Chapter 4 Three phase development of a fear potentiation model for sheep

4.1 Introduction

Boissy (1995) described fearfulness as an individual trait influencing an animal's reaction to a variety of fear provoking situations and as such, can be considered a basic feature of an animal's temperament. Fearfulness is controlled by a complicated mix of genetic, environmental and social factors. The induction of fear is pivotal when the animal is faced with an unpredictable or threatening situation as it motivates the animal to either avoid or try to avoid the situation or threat (e.g. fight or flight) and most importantly, facilitates a learned response. This allows the identification and assessment of new sources of danger (Boissy 1995), consequently fear responses are generally adaptive. However, if a sustained response does not obviate or negate the threat, a maladaptive state can arise. In addition, the capacity of the animal to learn and process the information will be influenced by the magnitude of fear response, impairing cognitive function. Through the process of conditioned learning, farm animals can associate humans with aversive handling or husbandry events, and consequently develop a fear of humans (see review by Rushen *et al.* 1999). Chronic stress has also been shown to cause excessive deformation to the hippocampus (Fuchs and Flügge 2003), which is critical to learning and memory processes. McEwen (2000) showed that the application of a daily stressor over a 3-4 week period was able to instigate learning deficits in experimental animals. However, from other research, there is also evidence to suggest individual variation in vulnerability to stress, based on research showing non-significant, as well as positive effects of hippocampal changes from administration of stressors (Bartolomucci *et al.* 2005). Excessive fear reactions can at worse, lead to injury or death of an animal or in some circumstances, the death of many, as can be the case for domestic poultry (Mills and Faure 1990). Similarly for cattle, excessive fear responses can result in injury to themselves, other cattle and/or stockpersons responsible for handling the animals (Grandin 1988).

Extensive research has been conducted to assess behavioural and physiological responses of fear, particularly in rodents. Consequently, many of the tests used for investigation of fear in rodents were later adapted for use in other species, such as livestock. For example, the open-

field test was used first by Hall in rodents (1934), in pigs (Beilharz and Cox 1967), dairy cows (Kilgour 1975) and later modified by Moberg *et al.* (1980) for use in sheep. A range of different, largely behavioural, tests have been developed to assess fearfulness and temperament including non-restrained (e.g. the docility test, (Le Neindre *et al.* 1995)) and restrained tests (e.g. the bail test (Fordyce *et al.* 1982)), movement tests to assess differences in ease of handling (Hinch and Lynch 1987), social dominance tests (Beilharz and Cox 1967), maternal temperament tests (sheep; Putu 1988; Murphy *et al.* 1994), exposure to predator stressors (Beausoleil *et al.* 2005; Blanchard *et al.* 2003) and fear potentiation tests (Korte and De Boer 2003), just to name a few. Each test has its own strengths and weaknesses. For example, choice tests are an example of non-restrained testing and have been shown to be unreliable when comparing mildly aversive procedures. Grandin *et al.* (1994) showed that cattle are hesitant to change a previously learnt choice, if the other choices are only mildly aversive. Grandin *et al.* (1986) also showed that sheep quickly changed their choice preference when presented with a highly aversive procedure (i.e. electro-immobilization). Burrow's (1997) provides an excellent review of the description and utility of the various temperament tests that have been applied in cattle. Additionally, Boissy's (1995) review on fear and fearfulness discusses other types of fear and anxiety assessments in animals.

Fear potentiation models are widely used in rodent-based research where prior exposure to a stressor in one specific context can enhance fear when animals are later assessed in a different context, such as a traditional anxiety test used for rodents (Korte and De Boer 2003). Korte *et al.* (2003) assert one of the advantages of using the fear potentiation model is that it facilitates the investigation of an animal's response to a perceived threat or challenge, under an increased state of anxiety. Contextual fear conditioning is another similar model designed for use in rodents to investigate fear and anxiety (Vieweg *et al.* 2006). Blanchard and Blanchard (1969) investigated contextual fear conditioning in rats where they received footshocks in a chamber, resulting in exhibition of freezing behaviour. On subsequent re-exposure to the same chamber, but in the absence of footshocks, the rats responded again by freezing, indicating a conditioned response to the context. Moreover, additional studies, have shown that even a single exposure to an aversive stimulus can trigger long-lasting effects on the hypothalamic-pituitary adrenal (HPA) axis (Dal-Zotto *et al.* 2003; Cordero *et al.* 2003). Essentially the models of fear potentiation, contextual and fear conditioning are similar, in that they are based on Pavlovian associative learning principles.

As argued previously, there is good evidence to show that a single exposure of an unavoidable stressor can enhance an animal's fear response. The majority of this research has been conducted using the elevated plus maze (EPM) in rodents (Korte and De Boer 2003; Martijena *et al.* 1997). In rodents, several stressors were applied, including isolation plus exposure to a predator, predator odour and social stressor tests. Of these, one initial exposure to the predator produced more consistent, robust results when animals were later naively tested in the EPM. Ruis *et al.* (1999) found isolation in combination with social defeat was effective in potentiating subsequent anxiety responses in rats. When rats were housed individually after exposure to a single social defeat, they exhibited more anxious behaviour compared with group-housed rats when later tested in the EPM after exposure to a mild stressor. Exposure to predator stress also results in more effective and sustained anxiety in rodents, lasting up to three weeks. Moreover, the odour of a predator has been shown to increase anxiety when tested at a later date (up to a week) (Adamec *et al.* 2004; Adamec and Shallow 1993). Negative results have also been attained using this type of model. Bramley *et al.* (2001) tested behavioural responses of Black (*Rattus rattus*) and Polynesian (*Rattus exulans*) rats towards real and synthetic predator odours within a Y-maze. The predator odours (cat urine and faeces, mongoose faeces and three different synthetic odours) were not found to elicit any aversion by the rats within the testing context. Bramley *et al.* (2001) then suggested that various factors for the negative findings were difficult to explain, but suggested that species, context and testing differences could be involved.

Sheep are a highly sociable, gregarious species and are therefore susceptible to isolation stress when separated from conspecifics (Cockram *et al.* 1994; Degabriele and Fell 2001; Vandenheede and Bouissou 1998) and when their movement is restrained (Hargreaves and Hutson 1997). Exposure to a dog has also been shown to be a highly aversive stressor (Niezgoda *et al.* 1987) causing changes in behaviour (Beausoleil *et al.* 2005), cortisol concentration (Cook 2002; Komesaroff *et al.* 1998), heart rate, ACTH, adrenaline and noradrenaline concentrations (Komesaroff *et al.* 1998; Baldock and Sibly 1990). Additionally, Cook (2002 and 2004) reported neurophysiological changes within the amygdala when sheep were exposed to a dog, where increases in corticotropin releasing hormone (CRH), cortisol and gamma amino-butyric acid (GABA) where observed. In view of these findings, it is likely that the combination of isolation in the presence of a dog would be a highly effective fear challenge for sheep.

The objective of this series of experiments was to develop a fear potentiation model for sheep, based on exposure to isolation, either in the presence or absence of a dog. The development of the fear potentiated model was undertaken in three phases in an effort to manipulate the different aspects of the model. Phase 1 was undertaken to assess the fear responses of sheep after exposure to isolation or isolation plus a dog and to determine the effect of presentation order of these stressors. In the second phase the effect of reinforcement of the isolation plus dog challenge on the fear potentiated response was tested. Secondly, the longevity of the fear potentiated response was determined. Finally, in the third phase in the development of the model it was necessary to determine whether sheep could detect the presence of the dog in the absence of visual or auditory cues.

4.2 Hypotheses

Experiment 1: *Fear response after one exposure to a dog*

Animals with prior exposure to a fear-eliciting context, isolation and a dog, (**Iso+Dog**) would display a potentiated fear response during a subsequent exposure in the same context, in the absence of the dog (**Iso**)

Experiment 2: *Fear potentiation after two exposures to a dog*

Double exposure of the **Iso+Dog** challenge would result in a greater potentiated response during a subsequent exposure to Isolation alone (**Iso**)

Experiment 3: *Fear reaction to the presence of a dog without visual or auditory contact*

Sheep would express fear in response to a dog during isolation without visual or auditory contact

4.3 Materials and methods

The experiments were conducted at the FD McMaster Laboratories, Chiswick, Armidale and were approved by the sites Animal Ethics Committee (AEC no: 04/17 and 04/45).

4.3.1 Animals

Animals were sourced from the Chiswick research station flock. Merino wethers aged 12 months were used for all experiments. For experiments 1, 2 and 3, 40 (liveweight range 25 -

32 kg), 60 (liveweight 30 – 36 kg) and 40 (liveweight range 28 – 38 kg) sheep were used, respectively. Additional animals were used in each experiment as companions to prevent test animals being isolated prior to the challenges. Sheep were randomly assigned to each stress challenge group (**Iso** or **Iso+Dog**) after stratification by liveweight.

4.3.2 Challenges

The two challenges consisted of 10 minutes of isolation, in either the presence (**Iso+Dog**) or absence (**Iso**) of a dog (Figs. 4.1: a, b and c). Sheep were moved into the isolation box for 10 minutes (Phases 1 and 2) or for 1 min (Phase 3) and the degree of agitation (movement) within the box was objectively measured during each minute. Vocalisations were also recorded throughout (see chapter 2 for details).

For experiments 1 and 2, the challenge involved moving the sheep into the isolation box and after 1 min the exit door was opened to reveal either the presence (**Iso+Dog**) or absence (**Iso**) of the dog. At 1 min intervals, the agitation score was recorded. After 9 minutes, the door was closed and at 10 min, the sheep was released back to its flockmates. During experiment 3, the sheep were isolated for 1 minute with the exit door closed, and at no time was the dog visible or audible.

The agitometers on the isolation boxes were calibrated before, during and at the end of each measurement period (see chapter 2 for details).

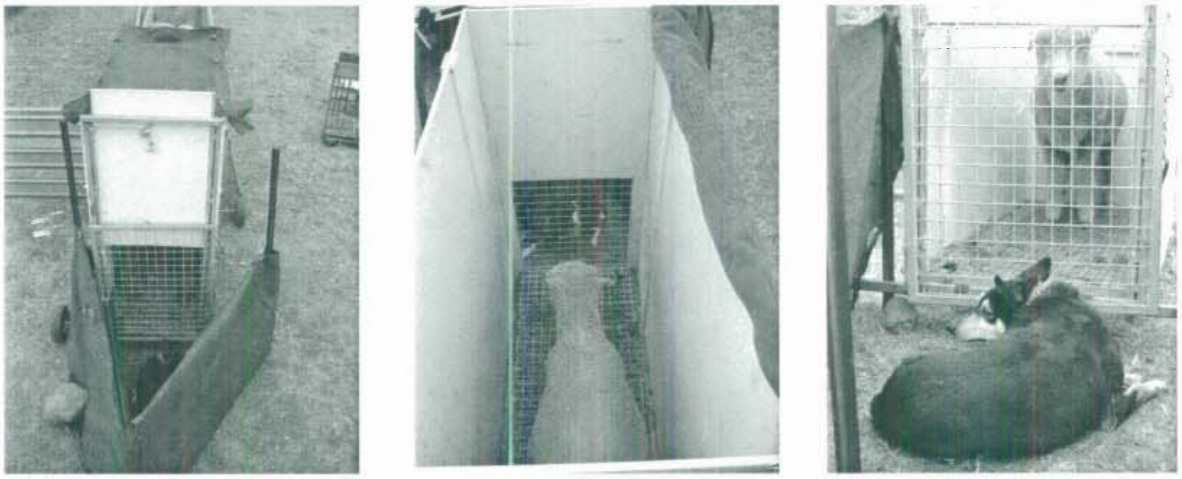


Figure 4.1: a) Overview of the isolation + dog challenge, b) view from inside the isolation box and c) front view into the isolation + dog challenge

4.3.3 Blood sampling

During experiment 1, blood samples were taken by jugular venepuncture at -20, 0, 15, 30, 60 and 90 minutes relative to the commencement of the challenge for analysis of blood cortisol. For experiment 2, animals were sampled at 0, 15 and 30 min relative to the start of the challenge. All blood samples were collected in 10 ml serum separator vacutainers (Becton Dickinson Ltd). On return to the laboratory the samples were centrifuged at 3000 rpm for 10 min and duplicate 3-4 ml aliquots of serum were stored at -20 ^{o}C until analysis.

4.3.4 Experimental procedures

Animals were bought into the treatment area approximately 1 h prior to commencement of the challenges and were left to settle for 40 min before testing commenced. The order of testing for each sheep was the same for each exposure during phases 1 and 2.

4.3.4.1 Experiment 1: Fear response after one exposure to a dog

A crossover design was used for this experiment. Ten animals a day (for two days) were exposed initially to either **Iso+Dog** or **Iso** challenges. Seven days later, the animals were subjected to the alternate challenge to that received during the initial exposure (Iso+Dog followed by Iso or Iso followed by Iso+Dog).

4.3.4.2 Experiment 2: Fear potentiation after two exposures to a dog

In experiment 2, the design was modified to determine the effect of reinforcement of the challenge context on the potentiated response. Thirty sheep were exposed twice to either the **Iso** or **Iso+Dog** challenge with two days separating each exposure. On the third exposure, both groups received the **Iso** challenge to assess the potentiated response. To test the longevity of the potentiated response, the animals were then re-exposed to the **Iso** challenge on three further occasions; one, three and six months after the third exposure.

For each stage of the final model investigated in experiment 2, it was predicted that specific behavioural and physiological responses would be linked with each exposure to the challenge, namely:

- First exposure: Initial response to exposure of sheep to the Isolation and dog challenge

A decrease in the behavioural response in the Isolation box test (IBT), concomitant with an increase in cortisol and heart rate

- Second exposure: Reinforcement of prior exposure to the Isolation and dog challenge

  Similar or further decrease of the behavioural response in the IBT with similar or higher increases in cortisol and heart rate

- Third exposure: Expression of a fear-potentiated response to prior Isolation and dog challenges, on subsequent presentation of Isolation only

  Memory of the initial Isolation and dog exposures resulting in similar low behavioural responses in the IBT and increases in cortisol and heart rate, similar to the previous exposures, even though the dog has been removed and practically the situation is less challenging

4.3.4.3 Experiment 3: Fear reaction to the presence of a dog without visual or auditory contact

Experiment 3 was conducted to test whether the sheep could sense the presence of the dog. This was predicated on the results from phase 2 which suggested that the sheep may have sensed the presence of the dog prior to the door being opened.

A cross-over design was employed in this phase. One group of sheep was exposed to the **Iso** challenge, whilst the second group was exposed to the **Iso+Dog** challenge, but in this instance the door was not opened. The test was performed over 1 min, after which the sheep exited the isolation box. The dog was present on the other side of the closed door and at no time did the dog vocalize. After two days the procedure was repeated, except that each group was presented with the alternate challenge.

4.3.5 Cortisol assay

Serum concentrations of cortisol were measured according to the procedures described in Chapter 2. For phase 1, the intra- and inter-assay coefficients of variation were 0.72 % and 5.83 % for high, 0.81 % and 6.94 % for medium and 4.49 % and 11.93 % for low control samples. For phase 2, the intra- and inter assay coefficients were 4.3 % and 8.25 % for high, 3.07 % and 7.84 % for medium and 3.55 % and 10.45 % for low control samples.

In phases 1 and 2, the 15 min blood sample was chosen to approximate the peak cortisol response. The integrated cortisol response (AUC) was determined according to the procedures described in Chapter 2.

4.3.6 Statistical analysis

All statistical tests were performed using SAS Version 8.2 (SAS Institute Inc., USA 1999). The homogeneity of variance was tested for all variables and for some measures logarithmic or square root transformations were required to normalize the data prior to analysis.

4.3.6.1 Experiment 1: Fear response after one exposure to a dog

The dependent variables included serum cortisol concentration at 15 min (peak cortisol), integrated cortisol response (AUC), agitation score during the first min in the box (IBT min1), cumulative agitation score from 2-9 minutes when the door was open to reveal the presence/absence of the dog (IBT 2-9min), during the last minute in the box (IBT min10) and number of vocalisations over the entire 10 min period (Vocs). The integrated cortisol response (AUC) was determined over the entire sampling periods (-20 to 90 min). The general linear model (GLM) procedure in SAS was used to analyse the data with the initial statistical model including the main effects of challenge (**Iso** and **Iso+Dog**) and sequence the animals received their challenges (**Iso** then **Iso+Dog** or **Iso+Dog** then **Iso**), day of testing (1 and 2), plus the interactions. Non-significant interactions were sequentially removed until the simplest significant ($P<0.05$) models were achieved.

4.3.6.2 Experiment 2: Fear potentiation after two exposures to a dog

The dependent variables included AUC, IBT min1, IBT min2-9, IBT min10 and Vocs. For the data from the initial three exposures, a MIXED model procedure in SAS was used to analyse the data. The full model contained the terms, challenge (**Iso+Dog** or **Iso**), exposure (Exposure 1, 2 and 3) and the interactions. For the above model, animal was fitted as the random term. To examine the effect of time of the potentiated response a repeated measures model in SAS (Proc Mixed) was used. The full model contained, challenge (**Iso+Dog** and **Iso**), time (Exposure 3, 1, 3 and 6 months), plus the first and second order interactions. Animal was fitted as the random term. Non-significant interactions were sequentially removed until the simplest significant models were obtained.

4.3.6.3 Experiment 3: Fear reaction to the presence of a dog without visual or auditory contact

The dependent variables used in this analysis were IBT min1 and vocalisations (Vocs). The GLM procedure in SAS was used to analyse data with the full model containing main effects of challenge model (**Iso** and **Iso+Dog**), challenge sequence (**Iso** then **Iso+Dog** and **Iso+Dog** then **Iso**), plus the interaction.

4.4 Results

4.4.1 Experiment 1: Fear response after one exposure to a dog

Significant interactions between challenge and sequence were observed for the IBT min 2-9 agitation score and vocalisations, as well as the cortisol responses (Table 4.1).

Table 4.1: Least square means for agitation scores at IBT min1, IBT min2-9, IBT min10, number of vocalizations (Vocs), cortisol at 15 min and total integrated cortisol response (AUC)

Main effects	**Isolation box test**				**Cortisol**	
Challenge	***Min 1**	***Min 2-9**	***Min 10**	**Vocs**	**15 min (nmol/L)**	**AUC (nmol/L/min)**
Isolation	39.60	238.02	24.09	3.36	30.26	2615.61
Isolation + Dog	33.41	34.67	4.09	1.58	42.86	2974.51
sed	3.2	30.1	4.5	0.2	0.9	207.3
Significance	ns	*P<0.001*	*P<0.001*	*P<0.01*	*P<0.001*	ns
Sequence						
Isolation – Isolation + Dog	35.52	101.75	8.44	2.08	36.16	2769.63
Isolation + Dog – Isolation	36.97	170.95	11.69	2.55	35.87	2820.48
sed	3.2	30.1	4.5	0.2	0.9	207.3
Significance	ns	*P<0.05*	ns	ns	ns	ns
Day						
1	35.16	123.72	9.84	2.35	37.67	2963.00
2	37.34	148.97	10.02	2.26	34.43	2627.10
sed	3.2	30.1	4.5	0.2	0.9	207.3
Significance	ns	ns	ns	ns	ns	ns
Interactions						
Challenge x Sequence	ns	*P<0.05*	ns	*P<0.05*	*P<0.001*	*P<0.05*
Challenge x Day	ns	ns	ns	ns	ns	ns
Sequence x Day	ns	ns	ns	ns	ns	ns
Challenge x Sequence x Day	ns	ns	ns	ns	ns	ns

*Backtransformed Log LSM

There was a significant interaction (P<0.05) between challenges x sequence of the challenges for IBT min2-9 agitation score (Fig. 4.2), Vocs (Fig. 4.3) and cortisol concentration at 15 min (Fig. 4.4a) and AUC (Fig. 4.4b) (Table 4.1). Higher agitation scores were evident for the subsequent **Iso** challenge following initial exposure to the **Iso+Dog** challenge. A similar pattern was evident for vocalisations (P<0.05; Fig. 4.3).

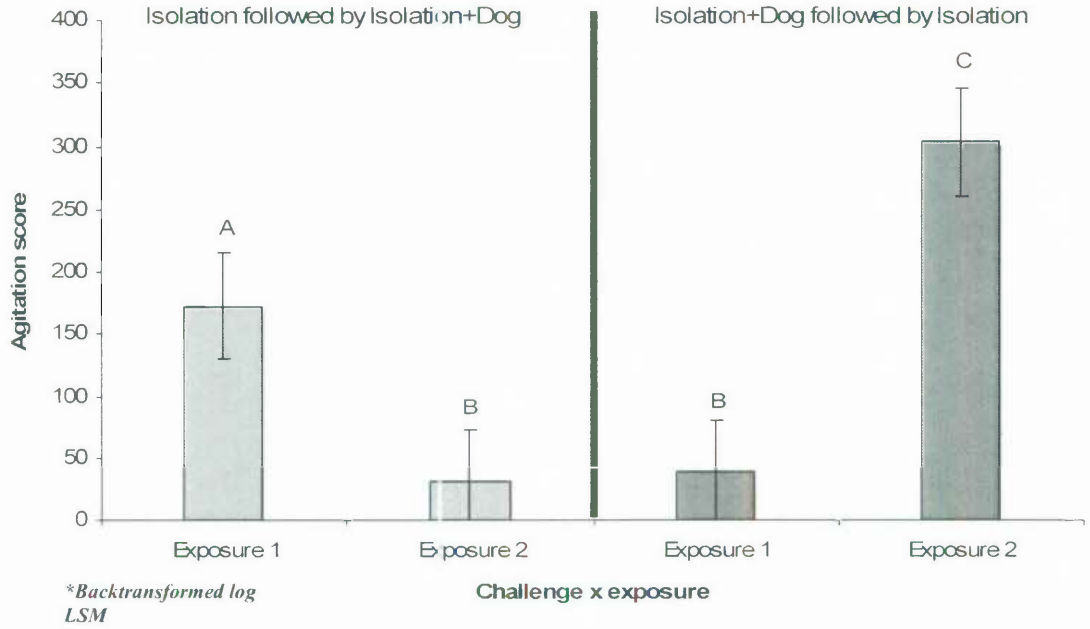


Figure 4.2: Least square means (±se) for the interaction between challenge x challenge sequence for IBT min2-9 agitation score

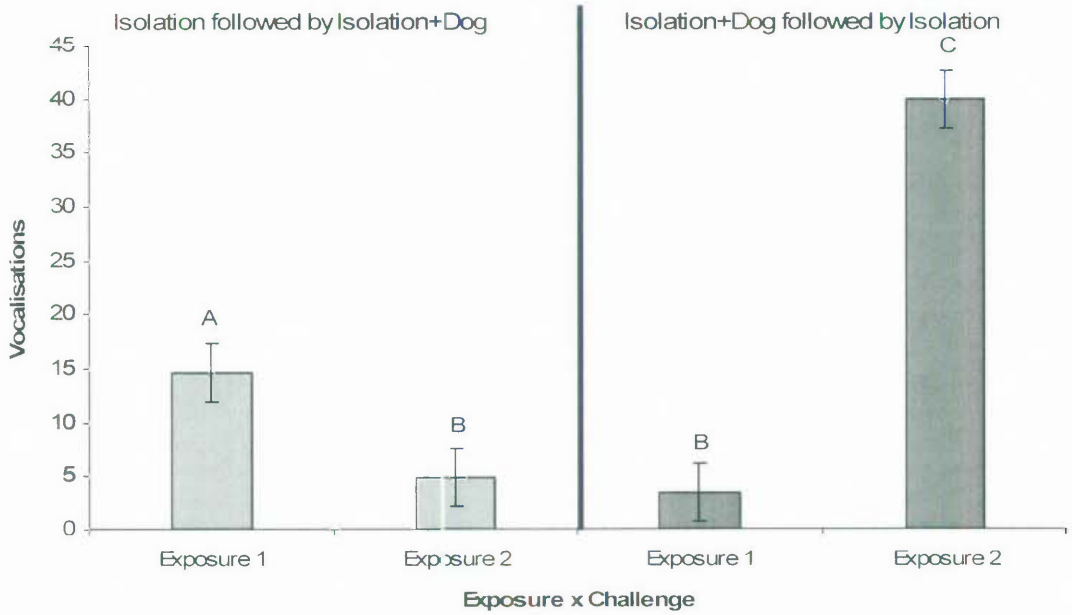
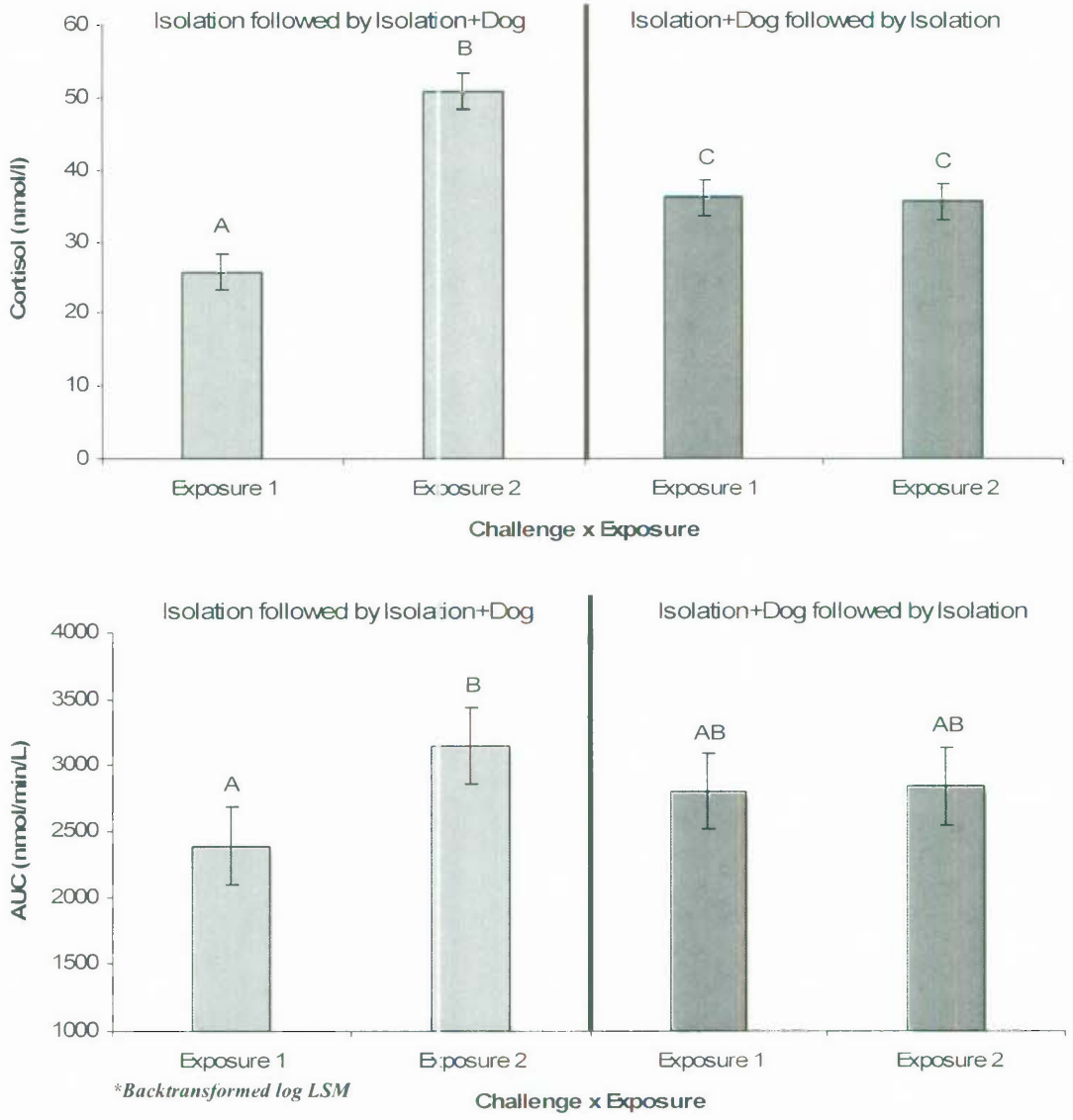


Figure 4.3: Least square means (±se) for the interaction between challenge x challenge sequence for number of vocalisations

Sheep initially exposed to the **Iso+Dog** challenge had significantly higher cortisol levels at 15 min during the subsequent **Iso** challenge compared to those observed for the group receiving the **Iso** challenge on the first exposure (Fig 4.3a). A similar pattern was evident for the AUC, although the difference in the least square means for the specific contrast between Iso (exposure 1) and Iso+Dog (exposure 2) was not significant (Fig. 4.4b).



Figures 4.4 (a and b): Least square means (±se) for the interaction between challenge x challenge sequence for cortisol a) Cortisol concentration (nmol/L) immediately after the challenge (15 min) and b) AUC

Figure 4.5 shows the unadjusted mean agitation scores for each minute of the 10 minutes of isolation for each challenge and exposure. Animals presented with the **Iso+Dog** challenge, regardless of exposure responded with low agitation scores, when the door was opened to present the predator, compared with the **Iso** challenge groups.

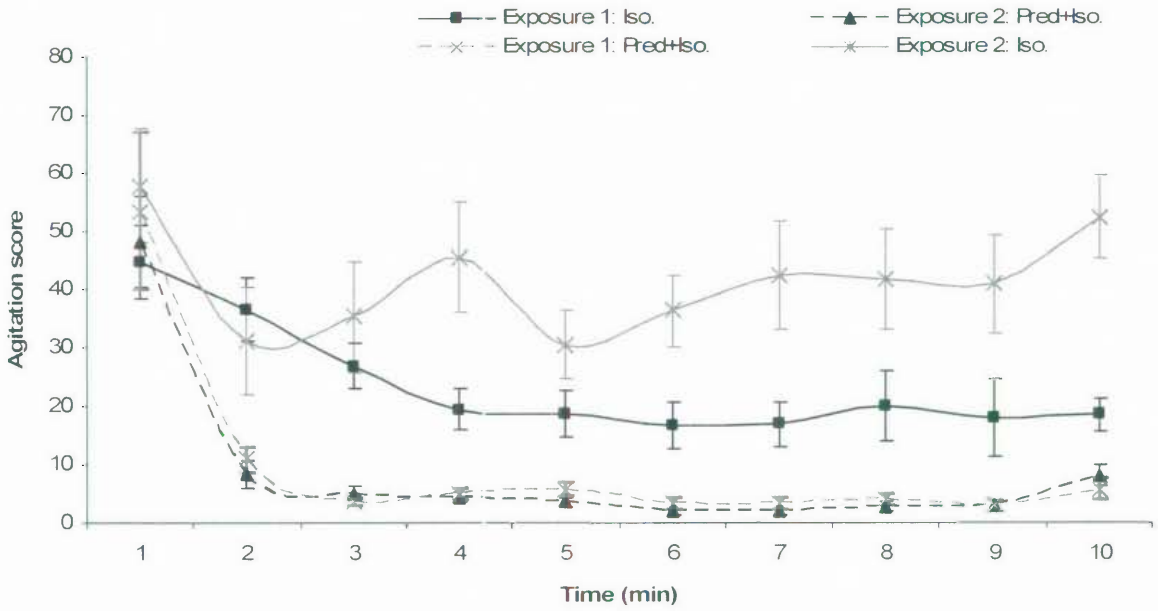


Figure 4.5: Unadjusted agitation scores over the 10 minutes during the **Iso+Dog** and **Iso** challenges for exposure 1 and 2

4.4.2 Experiment 2: Fear potentiation after two exposures to a dog

4.4.2.1 Agitation score and cortisol responses

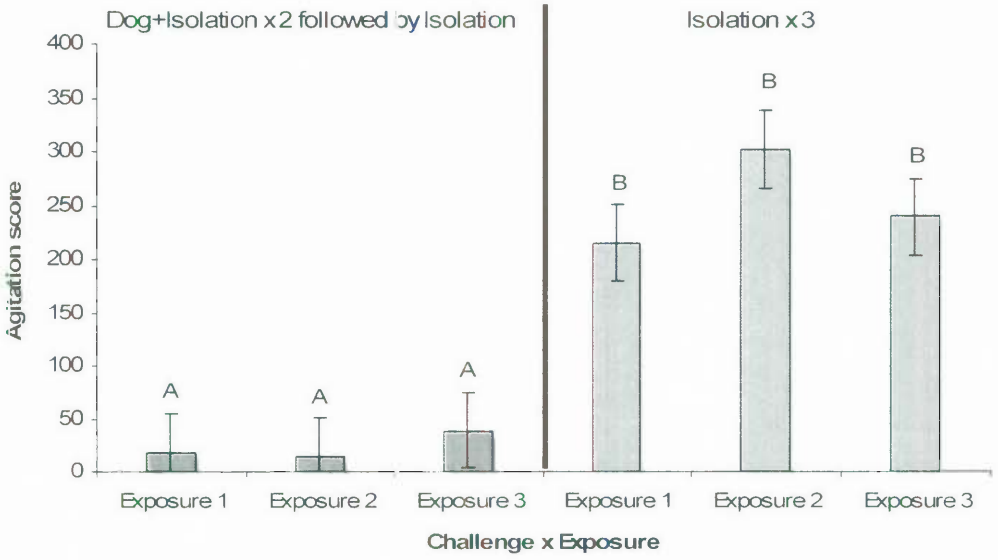
The agitation scores and number of vocalizations were higher for the **Iso** challenge compared with the **Iso+Dog** challenge (Table 4.2). Additionally, IBT min2-9, min10 and vocalisations increased from exposure 1 through to exposure 3.

Table 4.2: Least squared means for responses measured in the Isolation box test (IBT min1, IBT min2-9, IBT min10, vocalisations) and total integrated cortisol (AUC) for the three exposures

Main variables	**Isolation box test**				***Cortisol (nmol/L)**
Challenge	***IBT min1**	***IBT min2-9**	***IBT min10**	**Vocs**	**AUC**
Isolation	72.97	31.50	249.64	13.46	615.09
Isolation + Dog	29.96	6.62	21.98	4.14	709.38
Sed	8.2	7.4	43.4	4.0	33.2
Significance	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.05*
Exposure					
1	48.42	9.97^{A}	62.80^{A}	5.64^{A}	787.2^{A}
2	47.94	13.87^{B}	68.03^{A}	6.36^{A}	634.0^{B}
3	44.26	21.76^{C}	95.58^{B}	11.47^{B}	565.4^{C}
Sed	8.2	4.5	25.1	2.3	28.3
Significance	ns	*P<0.001*	*P<0.05*	*P<0.01*	*P<0.001*
Interactions					
Challenge x Exposure	ns	*P<0.01*	*P<0.001*	*P<0.001*	*P<0.01*

****Backtransformed log LSM***

The interaction between challenge x exposure was significant for the agitation scores IBT min2-9 ($P<0.01$) and IBT min10 ($P<0.001$) and number of vocalisations ($P<0.001$) (Table 4.2, Figs. 4.6 a - c). Relative to the **Iso** challenge, the **Iso+Dog** challenge resulted in a significant reduction in IBT min2-9 agitation scores on all exposures, including exposure 3 when the dog was not present (Fig. 4.6a).



Figures 4.6a: Least square means (±se) for the interaction between challenge x exposure for IBT min2-9

During the last minute of the challenge when the door was closed (IBT min10), the agitation score was significantly lower for animals presented with the **Iso+Dog** challenge during the first two exposures, compared with agitation scores for the **Iso** challenge (Fig. 4.6b). On the third exposure when the dog was absent a similar trend was observed however the difference between the challenges was not significant.

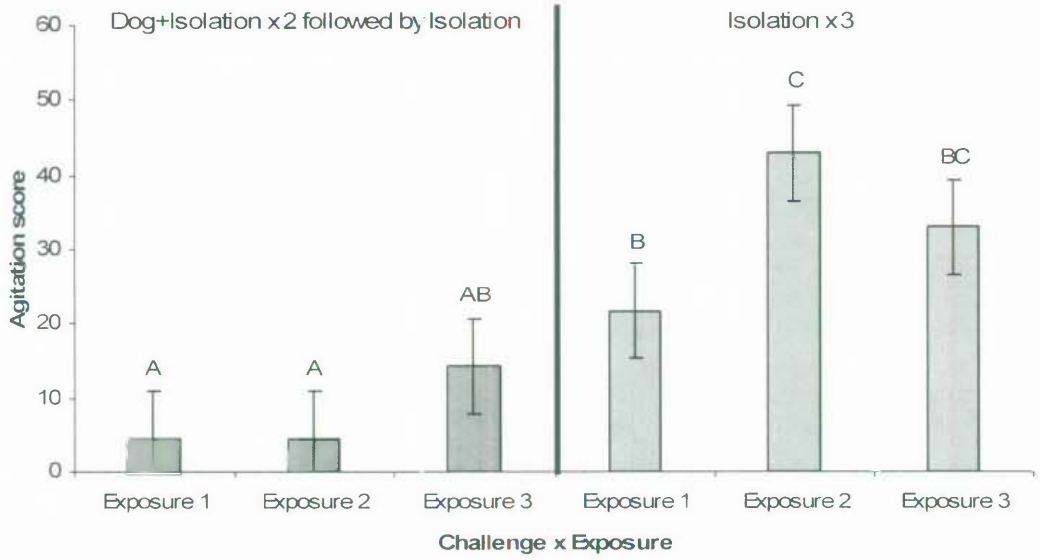
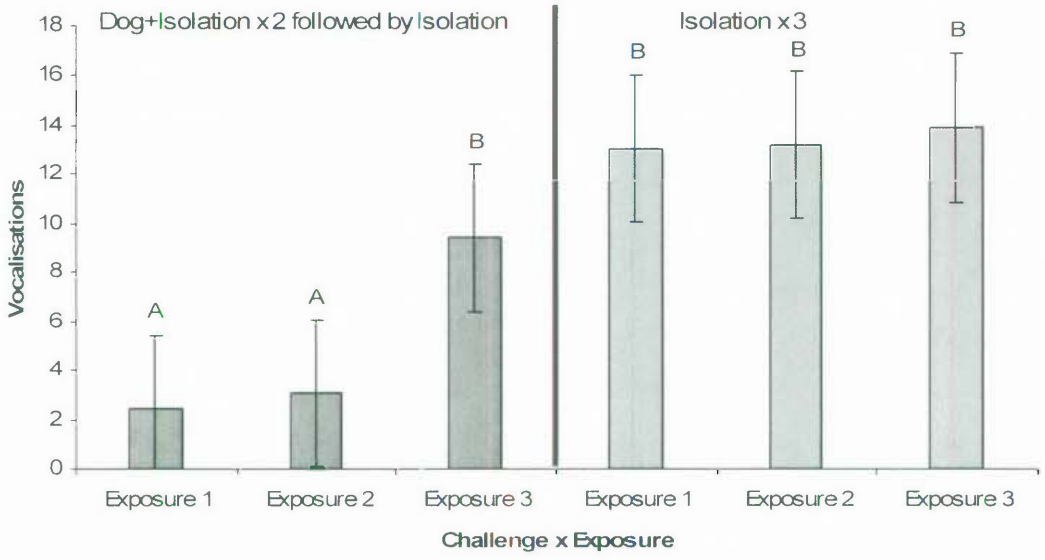


Figure 4.6b: Least square means (±se) for the interaction between challenge x exposure for IBT min10

The number of vocalisations were higher during all exposures to the **Iso** challenge compared to the **Iso+Dog** challenge. The differences were significant in exposures 1 and 2. During the third exposure to the **Iso+Dog** challenge (i.e. absence of the dog) the number of vocalisations increased compared to first two exposures, furthermore (Fig. 4.6c).



Figures 4.6c: Least square means (±se) for the interaction between challenge x exposure for vocalisations

The interaction between challenge x exposure was also significant ($P<0.01$) for the total integrated cortisol response (AUC). There was a decline in the AUC (by approximately half) after the first exposure to the **Iso** challenge compared to the subsequent last two exposures, which were similar. After the first exposure to the **Iso+Dog** challenge, AUC decreased in step-wise increments during the subsequent exposures. The AUC response was generally higher for the Iso+Dog challenge however, only the contrast between them on exposure 2 was significant (Fig. 4.7).

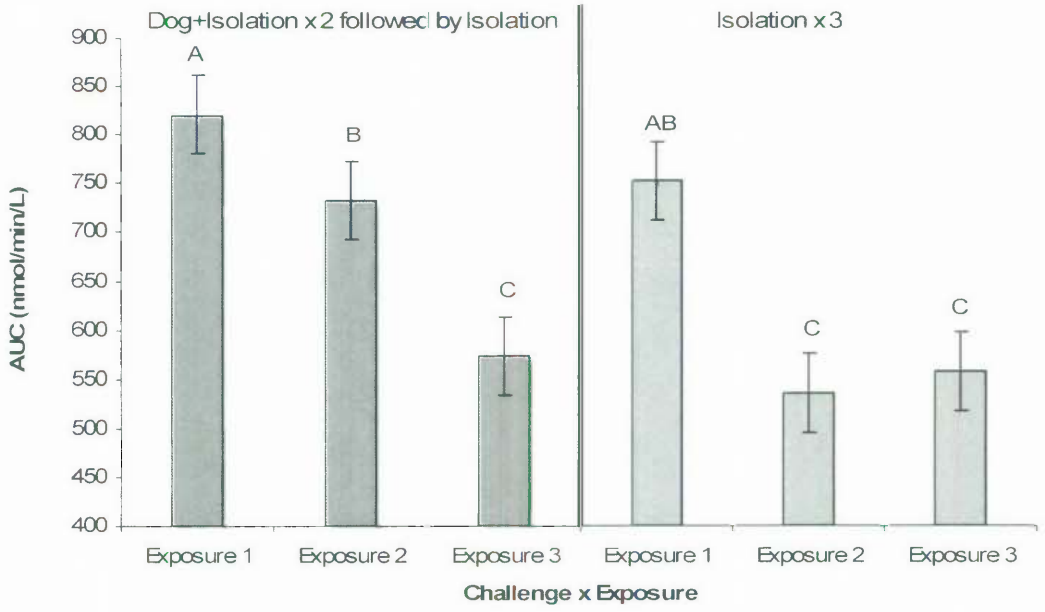


Figure 4.7: Least square means (±se) for the interaction between challenge x exposure for AUC

4.4.2.2 Longevity of potentiated fear response

In the analysis of the effect of time on the potentiated fear response a significant interaction between challenge x time was observed for all of measures (Table 4.3).

Table 4.3: Least square means for agitation scores and vocalizations measured in the IBT and total integrated cortisol response (AUC) in response to the challenges over time

	Isolation box test				**Cortisol**
Challenge	**IBT min1**	**IBT min2-9**	**IBT min10**	**Vocs**	**AUC (nmol/L/min)**
Isolation + Dog	62.80	249.01	35.16	15.76	464.05
Isolation	38.09	164.61	26.58	11.09	497.70
Sed	9.2	52.2	8.3	4.6	38.80
Significance	*P<0.001*	*P<0.05*	ns	ns	ns
Time					
Exposure 3	44.26^{AB}	156.50^{A}	21.76^{A}	17.31^{A}	533.79^{A}
1 month	58.56^{A}	250.91^{B}	37.34^{B}	10.18^{B}	464.05^{B}
3 months	42.52^{B}	$173.19B^{A}$	29.16^{C}	21.81^{C}	437.03^{B}
6 months	52.4^{A}	246.8^{AB}	35.8^{BC}	6.7^{D}	492.75^{A}
Sed	5.5	26.1	4.3	2.3	25.6 – 26.1
Significance	*P<0.01*	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*
Interactions					
Challenge x Time	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.001*	*P<0.01*

The IBT min1 agitation score for the **Iso+Dog** challenge were similar and consistently lower compared with **Iso** challenge at the first 3 time points (exposure 3, 1 and 3 months). After 6 months, the first minute score for the **Iso+Dog** challenge increased to levels similar to those observed in the **Iso** challenge during exposure 3. The **Iso** challenge agitation score initially increased at 1 month, and then decreased to levels lower than that observed during the third exposure. The **Iso+Dog** challenge had low levels up until 6 months when they increased to levels similar to that observed with the **Iso** challenge during the third exposure (Fig. 4.8).

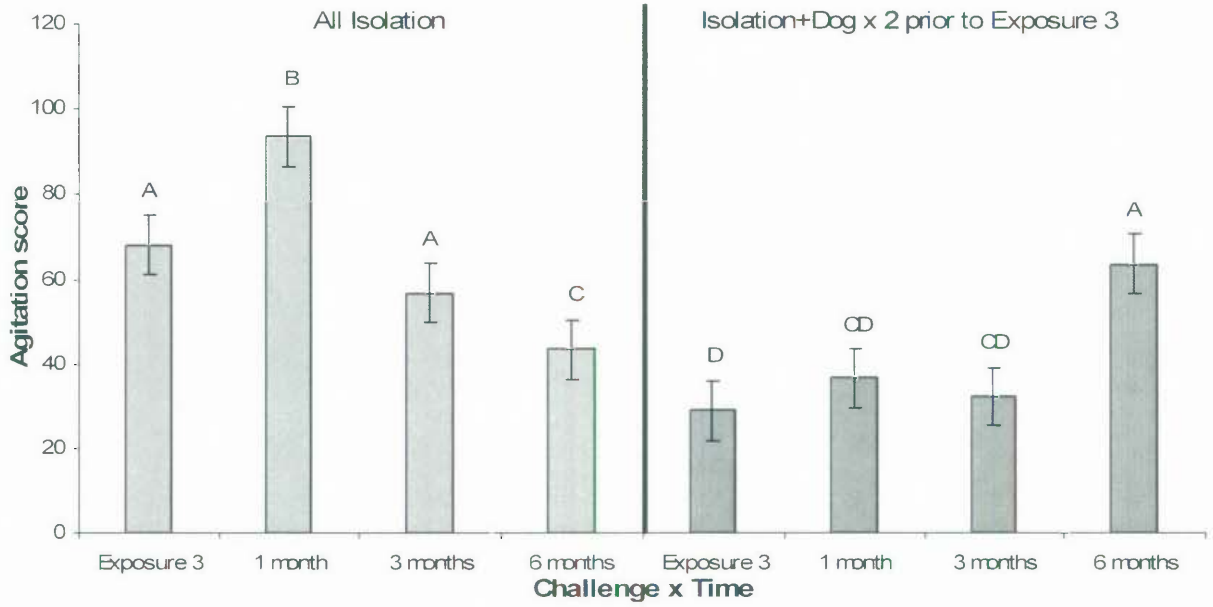


Figure 4.8: Least square means (±se) for the interaction between challenge x time for IBT min1

The challenge by time interaction for IBT min2-9 agitation score indicated a significant decrease during the third exposure to the **Iso+Dog** challenge (when the dog was absent) (Fig. 4.9). The agitation scores then increased during subsequent presentations at 1 and 3 months but the scores were still lower ($P<0.05$ at 1 month) than those observed for the **Iso** challenge during exposure 3 and at 1 month. After 6 months, there was a pronounced increase in the min2-9 agitation score for the **Iso+Dog** challenge and this was significantly higher than that observed for the **Iso** challenge at 3 and 6 months. For the **Iso** challenge, the agitation scores decreased after 1 month (Fig. 4.9).

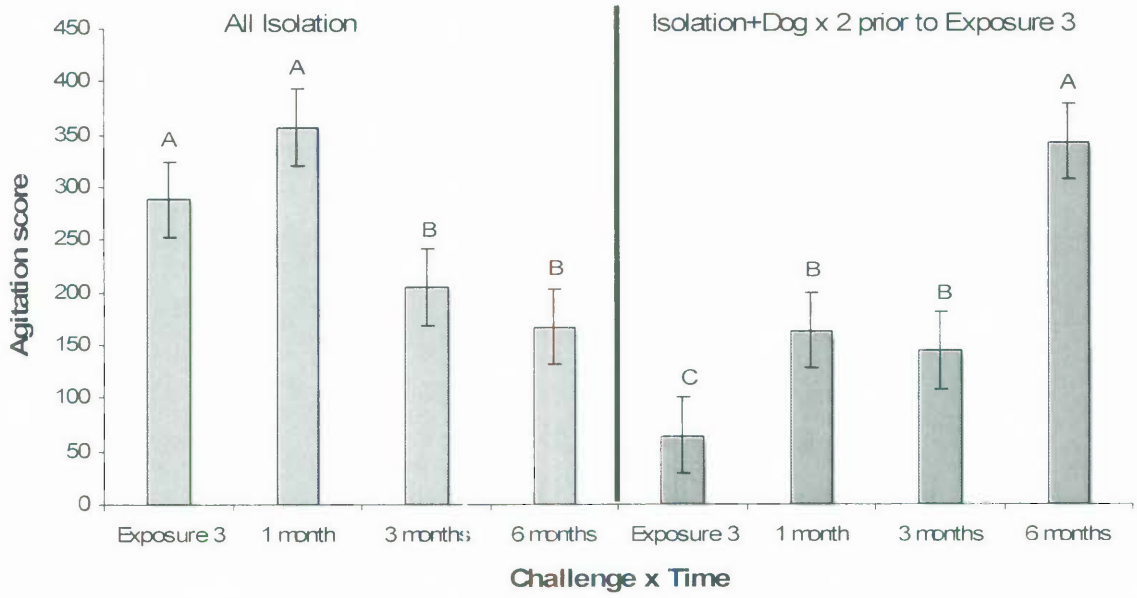


Figure 4.9: Least square means (±se) for the interaction between challenge x time for the IBT min2-9 agitation scores

Similar trends were observed for the IBT min10 agitation score (Fig. 4.10).

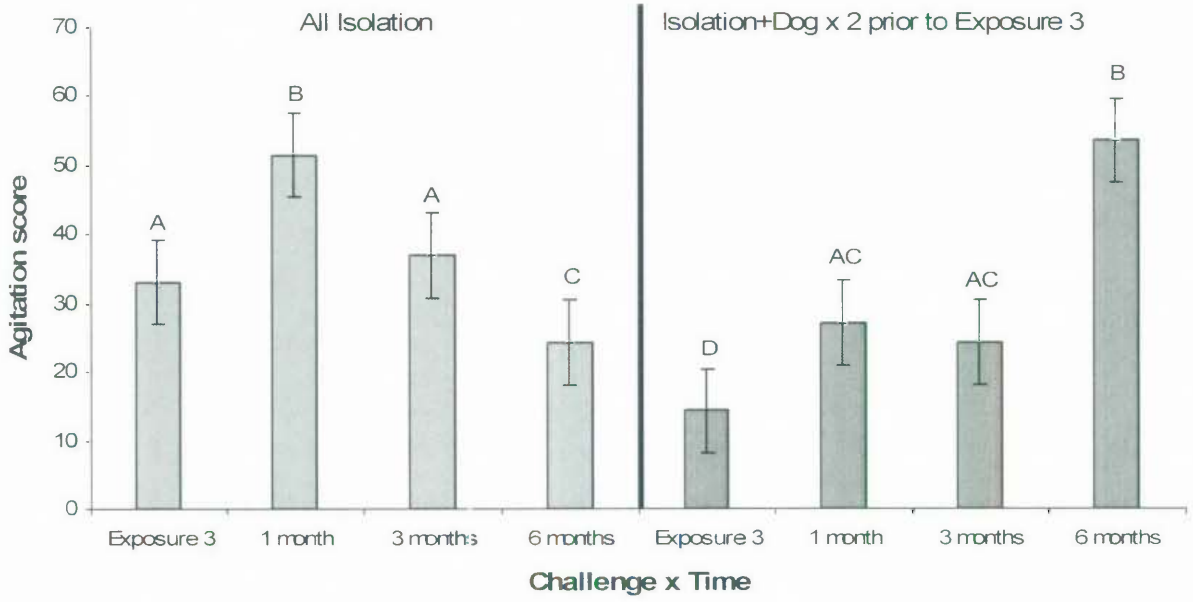


Figure 4.10: Least square means (±se) for the interaction between Challenge x Time for the IBT min10 agitation score

Over time, the number of vocalisations was similar for the **Iso** challenge with the exception of the 6 month period where there was a significant decrease. Sheep presented with the **Iso+Dog** challenge responded variably over time (Fig. 4.11). The differences between the challenges were only significant at 1 and 6 months.

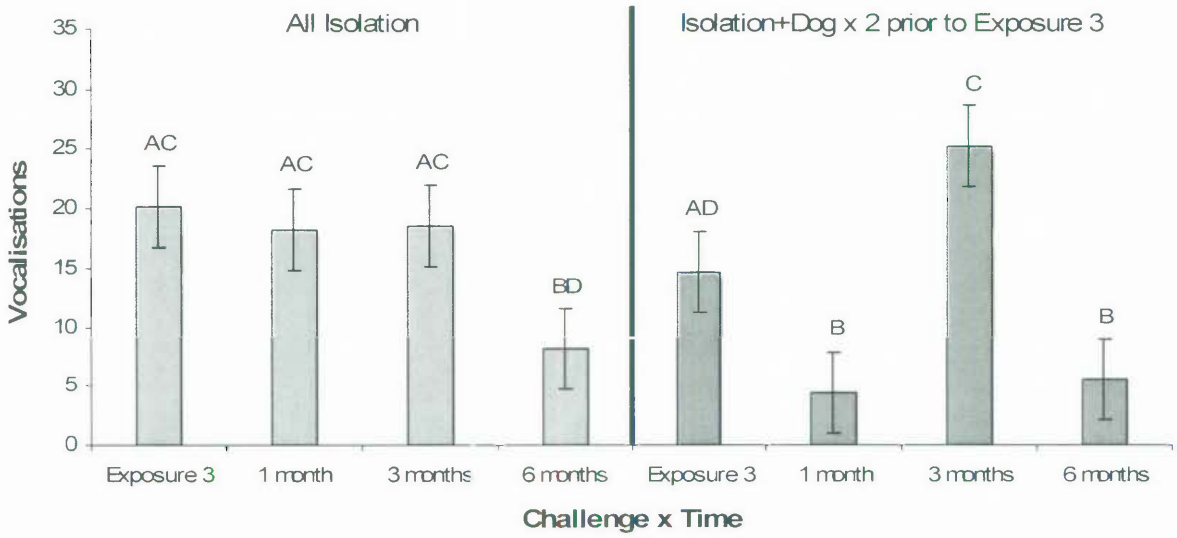


Figure 4.11: Least square means (±se) for the interaction between challenge x time for vocalisations

For the interaction between challenge x time, the AUC response showed that the sheep exposed to the **Iso** challenge during exposure 3 (and previous exposures) had lower AUC at subsequent time points (Fig. 4.12). Animals exposed to the **Iso+Dog** had similarly high responses at 1 and 6 months compared to the level observed during exposure 3. The reduction was significantly greater at 3 months. The AUC was generally higher, except at 3 months, for the **Iso+Dog** challenge compared to the **Iso** challenge. However, the differences were only significant at 1 month.

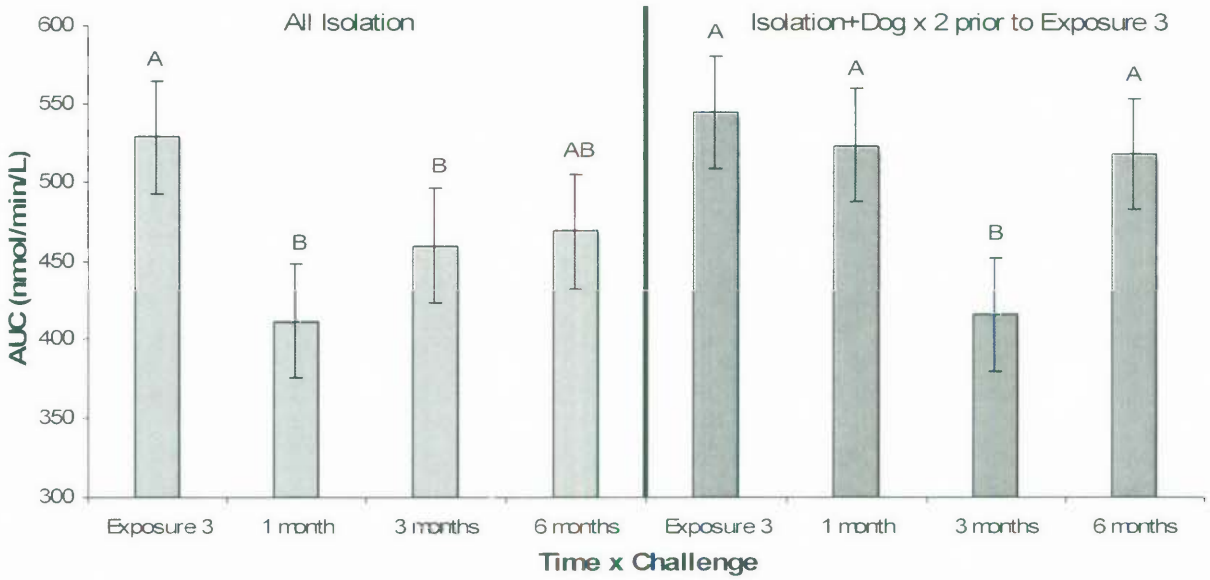


Figure 4.12: Least square means (±se) for the interaction between time x challenge for the total integrated cortisol response (AUC)

4.4.3 Experiment 3: Fear reaction to the presence of a dog without visual or auditory contact

There was a significant difference between the challenges on the agitation score during the first minute of the test (IBT min1) where the score was significantly lower for the **Iso+Dog** challenge compared with the **Iso** challenge (Table 4.4). The sequence of presentation of the challenges and the interaction between challenge x challenge sequence were not significant.

Table 4.4: Least square means for the agitation score and vocalisations during the first minute of the challenge

Main effects	**Isolation box test**	
Challenge	**IBT min1**	**Voc**
Isolation	115.46	6.41
Isolation + Dog	66.75	4.49
sed	0.13	0.15
Significance	*P<0.001*	ns
Sequence		
Isolation – Isolation + Dog	92.11	4.19
Isolation + Dog - Isolation	83.68	5.80
sed	0.13	0.15
Significance	ns	ns
Interaction		
Challenge x Sequence	ns	ns

4.5 Discussion

Collectively, the evidence, particularly from experiments 1 and 2, supports that a potentiated fear response in sheep was achieved. Korte (2001) suggested that if a psychological stressor was extreme enough, and the extinction period between exposures to the predator are sufficiently short, animals may become more sensitized to the stressor. The evidence from experiment 1 (fear response after one exposure to the **Iso+Dog** challenge) indicates that although both behaviour and the HPA response were altered, the fear potentiation was more strongly apparent in the behavioural response. Glucocorticoid secretion has been shown to be sensitive to situations of unpredictable stress: however research in rodents has revealed equivocal results. Mormede *et al.* (1988) observed rats in a variety of psychosocial situations (inescapable footshock, signaled and un-signaled footshock, active avoidance and un-shocked) and found that the glucocorticoid profiles were not different between the challenge contexts. However, the immune parameters tested did show varying degrees of change between challenges indicating a stress response. This implies that parameters other than cortisol may possibly have been more informative in assessing a potentiated stress response. Additional reasoning behind a weaker HPA response as compared with the behavioural response could be that the period of time between challenge exposures (seven days) was too long. Alternatively, the one brief exposure to the **Iso+Dog** challenge may not have been sufficient to create a significant memory of the challenge, which was substantial enough to induce a pronounced HPA response on re-exposure to the context.

This issue was examined in experiment 2 where the sheep were given an additional exposure to the context with the dog and there was a shorter extinction period between exposures. Sheep exposed to the **Iso+Dog** challenge responded with reduced movement in the visual presence of the dog, similar to that observed in experiment one. Many ungulate species freeze or remain motionless when a predator approaches (Caro *et al.* 2004), which this reiterates. Furthermore, this was associated with higher cortisol responses. When the dog was not present, the agitation scores still reflected a reduced behavioural response which could be interpreted as a conditioned behavioural response. The sheep possibly responded with increased caution of the situation, resulting in reduced activity because of the perceived threat of the dog. However, the HPA response (cortisol) was not elevated during the third exposure. The conditioned response to the **Iso+Dog** context in the absence of the dog appeared to be

managed more by an altered behavioural response as opposed to a full scale behavioural and physiological response.

Why the HPA response was not markedly stronger during the third exposure to the **Iso+Dog** context is unclear. Perhaps the lack of HPA response is indicative of some conservative adaptive stress response in the face of a perceived rather than real threat. Moreover, the expression of an altered behavioural response (in this case a reduction in movement within the box), may be the most appropriate and efficient defense. The possibility of a "sensitive" period after an aversive event may cause sheep to act more unpredictably because of the original aversive situation, until such a time as to when the associated memory is lost. Being able to respond appropriately to a potentially threatening situation requires an animal to take action to avoid the situation. However, being able to respond appropriately when the situation is revealed as less threatening is just as important. If a continual HPA axis response is maintained in non-threatening situations, pathologies can result, which can be likely to impair welfare.

In the wild, after a predator or threat has disappeared, acquired information, such as memory of the event, can help in the prediction of the possibility of subsequent encounters. This response implies that the animals related the context with the dog, but because the threat (dog) was not immediately visible, the sheep displayed more agitation. This may indicate a response more akin to flight, not fight, resulting in higher agitation scores possibly due to the unpredictability of the situation. Unpredictability has been shown to affect fear responses of animals in their ability to determine the influence of a situation (Désiré *et al.* 2002). Weiss (1972) identified three causal factors that play important roles in stress and fear; i) controllability of a situation, ii) feedback on the context iii) and unpredictability.

The evidence also supports the question that the fear potentiated response was sustained over time. After one month from the third exposure to the challenges, the animals initially exposed to the dog had a higher HPA response, as well as a conditioned behavioural reduction in agitation/movement. This indicates a memory of the context. It is plausible the effect may be longer lasting although the evidence here suggests it dissipated over time (from 1 month). A further reinforcement of the dog may be required to produce a more sustained effect.

Agitation scores in the **Iso** challenge animals decreased over the six month period, indicating habituation. Physiologically, the cortisol responses indicated that there was no effect after about 1 month, as the **Iso+Dog** challenge animals only had an increased cortisol concentration during the first month test. This in some way parallels the earlier observations that behavioural responses are perhaps the frontline defense to fear-eliciting situations and as memory of the fear diminishes or the animal adapts to the challenge then the potentiated cortisol response will become less at a greater rate than the behavioural responses. There also may be subtleties within behaviours with declining levels of agitation and increased levels of 'normal' isolation vocalisations.

The combined challenge of isolation and the presence of a dog (**Iso+Dog**) resulted in a marked reduction in the agitation score, where there was a distinct lack of movement within the box when sheep were faced with the challenge. The behaviour was typical of 'freezing' behaviour observed in some animals when exposed to specific stressors, particularly predators. The combination of the two stressors, with no obvious escape route facilitated the freezing response, as opposed to the expression of increased agitation. The expression of this behaviour was not surprising as it has been observed in sheep, where they remain alert/vigilant when a predator is detected in an attempt to evade or reduce the likelihood of an attack (see review by Eilam 2005 and Howery and DeLiberto 2003.

During experiments 1 and 2, the number of vocalisations was higher in the group exposed to the **Iso** challenge, compared with the **Iso+Dog** challenge. This was not unexpected as sheep are known to vocalize when isolated in a number of different situations (Boivin *et al.* 1994; Carbajal and Orihuela 2001; Romeyer and Bouissou 1992; Torres-Hernandez and Hohenboken 1979). This behaviour then fits well with the hypothesis that vocalisations were thought to have evolved to alert conspecifics to danger (Dennis and Melzack 1983) or to elicit the help of others (Pratt 1980). The presence of the dog took priority over the impact of isolation in the behavioural expression of the animals. However, the number of vocalisations increased significantly when sheep were presented with isolation and dog context but in the absence of the dog. This suggests that the animals are more aware of their isolation when the dog was not present, and the potentiated behavioural response was combined with the "normal" vocalization pattern of the isolated sheep.

Vocalisations can be interpreted in a number of ways depending on the context, situation and species. In this thesis, vocalization were interpreted as being associated with a fear response as illustrated by Kilgour and Szantar-Coddington (1997) {Kilgour & Szantar-Coddington 1997 #2240} who showed that in an arena test of isolated animals vocalized significantly more than those tested in groups. They postulated vocalization was related to increased nervousness.

In the first experiment, when the dog was not visible to the sheep during the first minute of isolation, no difference in agitation was observed, implying a visual link was necessary to establish the presence of the dog. However, this was not observed in the experiments in 2 and 3. Sheep exposed to the **Iso+Dog** challenge had higher agitation scores during the first minute, when the door was closed compared with that found for the **Iso** challenge. The main difference between experiment 1 and experiments 2-3, was that in experiment 1, the experiment was conducted outside in sheep yards and the latter experiments were conducted in a shed environment. Therefore, it is plausible that under the latter conditions the sheep may have detected olfactory cues of the presence of the dog, even though visual contact had not been established. Sheep have an acute sense of vision associated with the evolutionary process of being a prey species and for social recognition, (Clarke and Whitteridge 1973; da Costa *et al.* 2004), as well as a very well developed olfactory system (Hargreaves and Hutson 1997). They incorporate both these senses into interactions with the everyday environment (e.g. recognition of predators, detection of ewes in heat, maternal cues and identification of lambs (Levy *et al.* 2004; Dwyer 2004)). The likelihood that olfactory as well as visual cues are being used by the sheep is relatively high in this instance. It would be interesting to retest the animals using odour cues to see if a fear potentiated behavioural response could be induced, as research has shown that feeding behaviour in sheep can be highly suppressed when associated with either dog or wolf faeces (Arnould *et al.* 1998). Adamec *et al.* (2004) showed lasting anxiety-like behaviour in rats exposed to cats, and found responses were more intense after exposure to the cat, but lasting intermediate effects were also observed following exposure to the cat odour and to the testing room. However, in a review by Apfelbach *et al.* (2005), several studies showed predator odours to be ineffective in averting prey species from particular areas, suggesting further research is needed into factors mitigating the response.

The fear development model was developed in three experimental phases with different exposures dependent on the pharmacological treatment administered at the time. However,

because of the complexity of the test, it cannot be stated that the design was based only on social motivation (isolation) or predator avoidance (inclusion of the dog within the test), but also has other parameters, such as the novelty of the box, restraint within the box, manipulation of the sheep leading up to being put into the box, noise of the box opening or attachment of the HR monitors. None of these parameters can be separated within this model, as to which was more or less frightening or of novelty for the sheep within the testing parameters. This potentially could lead to the development of a slightly less complex model for further development at a later stage.

4.6 Conclusion

Fear potentiation in sheep can be achieved when animals are re-exposed to an isolation context where previously a dog was present. Furthermore, this conditioned effect appears to be more behavioural in nature. This behavioural response was still evident after one month, implying memory the original fear-eliciting situation, but dissipated beyond that. It is recommended the model include two initial exposures to the **Iso+Dog** context followed by a third exposure with the dog absent to facilitate the conditioned response. Furthermore, it may be desirable to conduct the test in well ventilated or open environments to minimise the olfactory detection of the presence of a dog.

Further assessment using the model and particularly its relevance in regards to assessing temperament *via* pharmacological, behavioural and physiological measures is continued in the following chapter, with additional manipulation through the use of a temperament selection line. Administration of the pharmacological treatments tested in Chapter 3's dose response experiments are examined using the fear potentiation challenge to assess the neurophysiological pathways hypothesized to underpin differences in the temperament of the selected lines.