannot be interpreted as either anxiogenic or anxiolytic.

Despite clinical efficiency of the depression treatments used, anxiogenic and anxiolytic responses using serotonergic ligands have been reported in the literature, although the majority of studies using 8-OH-DPAT reported a reduction in fear and anxiety. Pharmacological agents can act both pre- and post-synaptically on receptors, and administration of 8-OH-DPAT (and other partial agonists) administration can result in anxiogenic, anxiolytic and in some cases, inconsistent results (Gonzalez *et al.* 1996). File *et al.* (1996) found stimulation of the pre- synaptic 5-HT receptors with direct brain administration of 8-OH-DPAT to produce an anxiolytic effect in rats, whilst post-synaptic stimulation resulted in an anxiogenic-like response when administered before testing in the social interaction test and the elevated plus maze test. Bilkei-Gorzó (1998) found 8-OH-DPAT elicited no effect in the response to the light-dark box test for rats, whilst Griebel *et al.* (1995) and Carli and Samanin (1988) found it caused inconsistent effects. Environmental conditions such as changes in lighting have been also shown to influence the drug action, moving from anxiogenic to anxiolytic (Handley and McBlane, 1993). Additionally, it has also been suggested that previous experience plays a major role in determining whether anxiogenic or anxiolytic behaviours are observed (File and Gonzalez 1996). File and Gonzalez (1996) found no observable anxiolytic effects of 8-OH-DPAT in a maze in naïve rats when given 200 ng, whilst 100 ng was sufficient to achieve anxiolysis in rats with one previous 5 min exposure to the maze. Griebel *et al.* (1995) tested a number of pharmacological treatments in several animal anxiety tests and achieved inconsistent results. Although it has very strong affinity for the 5-HT_{1A} receptor, for unknown reasons it appears to shown inconsistent effects in anxiolytic tests (Carli and Samanin 1988; Griebel 1995). One suggestion put forward for the inconsistencies was the administration route. Central administration directly into certain parts of the brain is considered to be the most effective compared with subcutaneous or intraperitoneal injections. Within the literature there is a significant lack of information on both the behavioural and physiological responses

of sheep when presented with acute challenges to different types of ligands used in this experiment.

The serotonergic agonist, m-CPP consistently produces anxiety-like behaviour in rats (Rodgers et al. 1995; Kennett et al. 1989; Griebel et al. 1997) in a variety of test contexts. It is a major metabolite of the antidepressant drug trazodone, which causes significant activity in serotonergic neurons (Bilkei-Gorzó 1998) In humans, m-CPP elicits various neurophysiological effects, including increased plasma cortisol, ACTH and prolactin concentrations, blood pressure, temperature and heart rate (Broocks et al. 1997). In this experiment, m-CPP significantly increased the cortisol response in a dose-dependent manner, but most notably at the highest dose (2 mg/kg). The administration of m-CPP at the medium dose (1 mg/kg) elicited behavioural changes in the arena test including increased locomotion and decreased vocalisations. This correlates well with findings of Takamatsu et al. (2003), where administration of m-CPP to rhesus monkeys caused increases in plasma cortisol during handling. Additionally, research by Broocks et al. (1997) on human subjects and by Bagby et al. (1989) with rodents revealed increases in cortisol, similar to that seen in the present experiment. Kennett and Blackburn (1989) found that m-CPP when administered systemically caused suppression of social interaction in rodents; this is possibly similar to that seen in the arena test with the medium dose of m-CPP, which caused a marked increase in latency on approach of the test sheep to its flockmates, however because of the lack of consistent results from data from the arena test, this is not conclusive. Alternatively, the treatment may have also caused a marked aversion of the test sheep, when approaching the observer in the arena. In conclusion, m-CPP was associated with an increase in fearfulness, as indicated by the marked increase in cortisol and increased locomotion n the arena test, supporting previous research that m-CPP is an anxiogenic stimulus in animals, and in this case, sheep. This suggests that the serotonin pathway, in conjunction with these types of challenges is a reasonable target to pursue as it might account for some of the variation in fearfulness between animals.

Diazepam had an effect of reducing fearfulness in sheep when exposed to the isolation challenges, whilst the effect of PTZ was inconclusive. Both the serotonergic ligands caused significant changes in the cortisol responses and in some of the variables measure during the arena test, indicating a fearful response for both ligands, although 8-OH-DPAT has supposed

anxiolytic traits. There are several factors which may be relevant with regard to the observed results notably, the type of challenge used and factors such as the administration route of the pharmacological treatment, through to the behavioural repertoire of the animal being tested, as well as the pharmacokinetics of the drugs administered. Of these, the challenge model may be the central factor here as different models have been shown to affect the expression of anxiety and fear, and some tests are possibly more sensitive to particular agents. In this case, the small size of the treatment groups may have also contributed to the lack of consistent or significant effects. However, whilst more animals/treatment would have been useful, at the time, there was no available evidence for the actions of these drugs in sheep, therefore making it difficult to estimate experimental power and therefore sample size. Therefore in any future research of this nature, larger samples sizes/treatment is recommended.

There are a variety of potential motivations in every testing situation, including the arena test. Specific motivations within the arena test could include, disregarding the social motivation to locate flockmates and avoidance of the person in the arena test, the exit door as a potential positive motivation, whilst the entrance door has several potential negative motivations, including avoidance of the person moving the sheep into the arena test, removal from flockmates in the starter pen and remembering the person already in the arena test. Therefore these factors should be taken into consideration when discussing results pertaining to testing in the arena and it cannot ignored that the differences in responses between exposure 1 and 2, and 2 and 3 could also simply be due to habituation/attenuation to repeated exposure to the challenge context.

Sociability can be considered as an individual's reaction to the presence or absence of conspecifics, so that sociable animals would tend to seek out others. Sheep are generally recognized as highly social animals as assessed behaviourally {Sibbald, Elston, et al. 2005 #13370}. Studies investigating sociability of animals, particularly rodents have shown that selection for low or high sociability in early life can affect later behavioural assessment suggesting the possibility of a genetic basis for this trait (Mignon-Grasteau et al. 2005, {Mignon-Grasteau, Boissy, et al. 2005 #13350}. Studies by Mills and Faure investigated sociability in Japanese quail (*Coturnix japonica*) and. Boissy and Le Neindre (1997) in cattle have both suggested genetic variation in sociability.

Neurophysiological associations with sociability and fearfulness have also been studied particularly in relation to the serotonergic system. For example, Harro et al. (2006) ({Harro, Alttoa, et al. #13330} suggested that extra-cellular levels of 5-HT reflected the individual differences in sociability of rats. Pharmacological treatments have also been shown to affect sociability in animals. Glucocorticoid treatments have the ability to alter affect social play in juvenile rodents when administered in early life. {Kamphuis, Croiset, et al. 2004 #9440} and also impair coping ability in adult mice {Leshner & Schwartz 1977 #13290}. Chronic administration of DZP increases social interaction between rodents, however its acute administration reduces social interaction {File 1980 #13390}{File & Hyde 1978 #13400}. Both of these experiments illustrated a clear correlation between measurement of anxiety and sociability.

Serotonin systems are widely studied in regards to sociability, specifically social stress, and dependent on past history of the animal, administration of serotonin agonists or antagonists, can have a marked effect on the sociability of animals, in this case rats. The administration of 8-OH-DPAT, a 5-HT agonist, can cause a decrease in the corticosterone response in rats that have had been previously defeated in a social isolation test, which was suggested to be due to changes in receptor sensitivity {Korte, Buwalda, et al. 1995 #13410}. Additionally, the GABA system can be moderated by social stress, as shown by {Kang, Thomson, et al. 1991 #13420}, where social defeat in rodents resulted in increases in mRNA levels of $GABA_A$ subunits.

Sibbald et al. (2005) {Sibbald, Elston, et al. 2005 #13370} indicated that measuring sociability in sheep can be assessed in a variety of ways involving both social isolation and measuring motivation to be with conspecifics. However, they also stated that there is a possibility of confounding of the data, as the sheep in thesesituation may also experience fear, stress and anxiety, therefore different methods of measuring specific traits must be considered carefully. For example Sibbald et al. (2005) {Sibbald Elston, et al. 2005 #13370} used nearest neighbour measurements in flocks of grazing sheep, thereby trying to avoid problems likened to fear responses.

3.7 Conclusion

This is the first available information of sheep responses to these particular pharmacological treatments when exposed to acute fear-eliciting challenges. In the case of 8-OH-DPAT and PTZ, the behavioural and physiological responses were not conclusive and the inconsistency may be a function of the tests used or other factors. The administration of DZP and PTZ gave the most consistent physiological responses and aligned well with data from mainly rodent literature. The interpretation of the behavioural responses was less clear, but the responses do generally align with expectations for each of the treatments. Further investigation using these pharmacological treatments, DZP and *m*-CPP is recommended, but requires an increase in number of animals used, to ensure consistency of the stress response when assessing fear responses. Extrapolation of results from human and rodent data were initially used to develop an understanding of the type of responses that have been observed, however because of the lack of specific research in sheep regarding this type of experimentation, similar results cannot be predicted.

The following chapter discusses the development of a fear-potentiation challenge to be used in conjunction with the pharmacological treatments identified in this chapter. The development of the model continues from that discussed in this chapter, which indicated that a longer period of time of isolation within the box may be warranted.