Behavioural and physiological responses of domestic sheep (*Ovis aries*) to the presence of humans and dogs

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Ngaio Jessica Beausoleil BSc [1]

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Abstract

Both humans and dogs are integral in sheep production systems; however, which is more aversive to sheep, or indeed, whether either causes significant stress, has not been shown experimentally. The aim of this thesis was to examine some behavioural and physiological responses of domestic sheep to the presence of humans or dogs. An arena test was used to measure the relative aversion of sheep to the presence of a human or dog, as well as to elucidate differences in the responses of flocks at the University of Western Australia (UWA) which were putatively selected for differences in fearfulness. A Y maze preference test was used to 'ask' sheep whether they preferred a human shaking a rattle or a barking dog. In both tests, adrenocortical responses were measured concurrently to support the interpretation of behaviour.

The presence of a human or dog in the arena elicited significantly more avoidance and vigilance behaviour and less exploration than did the presence of a control object. However, the dog elicited significantly more of this fear-related behaviour, and significantly larger adrenocortical responses than did the human. Sheep also expressed a clear preference for a human shaking a rattle over a barking dog in the Y maze test and exhibited larger adrenocortical responses to the dog than to the human in the Y maze facility.

The UWA flocks differed in their expression of locomotor and vocal activity; MA sheep were more active/vocal than the other flocks, not only in the presence of the human but also with the box or dog. MA sheep expressed less avoidance and vigilance and more exploration than the other flocks in the presence of the human and exhibited significantly lower plasma cortisol concentrations than LA sheep after exposure to the human (10-min sample). However, there were no inter-flock differences in fear-related behaviour or adrenocortical responses when the flocks were presented with the box or dog. The results do not support the notion that the UWA flocks have been selected for differences in a consistent predisposition to react fearfully.

The adrenocortical responses measured in these studies were only moderate in magnitude and duration, with peak plasma cortisol concentrations 2-3 times higher than pre-treatment values, and all concentrations returning to pre-treatment levels within one hour of the start of treatment. If these observations are confirmed in practical situations, the presence of humans and dogs during routine handling should cause little concern on the basis of animal welfare. However, limiting the presence of dogs in certain situations (e.g. before slaughter) may reduce stress in domestic sheep.

Significant methodological developments in this research include the use of multivariate statistical techniques to analyze arena behaviour, the concurrent measurement of adrenocortical and behavioural responses in the arena and Y maze tests, and the explicit testing of the effects of individual lateral biases on choice behaviour in a Y maze test. Future studies should measure sheep stress responses to the presence of humans and dogs in practical situations.

[1] 4 year degree from the University of Victoria, British Columbia, Canada.

Declaration

This is to certify that I have pursued this study in accordance with the requirements of Massey University's regulations including:

i. The research carried out for my Doctoral thesis has been used in whole or in part for this qualification only.

ii. The research is my original work, except as indicated by appropriate attribution in the text and/or acknowledgements. I claim full responsibility for the primary role in developing the original scientific ideas and experimental designs, collecting and interpreting the data, and writing all scientific documents associated with the research outlined in this thesis, with guidance from my academic supervisors.

iii. The text, excluding appendices and bibliography does not exceed 100,000 words.

iv. All ethical requirements relating to the research have been met as required by Massey University, and/or other organizations and committees, and under the relevant legislation.

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Publications

Publications related to thesis research

Beausoleil, N.J., Stafford, K.J., Mellor, D.J. 2005. Sheep show more aversion to a dog than to a human in an arena test. *Applied Animal Behaviour Science, 91:* 219-232 (**Appendix 1**).

Beausoleil, N.J., Stafford, K.J., Mellor, D.J. Does direct human eye contact function as a warning cue for domestic sheep, *Ovis aries? Journal of Comparative Psychology, 120 (3): 269-279* (**Appendix 2**).

Beausoleil, N.J. Stafford, K.J., Mellor, D.J. 2004. Can we use change in core body temperature to evaluate stress in sheep? *Proceedings of the New Zealand Society of Animal Production, 64:* 72-76 (**Appendix 3**).

Other publications completed in parallel with thesis research

Beausoleil, N.J., Mellor, D.J., Stafford, K.J. 2004. Methods for marking New Zealand wildlife: amphibians, reptiles and marine mammals. Department of Conservation, Wellington, New Zealand 147p. ISBN 0-478-22631-4 (**Appendix 4**).

Mellor, D.J., Beausoleil, N.J., Stafford, K.J. 2004. Marking amphibians, reptiles and marine mammals: Animal welfare, practicalities and public perceptions in New Zealand. *Miscellaneous Publication,* Department of Conservation, Wellington, New Zealand 55p. ISBN 0-478-22563-6 (**Appendix 5**).

Table of Contents

List of Tables

List of Figures

List of Equations

CHAPTER 1

General Introduction

The aim of this thesis was to examine some behavioural and physiological responses of domestic sheep, *Ovis aries*, to the presence of humans or dogs. This introductory chapter is brief, as each of the following experimental chapters begins with a comprehensive introduction focussed on the specific experimental topic to be addressed. Likewise, each experimental chapter contains a detailed discussion of the results achieved, including the relationships to existing knowledge of the subject, limitations of interpretation based on the experimental methodology employed, implications of the results in terms of animal welfare and management, and proposals for future study. Accordingly, Chapter 8, the general discussion, is relatively brief, primarily integrating the results of the different experimental studies to draw overall conclusions about the relationships between domestic sheep, humans and dogs. A list of the relevant references is provided at the end of each chapter.

1.1 Background

Much research has been carried out to address the physical needs of production animals. However less emphasis has been placed on the emotional, social and behavioural effects of common production practices on livestock (Rushen, 2000). Groups of sheep are routinely mustered and yarded as part of management procedures. In New Zealand, commercial flocks of sheep are large, necessitating the use of dogs to move them efficiently. This system relies on the flight response of sheep to the dog, together with avoidance of the human handlers (Lynch et al., 1992).

The fact that domestic sheep move away from dogs is what makes the dog such an effective management tool. However little is known about the relationship between the two species, except that it is typically defined as a predator-prey association. Although many studies have used the presence of a dog or dog-related cues (e.g. barking) to elicit a physiological stress response in sheep (Harlow et al., 1987; Engler et al., 1988; Baldock and Sibly, 1990; Canny et al., 1990; Kilgour and Szantar-Coddington, 1997; Komesaroff et al., 1998; Cook, 2004), few have explicitly addressed the behavioural and physiological responses of sheep to the presence of dogs (MacArthur et al., 1979; Torres-Hernandez and Hohenboken, 1979; Kendrick et al., 1995).

The nature of the relationship between humans and domestic sheep is also uncertain. Some researchers postulate that humans are viewed as dominant conspecifics, while others argue that humans are recognized as predators (Rushen et al., 1999). Domestication has, by definition, reduced the fear of humans in domestic species (Price, 1984; Zohary, 1998; Price, 1999). However, domestic sheep still show behavioural and physiological responses indicative of fear, stress or aversion to the presence of humans (e.g. Pearson and Mellor, 1976; Harlow et al., 1987; Baldock and Sibly, 1990; Romeyer and Bouissou, 1992; Bouissou and Vandenheede, 1995; Goddard et al., 2000; Hansen et al., 2001; Erhard, 2003; Vierin and Bouissou, 2003).

Both humans and dogs are an integral part of sheep production systems, however, little is known about their relative aversiveness to sheep. Based on neurophysiological and behavioural evidence, Kendrick and Baldwin (1987) suggested that dogs and humans are recognized by sheep with similar emotional significance. However, Rushen (1990) stated, with respect to sheep that, 'whether humans or dogs cause more distress has not been shown', and there has been little evidence of progress in this field since this statement was made.

1.2   Thesis Rationale

Whether humans or dogs are more aversive to domestic sheep, or indeed, whether either causes significant stress at all, is an important issue in several respects. Firstly, stress and fear in production animals can cause economic loss by negatively impacting on productivity, immune functioning, reproduction and product quality (e.g. Sefton and Crober, 1976; Hutson, 1980; Craig et al., 1983; Mills and Faure, 1990; Barnett et al., 1992; Jones, 1996; Grandin, 1997; Jones, 1997; Jones et al., 1997; Voisinet, 1997; Hemsworth and Coleman, 1998; Murphy, 1999; Hemsworth, 2000; Geesink et al., 2001; Ruiz-de-la-torre et al., 2001). In addition, highly stressed or fearful animals are injured more frequently during handling, and are often more difficult to move through handling facilities, increasing the time required to process them (Hutson, 1980; Grandin, 1997).

Secondly, stress can have deleterious effects on the welfare of individual animals (Broom and Johnson, 1993; Moberg, 2000), and animal producers have a responsibility

4

to minimize animal welfare compromise, both for ethical and economic reasons. As well as being a concern in its own right, animal welfare compromise may limit access to international product markets. Some consumers are concerned about the processes involved in animal production, and the ethical treatment of production animals may be an important factor influencing their purchasing behaviour (Warris, 1995). Therefore, consumer preferences based on concerns about animal welfare have the potential to influence animal management practices, regardless of whether these perceptions are accurate or not (McInerney, 1997; 2004).

Understanding the welfare of production animals depends on a knowledge of the perceptions, and behavioural and physiological responses of these animals to common management procedures. By measuring changes in behaviour and physiology, we may be able to draw conclusions about the relative stress experienced by domestic sheep due to the presence of humans or dogs, and compare such stress levels to those elicited by other common sheep husbandry practices.

1.3 Measurement of animal stress and emotion

Negative emotional states relevant to the study of animal stress and welfare, such as fear or aversion, can only be inferred from observable and measurable parameters (Boissy, 1995). Animal stress has been measured using a variety of endocrine, behavioural, autonomic nervous system and immunological endpoints (Moberg, 2000). Each method has advantages and limitations, both in the assessment of animal stress and for making inferences about animal welfare (Broom and Johnson, 1993; Moberg, 2000).

Behavioural indices of stress are readily detectable in the field, and are often believed to be most closely related to the welfare of the animal (Fell and Shutt, 1989; Wemelsfelder and Farish, 2004). Behaviour is considered by some to be the physical manifestation of an animal's integrated physiological response to its environment (Clotfelter et al., 2004), and as such, behavioural responses may reflect internal motivational states or emotions (Paul et al., 2005).

However, others believe that behavioural responses are not so much an expression of an emotional state, as specific strategies to deal with particular problems, such as avoiding

5

predators or finding conspecifics (Rushen, 1990). Depending on the specific characteristics of the real or perceived threat, behavioural responses can vary greatly, while the underlying emotional state may be the same e.g. fear (Boissy, 1998). Because behavioural responses tend to be specific to the context in which they occur, it is unlikely that there are 'general' behavioural indicators of stress or fear. Therefore, the use of individual behaviours as indicators to judge the severity or intensity of different stressors may be problematic (Rushen, 2000).

While individual behaviours may be difficult to interpret, multivariate statistical techniques can reveal patterns of behaviour which may reflect underlying motivational states, such as fear, exploration or sociality (Maier et al., 1988; Markel et al., 1989; De Passille et al., 1995; Vierin and Bouissou, 2003). In addition, behaviours which occur consistently in a variety of contexts that appear to have the same affective valence (e.g. are all pleasant), and which do not appear in oppositely valenced situations are likely to be more reliable indicators of emotion (Paul et al., 2005). However, many behavioural responses consist of changes in the frequency, intensity or quality of certain behaviours, rather than just their presence or absence, making interpretation more difficult. Nevertheless, there are behavioural tests which minimize the degree of interpretation required (e.g. aversion learning techniques), and measurement of behavioural responses has contributed significantly to the study of fear, aversion and stress in domestic sheep (e.g. Cockram, 2004; Dwyer, 2004; Dwyer and Bornett, 2004; Wemelsfelder and Farish, 2004).

Because of the potential problems associated with interpretation of behavioural responses to challenging situations, wherever possible observation of behaviour should be complemented by the measurement of physiological stress indices, such as those representing hypothalamic-pituitary-adrenal (HPA) activity (Broom and Johnson, 1993; Ramos and Mormede, 1998). Briefly, stress-induced activation of the HPA axis, as indicated by plasma corticosteroid concentrations, reflects the individual's capacity to cope with environmental challenge (Carere et al., 2003). As such, relative plasma cortisol responses to fear-eliciting stimulation may reflect the intensity of the internal emotional state associated with the stressful experience, e.g. fear (Ramos and Mormede, 1998; Mellor et al., 2000; Paul et al., 2005). The non-specificity of HPA responses adds credibility to its use to assess subjective experiences associated with stressful situations,

6

as emotional state may be the variable common to a range of the different challenges, while behavioural responses may be more context-specific (Mellor et al., 2000).

1.4 Thesis methodology

More integrated studies are required to bring together physiological measures, direct observation of behaviour, and aversion learning techniques in the study of animal stress and welfare (Rushen, 1990). Therefore, the studies constituting this thesis are based on direct measurement of behaviour, together with concurrent measurement of a physiological stress indicator, as well as the use of a Y maze preference test, an aversion learning technique. Together, these studies have the overall aim of elucidating the potential for the presence of humans and dogs to cause stress in domestic sheep.

Each experiment was designed to allow assessment of the validity of the behavioural methodology for investigating the effects of fear-eliciting stimuli on sheep behaviour. Once satisfied of the suitability of the test for its intended purpose, it was used to evaluate differences in behavioural responses between treatments of unknown relative aversiveness e.g. human versus dog. Existing methodologies for testing fear in sheep have been modified as was considered to be appropriate, and in one particular case, I have identified, quantified and explicitly tested for the effects of a potentially confounding factor in a commonly used experimental procedure.

In Chapter 2, I tested whether a modified arena test could detect differences in the behavioural responses of sheep to stimuli differing greatly in aversiveness e.g. cardboard box versus dog. The arena test creates approach/avoidance motivational conflict in the test sheep by presenting a stimulus between the individual test animal and a group of flockmates. The standard arena test has been used to compare the behavioral responses of different groups of animals to the same stimulus (e.g. Fell and Shutt, 1989; Fell et al., 1991; Chapman et al., 1994; Kilgour and Szantar-Coddington, 1995; Wynn et al., 1995; Adams and Fell, 1997; Kilgour and Szantar-Coddington, 1997; Behrendt, 1998; Degabriele and Fell, 2001; Erhard and Rhind, 2004). At the outset of this work, it was believed that the arena test methodology could be modified to detect differences in stimulus aversiveness, and this view was subsequently supported by the results of Erhard (2003).

7

In Chapter 2, a multivariate statistical technique (canonical discriminant analysis) was used to identify patterns of behaviour relating directly to the presence of a predator or a non-predator in the arena test. This information was then used to compare the relative aversion of sheep to the presence of a dog or a human.

In Chapter 3, those behaviours identified in Chapter 2 were used to determine whether the arena test was sensitive enough to detect differences in behavioural responses to stimuli very similar in aversiveness. In order to vary the aversiveness of the stimulus only slightly, the same human was presented, either making direct eye contact with the sheep, or averting his eyes. Once again, multivariate statistical analysis was used to identify additional patterns of behaviour which differed in frequency in response to stimuli similar in aversiveness.

The major modification to the standard arena test was the use of the multivariate statistical technique which allowed discrimination of stimulus-related reactions, based on the integrated behavioural response, rather than by collating differences in individual behaviours. To my knowledge, multivariate techniques have not previously been applied to the analysis of livestock behaviour measured in an arena test, although similar procedures have been used to analyze behaviour and motivational states in open field and other behavioural tests (e.g. De Passille et al., 1995; Vandenheede et al., 1998; Vierin and Bouissou, 2003; Boissy et al., 2005).

Previous studies have shown that the behavioural and physiological fear responses of sheep to the presence of a human or dog are modulated by factors such as age, sex, breed/genotype and previous experience (Torres-Hernandez and Hohenboken, 1979; Mateo, 1991; Romeyer and Bouissou, 1992; Vandenheede and Bouissou, 1993; Kilgour and Szantar-Coddington, 1997; Lankin, 1997; Hall et al., 1998; Goddard et al., 2000; Hansen et al., 2001). In Chapters 4 and 5 of this thesis, the arena test was revisited to examine whether selection for differences in a heritable factor labelled 'temperament' led to consistent differences in behaviour and in adrenocortical responses in a range of contexts. The same stimuli were presented as were used in the first set of arena tests: box, human, dog (Chapter 2).

'Temperament' has been variously defined and described in the applied ethology literature, but is generally used to refer to the responses of animals to events or situations considered likely to elicit fear (Boissy and Bouissou, 1995; Grandin and Deesing, 1998; Erhard et al., 2004). Sheep at the University of Western Australia (UWA) have been differentially selected based on a factor labelled 'temperament' for at least 15 years. In this case, 'temperament' was defined according to the behavioural responses of the sheep to social isolation, and to the presence of a human in an arena test. Selective breeding has resulted in the creation of two behaviourally distinct flocks: a More Active flock (MA), which responded to the selection environments with more active behavioural responses, and a Less Active flock (LA), selected for less active responses (Murphy et al., 1994; Murphy, 1999). Previous researchers contended that selection had been based on differences in fearfulness or emotional reactivity.

In Chapter 4, I compared the behavioural and adrenocortical responses of the two UWA flocks during and after an arena test that included the presence of a human. The aim was to confirm, and expand upon, the reported inter-flock differences in behavioural response to a human, and to test, using a physiological stress indicator, the assumption that the More Active sheep experienced more fear than the Less Active sheep in the presence of a human in the arena test.

Once again, a multivariate technique was used to analyze behavioural responses in the arena test. In this case, factor analysis was used to identify potential motivational states underlying specific patterns of sheep behaviour e.g. fear of the stimulus. The flocks were then compared in terms of their relative expression of each behavioural pattern. Prior to this study, neither multivariate analysis of behaviour, nor measurement of physiological indicators of stress had been employed to validate the use of the behaviours included in the 'temperament' index to measure individual variation in fearfulness or emotional reactivity in the UWA sheep.

In Chapter 5, I compared the behavioural and adrenocortical responses of the UWA flocks to two stimuli which were not part of the selection environment. Presentation of the biologically insignificant control object (box) was used to examine inter-flock differences in response to the arena testing procedure itself, while presentation of a predator (dog) allowed evaluation of the effect of a stimulus with high biological

significance to sheep (Cook, 2004). The responses to the human, presented in Chapter 4, were also included for comparison.

The aim of this study was to determine whether inter-flock differences in behaviour and adrenocortical responses were consistently expressed in a range of potentially fear-eliciting situations, or whether such differences were specific to the selection environment (e.g. human presence). The impetus for this investigation was the idea that it is possible to simultaneously select for lower fear of humans and high levels of anti-predator behaviour in domestic livestock species (Lasater, 1972; Grandin and Deesing, 1998). The results would also provide further insight into the nature of the factor 'temperament' on which selection of the UWA flocks has been based.

Previous studies have used the arena test to compare the behavioural responses of groups of sheep differing in genotype/ breed or mothering ability to a single stimulus or situation (Murphy et al., 1994; Kilgour and Szantar-Coddington, 1995; 1997), but the study described in Chapter 5 is one of the first to examine whether such differences are consistently expressed by sheep in the presence of different stimuli in the arena test.

Using the same stimuli in the UWA arena tests (Chapters 4 and 5) as were used in the first set of arena tests (Chapters 2 and 3) allowed comparison of the effects of human and dog presence on the behavioural and physiological responses of sheep of different breeds (Merino versus Romney) and from different production systems (Australian wool versus New Zealand meat). It also allowed further validation of the use of the modified arena test for discriminating behavioural responses elicited by different stimuli.

In the final series of experiments, described in Chapters 6 and 7, a Y maze preference test was used to simply 'ask' individual sheep about their preferences between aversive stimuli, thereby minimizing the difficulties associated with interpreting behavioural responses in terms of fear or aversion of the stimulus. Choice behaviour in preference tests provides a common behavioural response which can be used to rank the subjects' relative aversion to different situations, allowing widely differing treatments to be compared on a single scale (Rushen, 1986; 1990; 1996). The Y maze test is an example of an aversion learning technique; to avoid exposure to a stimulus or treatment

10

considered aversive, individual animals must learn the association between that treatment and the side of the maze in which it is presented (Rushen, 1996). This methodology was used to measure the preferences of individual sheep between two stimuli commonly used to move sheep on farms and in the pre-slaughter environment: a barking dog or a human with a rattle.

The Y maze methodology was modified by concurrently measuring the plasma cortisol responses of the sheep to the two stimuli presented within the context of the Y maze testing procedure. Once again, the concurrent measurement of a physiological indicator of stress aided the interpretation of preferences expressed in the Y maze, and gave an indication of the aversion of sheep to the presence of these stimuli relative to other commonly used management procedures (Chapter 6).

This is also the first study in which individual lateral biases (or side preferences) expressed by sheep in a Y maze have been systematically characterized, and the potential effects of such biases on choice behaviour and the expression of stimulus preferences explicitly tested. The results of Chapter 7 demonstrate the importance of this development for the accurate interpretation of preferences expressed by individual animals in the Y maze and other preference tests.

A general discussion of the experimental results of this thesis is presented in Chapter 8. The conclusions drawn from this research about the relative aversiveness of humans and dogs to domestic sheep are more robust owing to the use of several different methodologies to address variations on the same theme. In addition, the use of an aversion learning methodology, which minimizes the necessary interpretation of behaviour, in conjunction with measurement of a physiological indicator of stress, allowed evaluation of previous and my own interpretations of sheep behaviour expressed in arena tests.

The measurement of plasma cortisol responses to humans and dogs in two very different experimental procedures also meant that the conclusions drawn about the aversiveness of each stimulus, independent of the testing procedure itself, were more reliable. However, it is acknowledged that differences in methodology, the prior experiences of the experimental groups of sheep with humans and dogs, and other factors may have

11

influenced the behavioural and physiological responses of the test sheep, and such comparisons have therefore been made with caution.

Chapter 8 also discusses some measures that could be implemented to improve the experimental protocols used in this thesis. Finally, I outline future experimental work which could contribute to the understanding of the perceptions, responses and potential stress of domestic sheep in the presence of humans or dogs.

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17

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CHAPTER 2

Sheep show more aversion to a dog than to a

human in an arena test

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2.1 Abstract

An arena test designed to create approach/avoidance motivational conflict in sheep has been shown to detect differences in stimulus aversiveness. Approach/avoidance conflict is created by presenting a stimulus between the individual test sheep and a group of companions. In this study, an arena test was used to assess differences in the aversiveness of a Box, Goat, Human and Dog. In addition, differences in behaviours expressed in the arena test were used to speculate on the aversiveness of the Human, relative to the Dog and Goat. The behavioural responses of individual sheep were measured during a 10-minute exposure to one of the stimuli, and differences between treatment groups were analyzed using a Kruskal-Wallis test on ranked data ($P < 0.01$). An integrated index of fear-related behaviour was also calculated for each animal (Fearfulness Score). When the Dog was present, test sheep stayed furthest from the group sheep, were highly vigilant and explored very little. The distance maintained from the group sheep was intermediate and similar with the Goat and Human, as were vigilance and exploration. However, while sheep often sniffed the Goat, they rarely sniffed the Human. The Fearfulness Scores indicate that the Human elicited more fear-related behaviour than the Goat ($P < 0.001$). The results of this experiment suggest that the arena test is an appropriate tool to test the relative aversiveness of different stimuli for individual sheep, that the Dog was the most aversive stimulus presented, and that the Human may have been slightly more aversive than the Goat.

2.2 Introduction

The behavioural responses of domestic sheep to dogs (Torres-Hernandez and Hohenboken, 1979; Hansen et al., 2001), humans and other stimuli or situations have been tested and interpreted in terms of fear (Romeyer and Bouissou, 1992; Vandenheede and Bouissou, 1993; Bouissou and Vandenheede, 1995; Vandenheede and Bouissou, 1996; Vandenheede et al., 1998; Murphy, 1999; Erhard, 2003). However, the relative aversion of sheep to humans and dogs, expressed behaviourally, has not yet been compared in a single experiment.

21

The arena test is a combination of motivational choice test and open field type test, and appears to be a sensitive index of changes in complex behaviour (Gray, 1971; Fell et al., 1991). In such a test, individual sheep exhibit behaviours that reflect competing motivations to join a group of conspecifics, and to avoid a stimulus presented between the test sheep and its companions. The arena test measures unconditioned behavioural responses, and such tests are generally used to measure negatively valenced states such as fear or aversion.

Arena tests have been used to compare the behavioural responses of different groups of animals to the same stimulus, and sheep were originally tested in groups of 3-5 animals. However, Kilgour and Szantar-Coddington (1997) found that a single-animal test may be more appropriate for detecting differences in behavioural response between groups of sheep. The standard and single-animal methodologies have been used to detect differences in sheep behaviour between groups differing with respect to surgical and hormonal treatments, exposure to environmental pollutants, chronic stressors and parasite loads, and between different breeds, and groups selected for differences in characteristics such as mothering ability (e.g. Fell and Shutt, 1989; Fell et al., 1991; Chapman et al., 1994; Kilgour and Szantar-Coddington, 1995; Wynn et al., 1995; Adams and Fell, 1997; Kilgour and Szantar-Coddington, 1997; Behrendt, 1998; Erhard and Rhind, 2004).

The arena test methodology was modified in order to detect differences in stimulus aversiveness. The distance maintained from a stimulus presented in the arena test is taken as an integrated reflection of the aversion elicited by that particular stimulus (Fell and Shutt, 1989; Erhard, 2003). The behavioural response measured (distance) is the same, regardless of the stimulus, which enables the comparison of different stimuli to be made on one scale. Therefore, such a test can be used to compare the effects of different stimuli on individual sheep, and has been shown to detect differences in stimulus aversiveness (Erhard, 2003).

The objectives of this experiment were to determine whether the arena test could detect differences in aversiveness between the different stimuli presented (Box, Goat, Human, Dog) and to determine, based on behaviours related to aversion, whether sheep found the Human less aversive than the Dog. The hypotheses to be tested were that individual

sheep would stay further away from the group sheep when a live stimulus (Goat, Human, Dog) was present than when a biologically insignificant novel object (Cardboard Box) was present, and that they would stay further away from the group sheep when the Dog was present than when the Goat was present. In addition, it was hypothesized that differences in other behaviours would be observable when different stimuli were presented. Finally, it was hypothesized that observed differences in distance from the group sheep and other behaviours would indicate that the Human was less aversive than the Dog, but more aversive than the Goat.

2.3 Methods and Materials

Animals

80 Romney hoggets (16 months old) were used in this study. The hoggets had been raised together since weaning on a Massey University farm in New Zealand. These sheep would have had some regular contact with the farmer and his dogs for most of their lives e.g. moved between paddocks, shearing, drenching. However, they would have had little experience with goats, and then only visual contact. Ten East Friesen hoggets, kept with the Romneys since weaning, were used as Group sheep. The East Friesens were used so that all test sheep were presented with 'strange conspecifics', as sheep from within a mixed mob prefer to associate with others of their own breed (Arnold and Pahl, 1974). This minimized the potentially confounding factor of personal associations between test and group sheep altering their motivation to join the group sheep. Sheep were randomly assigned to a test day and treatment, based on presentation order in the race. Extra sheep were assigned to each test group to keep the first and last test animals from being isolated in the post-test and pre-test pens, respectively. The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (protocol 01/84).

Experimental setup

Behavioural responses to the four stimuli were tested using a modified arena test (Fell and Shutt, 1989; Kilgour and Szantar-Coddington, 1995). All tests took place in November 2001 and were conducted between one and three pm. The arena was constructed in a shed with an open side facing southeast. The arena measured 8m x

23

3.5m and a grid of squares (approximately 1m x 1m) was painted on the slatted wooden floor (Figure 2.1). Test sheep could be visually separated from the testing area, but were not outside the audible range of each other. The stimulus was placed in square 2 with a wooden cabinet unit (approximately 1m wide x 1m tall) behind it, to create a visual barrier between the stimulus and the group sheep. This was found to be necessary in a pilot trial, as the dog became agitated when it was unable to watch the group sheep behind it, and the test sheep in the arena. The cabinet unit was left in place for all trials.
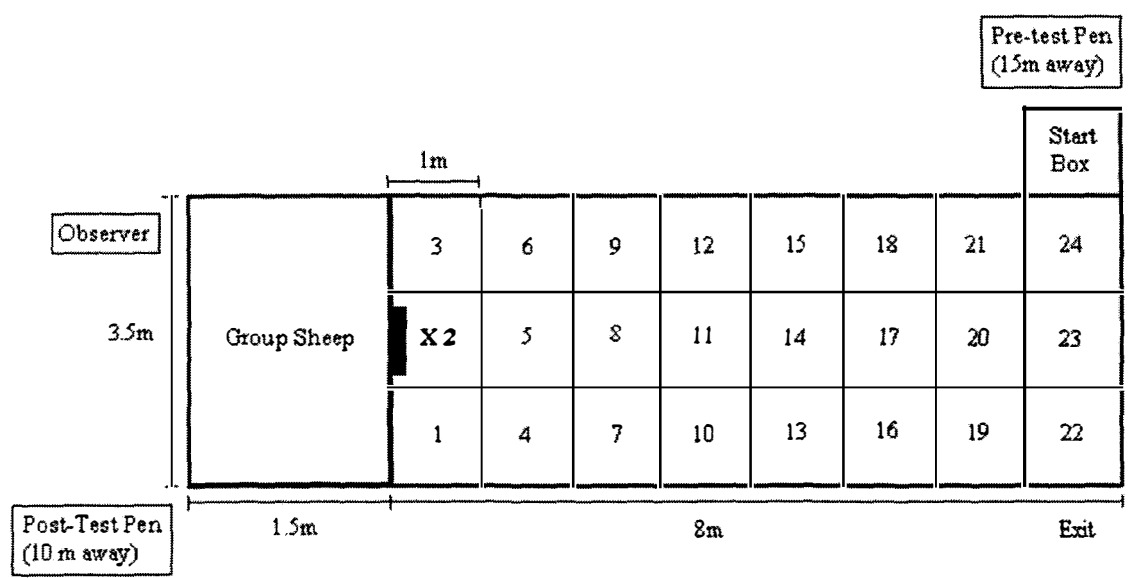
Figure 2.1 Test arena dimensions. X marks the position of the stimulus. The cabinet is represented as the black rectangle behind the stimulus in square 2. The sides of the arena were covered in shade cloth.

The four stimuli were:

    1. Cardboard box

    2. Goat

    3. Human

    4. Dog

The box control (plain cardboard, placed on end to stand about 0.5m tall) was used as a novel object with no biological significance to the sheep. All live stimuli were unfamiliar to the test sheep. The same human (male, wearing the same blue overalls each day) was used on all days, and remained standing immobile during the test, while

following the sheep with his eyes. This person was not involved in handling the sheep in any other way. The dog was a 6-year-old spayed female border collie experienced in working sheep. This type of dog was selected because of its confidence around sheep, and because a barking dog (e.g. huntaway breed) may have agitated the sheep waiting to be tested. The goat used was an adult female Angora goat with horns. The dog and goat were secured in place with a collar and short leash attached to the floor. Although the leash markedly limited movement, the goat was still seen to move outside square 2 into squares 1 and 3 on occasion. The dog generally stayed sitting in square 2.

Procedure

The sheep were familiarized with the yards, races and test arena before testing, in order to reduce the confounding effects of novelty on the behavioural responses of the test sheep. The whole mob was moved through the facility, and groups of about 20 animals were left in the arena for half an hour. This procedure was repeated twice in the week prior to testing. The goat and dog were also tied up in the arena for 15 minutes on two occasions in the week prior to testing to familiarize them with the facility; no sheep were present on these occasions.

On the day of testing, the test sheep were drafted, as a group, out of the mob. Five of the East Friesen sheep were placed into the Group Sheep pen (Figure 2.1), which was separated from the arena by a metal farm gate. Test sheep had visual (and limited physical) access to group sheep at all times during the test (never visually isolated). Each stimulus was presented twice on each afternoon, with the order of presentation being randomized for each day (8 sheep tested per day). Ten non-consecutive days of testing allowed 20 sheep to be observed in the arena with each stimulus. When the stimulus and observer were in place, an individual sheep was pushed gently up the race into the start gate of the arena by a handler. Once the sheep entered the arena, the observer began timing the 10-minute test period. When the test period was over, the sheep was moved by the handler towards the exit gate and into a post-test pen. The stimulus was replaced, and the next sheep was moved into the arena. Each sheep was tested once, being exposed to only one of the four stimuli.

Behaviour measurement

The observer sat in an adjacent room behind a window blocked with shade cloth, except for a small viewing slot. All behaviours were recorded on a tape recorder during the test period. In addition, every 15 seconds the square occupied by the sheep's left front foot was recorded. If the foot was on a grid line, the position of the right front foot was used, as this would best represent the position of the sheep's body. Table 2.1 presents the behaviours measured in the arena test, and when necessary, gives an explanation of how the behaviour was defined or measured in this experiment. In Table 2.2 (Results) the behaviours have been grouped according to their most likely function e.g. vigilance, exploration, or other, if their function was not clear.

Table 2.1 also shows 11 behaviours included in the synthesis of the Fearfulness Score, and their relationships to fear (Romeyer and Bouissou, 1992). Fearfulness Scores were calculated for each animal in this study by modification of the procedure of Vandenheede and Bouissou (1993). The Score was calculated by finding the mean of the ranks obtained by each animal for the 11 behaviours. For example, in a group of 80 sheep, the animal showing the highest number of escape attempts (sign of fear) is given a rank of 80, while the animal showing the lowest number is ranked 1 (less fear) for this behaviour. Conversely, an animal sniffing the stimulus many times (absence of fear) is given a rank of 1, while the animal sniffing the stimulus least is given a rank of 80 for this behaviour. These ranks were summed for each animal and the results divided by the number of behavioural items (11). The higher the score, the more fearful the behaviour the animal exhibited in this arena test.

Several behaviours (Vocalizations, Defecations) used in Fearfulness Scores in previous studies (Vandenheede and Bouissou, 1993; Bouissou and Vandenheede, 1995; Bouissou et al., 1996; Vandenheede and Bouissou, 1996; Vandenheede et al., 1998) were excluded in this study. This was done because of concerns that their relationships to fear probably vary according to the stimulus present, which could confound the index. Therefore, six behaviours previously included and five other derived behaviours were used to generate Fearfulness Scores.

Table 2.1 Parameters of sheep behaviour measured in response to stimuli presented in an arena test.

Parameter of Behaviour	Relationship to fear	Definition of behaviour
Mean distance to group sheep [ab]	+	The mean distance test sheep maintained from group sheep over 10 minutes. Calculated by multiplying the distance from the group pen by the proportion of time spent there
Immobilization frequency [a]	+	Sheep stood still with no movement of any body part (including ears) for three seconds or more
Trot frequency [a]	+	Sheep broke out of a walk in any direction. Trots into the arena at the beginning of the test were discounted, as these were related to the handler, not the stimulus
Escape attempts [a]	+	Any attempt to escape from the arena, including jumping or rearing against the walls
Baulks [ab]	+	A sharp, sudden movement while remaining with all feet in the same spot ('flinch' from Romeyer and Bouissou, 1992)
Glances at stimulus [a]	+	Sheep looked towards the stimulus for less than three seconds before re-directing its gaze
Stares at stimulus [ab]	+	Sheep maintained its gaze at the stimulus for 3 seconds or more
Sniff stimulus [a]	-	Sheep brought its nose very close to stimulus and sniffed
Latency to sniff stimulus [a]	+	The 15-second interval in which the sheep sniffed the stimulus for the first time
Sniff in Square 2 [ab]	-	Sheep sniffed the ground in square 2 where the stimulus was located
Sniff cabinet (in square 2) [ab]	-	Sheep sniffed the cabinet located in square 2 behind the stimulus
Bleats	?	Only bleats that could be heard by the observer in the next room were recorded (high-pitched bleats)
Foot stamps	?	A front foot (or feet) was brought up off the ground in a controlled manner, and brought down with force to produce a sound on the ground
Lip licks	?	Sheep's tongue came out of its mouth in a licking motion. Each lick was counted as a separate event
Departures	?	The sheep turned its body to face away from the stimulus and moved away
Minimum distance to group sheep	+	Minimum distance between test sheep and group sheep during 10 minutes
Glances at group sheep	?	Glances directed towards group sheep
Sniff ground	?	
Sniff side of arena	?	
Urinations	?	
Advances	?	Forward movement in the direction of the stimulus
Sniff group sheep	?	
Latency to bleat	?	The 15-second interval in which the sheep bleated for the first time
Defecations	?	
Chew side of arena	?	
Duration chewing cud	?	Duration spent chewing cud in 10 minutes
Duration lying down	?	Duration spent lying down in 10 minutes
Duration spent in start gate	?	Duration spent in the start gate (out of sight of the stimulus) in 10 minutes

+ presence of fear for a high value of the parameter; – absence of fear for a high value of the parameter

[a] behaviour included in synthesis of Fearfulness Score

[b] derived behaviours; not explicitly related to fear by Romeyer and Bouissou, 1992.

Statistical analysis

None of the behavioural data were normally distributed, and they could not be transformed to approximate normality. Therefore, mean and minimum distance maintained from the group sheep, and the frequencies and latencies of other behavioural items and Fearfulness Scores were compared between the four treatments using the non-parametric Kruskal-Wallis test (one-way ANOVA of ranked data) followed by Bonferroni tests at levels of significance 0.01 and 0.001 (Conover and Iman, 1981). In addition, a canonical discriminant analysis on the raw data was performed, to determine which behaviours explained the variation between treatment groups (all behaviours in Table 2.1 included). All statistical tests were performed using SAS Version 8.0 (SAS Institute Inc., 1994).

2.4 Results

The expression of 17 behaviours measured in the arena test differed significantly between at least 2 treatment groups (Table 2.2).

Box

Individual sheep came and stayed closest to the group sheep (mean and minimum distance) when the Box was present. They exhibited little vigilance behaviour (glances, stares at stimulus, glances at group sheep) but a lot of exploratory behaviour, both toward the stimulus itself, and the arena in general (sniff ground, side, group sheep). Sheep never foot stamped in the presence of the Box, and vocalized very little.

Goat and Human

Individual sheep maintained an intermediate distance from the group sheep when the Goat or Human was present. They were more vigilant (glances, stares at stimulus), but explored the general arena as much as sheep presented with the Box (sniff ground, side). However, exploration of the arena near the stimulus (square 2 and cabinet) was inhibited by the presence of the Goat or Human. The Goat itself was sniffed as often as the Box; however, the Human was sniffed less often than the Box or Goat. Sheep vocalized more frequently when the Goat was present than with the Box.

28

Table 2.2 Mean frequency or duration (± SEM) of sheep behaviours measured in the Arena Test (raw data). Only behaviours found to be significantly different between at least two treatments using a Kruskal Wallis test on ranked data are presented. Treatments with different letters are significantly different at P < 0.01.

Behaviour	Box	Goat	Human	Dog	χ^2	P
			Treatment			
Distance from Stimulus						
Mean distance from group sheep (m)	2.19 ± 0.18^a	3.62 ± 0.29^b	3.96 ± 0.38^b	6.18 ± 0.39^c	36.71	*<0.0001*
Minimum distance from group sheep (m)	1.0 ± 0.00^a	1.17 ± 0.12^a	1.7 ± 0.22^a	4.1 ± 0.35^b	45.04	*<0.0001*
Vigilance Behaviours						
Glances at Stimulus	5.60 ± 1.10^a	30.72 ± 2.22^b	37.45 ± 5.06^b	52.45 ± 3.82^c	51.38	*<0.0001*
Stares at Stimulus	0.00^a	2.89 ± 0.62^b	3.4 ± 0.57^b	15.05 ± 1.94^c	59.13	*<0.0001*
Glances at Group Sheep	25.05 ± 2.21^a	31.17 ± 3.28^{ab}	40.55 ± 3.95^b	46.10 ± 3.79^b	20.57	*<0.0001*
Exploratory Behaviours						
Sniffs Stimulus	3.35 ± 0.50^a	3.28 ± 0.79^a	0.20 ± 0.14^b	0.00^b	49.79	*<0.0001*
Sniffs Square 2	1.70 ± 0.38^a	0.00^b	0.05 ± 0.05^b	0.00^b	48.77	*<0.0001*
Sniffs Cabinet in Square 2	3.05 ± 0.49^a	1.39 ± 0.75^b	0.30 ± 0.18^b	0.05 ± 0.05^b	44.36	*<0.0001*
Sniffs Group Sheep	3.10 ± 0.64^a	1.56 ± 0.43^{ab}	1.10 ± 0.35^{bc}	0.20 ± 0.16^c	26.80	*<0.0001*
Sniffs Ground	9.85 ± 1.21^a	5.72 ± 0.74^a	7.10 ± 0.93^a	1.10 ± 0.36^b	38.59	*<0.0001*
Sniffs Side	6.30 ± 0.87^a	3.89 ± 0.71^{ab}	4.80 ± 0.66^{ab}	2.20 ± 0.57^b	15.42	*0.002*
Latency to Sniff Stimulus (mins)	2.24 ± 0.77^a	3.38 ± 0.92^a	9.55 ± 0.32^b	10.0 ± 0.00^b	48.71	*<0.0001*
Other Behaviours						
Stamps	0.00^a	0.06 ± 0.06^a	0.05 ± 0.05^a	5.90 ± 1.71^b	37.47	*<0.0001*
Bleats	2.25 ± 0.85^a	18.61 ± 4.28^b	7.45 ± 2.56^{ab}	4.00 ± 1.88^{ab}	13.25	*0.004*
Urinations	0.90 ± 0.14^{ab}	1.17 ± 0.20^a	1.30 ± 0.16^a	0.30 ± 0.11^b	21.49	*<0.0001*
Lip licks	2.10 ± 0.40^a	5.17 ± 0.85^{ab}	7.05 ± 1.65^b	7.80 ± 1.23^b	15.85	*0.001*
Departures	5.80 ± 0.55^a	7.00 ± 1.03^a	5.05 ± 0.87^{ab}	2.20 ± 0.30^b	23.19	*<0.0001*

Dog

Individual sheep stayed furthest from the group sheep when the Dog was present. They were more vigilant than with the other stimuli (glances, stares at stimulus), never sniffed the Dog itself, rarely explored the area near it (sniff square 2, cabinet, group sheep), and showed little general exploratory behaviour (sniff ground). Sheep foot stamped frequently, but rarely urinated or departed when the Dog was present.
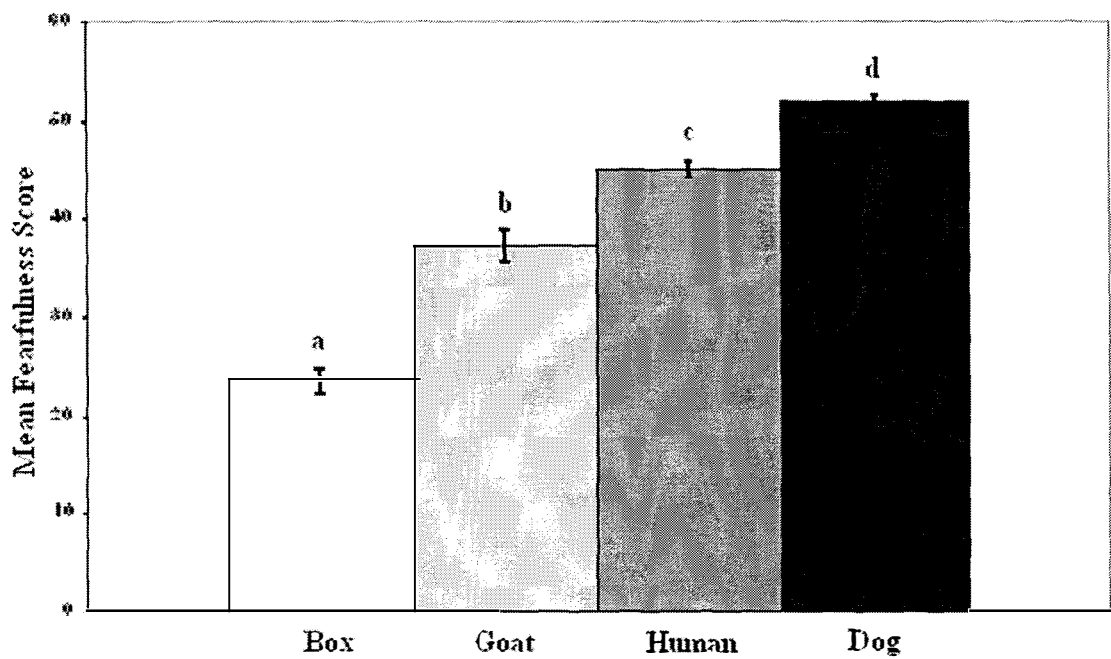
Figure 2.2 Mean Fearfulness Scores (± SEM) of sheep in the arena test. Bars with different letters are significantly different at P < 0.001.

The Fearfulness Scores were different for each treatment group, with Dog eliciting the highest scores, followed by Human, Goat and then Box (Figure 2.2). The canonical discriminant analysis (Figure 2.3, Table 2.3) showed that behaviours most important in separating the treatment groups were: mean distance maintained, glances and stares at the stimulus versus departures. Sheep presented with the Dog tended to stay further away, glance and stare more, and depart less than those with the other three stimuli.

Table 2.3 Results of canonical discriminant analysis. *A.* Squared distance between treatments, *B*. Behaviours important in explaining variation between treatment groups. Large positive loadings indicate that the behaviour has a large influence on the canonical discriminant score. Large negative loadings indicate that the behaviour has a large negative influence on the canonical discriminant score.

A. Squared distance between treatments

	Box	**Goat**	**Human**	**Dog**
Box	0	28.98	25.91	83.44
Goat		0	21.84	55.57
Human			0	43.41
Dog				0

B. Canonical Dimensions

	Dimension 1		**Dimension 2**	
Variation explained (%)	69		18	
	Behaviour	Loadings	Behaviour	Loadings
	Mean distance	1.43	Lying duration	0.97
	Stares at stimulus	0.89	Bleats	0.72
	Glances at stimulus	0.61	Mean distance	0.60
	Departures	-0.56	Duration in start gate	-0.71
			Sniff square 2	-0.70
			Chew side of arena	-0.70

The Human and Goat elicited intermediate responses, and the Box elicited more departures, shorter distances and less vigilance. This dimension could be interpreted as 'dog-specific responses' or perhaps 'response to a predator'. The second dimension separated the Goat from the other stimuli, with sheep lying down and bleating more, staying further away, sniffing square 2 less and spending less time in the start gate when the Goat was present. This dimension could be interpreted as 'goat-specific responses'.
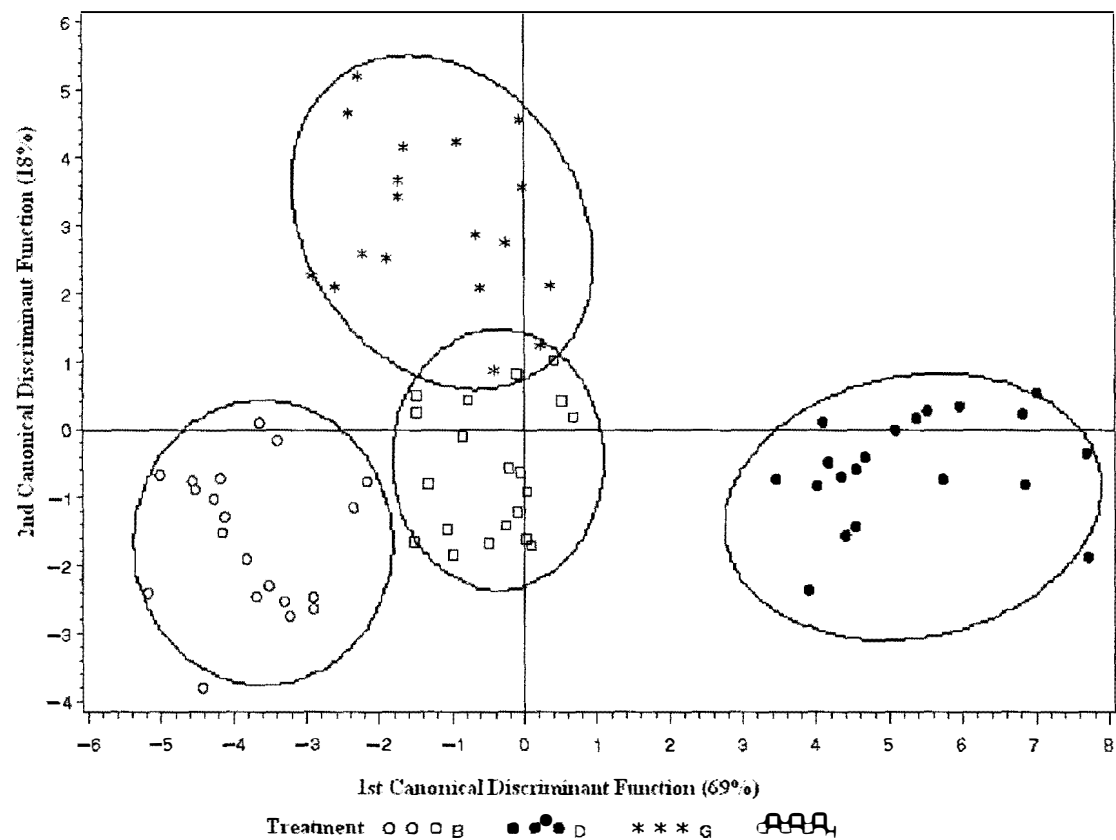
Figure 2.3 Canonical discriminant scores of individual sheep according to which stimulus was presented. Treatments: B = Box; D = Dog; G = Goat; H = Human.

2.5 Discussion

As anticipated in the first hypothesis, individual sheep did stay further away from the group sheep when a live stimulus was presented than with the less biologically significant novel object. This suggests that the distance maintained from the group sheep in the arena test can be taken to indicate the aversiveness of the stimulus to the sheep. Erhard (2003) also found that individual sheep stayed further away from their companions when a more aversive stimulus was presented (forward-facing human versus human facing away).

Sheep also stayed further away from the group sheep when the Dog was present than when the Goat was present, suggesting that they found the Dog more aversive. This result agrees with the traditional beliefs about the relationships between sheep and dogs, and sheep and goats. Domestic dogs are widely documented to be predators of sheep (e.g. Coll, 1922; Roy and Dorrance, 1976; Boggess et al., 1978; Robel et al., 1981; Schaefer et al., 1981). Experimentally, their presence has been shown to elicit physiological stress responses, including increases in heart rate (MacArthur et al., 1979; Harlow et al., 1987; Baldock and Sibly, 1990) and in plasma concentrations of cortisol, ACTH, adrenaline and noradrenaline (Harlow et al., 1987; Komesaroff et al., 1998). Goats are not predators and neurophysiological evidence suggests that they are recognized, along with conspecifics, as non-threatening by sheep (Kendrick and Baldwin, 1987).

It was also hypothesized that differences in other behaviours would be observed when different stimuli were presented. Differences in vigilance behaviours, exploration and other behaviours such as foot stamping, vocalization, urination, lip-licking and departures were observed. Individual sheep were highly vigilant and spent little time exploring the arena when the Dog was present. Baldock and Sibly (1990) found that sheep spent more time alert in the presence of both a dog and human, than with a human alone, and dogs have previously been reported to inhibit investigatory behaviour in sheep (Torres-Hernandez and Hohenboken, 1979). There is generally a trade off between exploratory and vigilance behaviour in prey animals. This is because these behaviours reflect competing motivations for resource acquisition and predator avoidance (Illius and FitzGibbon, 1994).

33

Sheep also glanced at the group sheep more often when the dog was present. This result agrees with evidence that Bonnet Macaques tend to look at nearby troop members when presented with leopard models, possibly to capitalize on their companions' assessment of any possible threat (Coss and Ramakrishnan, 2000). Griffin et al. (2000) speculated that social cues become more salient in a 'risky' environment.

Sheep foot stamped almost exclusively when the Dog was present, but its presence seemed to inhibit urination and departures. This pattern of behaviour may have evolved specifically in response to predators (Dwyer, 2004). For example, it may be dangerous to turn your back on a predator in order to walk away. Indeed, a sheep departing from a predator is reported to stimulate attack (Jansen, 1974; Connolly et al., 1976). Likewise, urination would provide olfactory cues to a potential predator in the vicinity.

While the presence of the human or dog elicited significantly more lip-licking than the presence of the box, the significance of this sheep behaviour in terms of fear or aversion is unknown. However, taken with the other results of this study, a higher frequency of lip-licking may reflect more fear or aversion in sheep in the arena.

Vocalization did not appear to be suppressed by the Dog or Human, as has been previously reported (Torres-Hernandez and Hohenboken, 1979; Romeyer and Bouissou, 1992). Rather, the Goat appeared to elicit more vocalization than the other stimuli. In addition, sheep sniffed the Goat more often than the Human or Dog. It is contended that the Goat was recognized as a strange conspecific, and the test sheep may have been contact calling. This idea is supported by the separation, along the second dimension of the canonical discriminant analysis, of the Goat from the three other stimuli, suggesting that the Goat was perceived in a different category. Neurophysiological evidence shows that the image of a horned goat stimulated the same cells involved in facial recognition as a horned sheep of a different breed to the test sheep, implying that goats may be recognized in a similar emotional category as strange conspecifics (Kendrick, 1991). It would have been interesting to present a 'Stranger Sheep' stimulus as well, to compare behavioural responses between a strange conspecific and the Goat.

It was hypothesized that observed differences in the distance maintained from the group sheep and other behaviours would indicate that the Human was less aversive than the

Dog, but more aversive than the Goat. Sheep presented with the Goat or Human came and stayed closer to the group sheep, were less vigilant and explored more, and showed less fear-related behaviour (Fearfulness Score) than when the Dog was present. Moreover, the distance maintained and almost all other behaviours were similar in response to the Goat and Human. These results suggest that the Human was less aversive than the Dog, and similarly aversive to the Goat. However, while sheep often sniffed the goat, they rarely sniffed the human. The integrated Fearfulness Scores also indicate that the sheep exhibited more fear-related behaviour in the presence of the Human than with the Goat, suggesting that the Human may have been more aversive than the Goat.

Whether dogs or humans cause more fear in sheep is not known, and the nature of the relationship between humans and sheep is uncertain (Rushen, 1990). Some postulate that humans are viewed as dominant conspecifics, while others argue that humans are seen as predators (Rushen et al., 1999). Based on neurophysiological and behavioural evidence, Kendrick and Baldwin (1987) suggested that dogs and humans are recognized with similar emotional significance. However, the results of this study suggest that this particular Dog was more aversive to individual sheep than the Human presented.

The use of live non-human animals in this experiment may be problematic, as inconsistencies in stimulus behaviour can influence the responses of the test sheep. For example, the Goat was somewhat aggressive towards test sheep, which may account for the fact that exploration of the area near the Goat was inhibited. In addition, while I could ask the human to maintain a non-threatening demeanour, I could not control the dog's behaviour, beyond keeping it in square 2. However, in order to determine the behavioural responses of sheep to a range of 'real' stimuli, the use of live animals was deemed most appropriate.

There may also be confounding effects of differences in experience with the stimuli on the behavioural responses of the sheep (Cockram, 2004). However, these test animals had been born on the same farm, and had run as one mob since weaning, and their experiences could be expected to be as consistent as any group of normal farm animals. These sheep were raised outdoors, with some regular contact with humans and dogs. Most of this contact would be considered aversive e.g. herding, yarding, docking,

drenching. However, this contact represents the normal experience of intensively managed sheep in New Zealand, the behavioural responses of which I wished to study in the arena test. Because experience with a specific human, dog or goat will influence a sheep's subsequent behavioural response to that stimulus, all stimuli used were unfamiliar to the test sheep. The sheep had had little, if any, experience with goats. Therefore, the goat may have been considered by the sheep to be somewhat novel, and subsequently, some of their behaviour towards the goat could have reflected this novelty e.g. increased vocalization.

2.6 Conclusions

The results of this experiment suggest that the arena test is an appropriate tool to test the relative aversiveness of different stimuli to individual sheep, as suggested by Erhard (2003). Distance from the group sheep, and levels of vigilance and exploratory behaviour differed between sheep presented with different stimuli. In addition, the Dog elicited a pattern of behaviours that may have specifically evolved in response to a predator. In this arena test, individual sheep found the Dog most aversive, and the Box least. The Goat and Human appeared similarly aversive, except that the sheep rarely sniffed the human. The integrated Fearfulness Score indicates that, overall, this Human elicited more fear-related behaviour than this Goat, suggesting that the Human may have been slightly more aversive. Future work should correlate physiological reactions to different stimuli with behavioural responses in the arena test.

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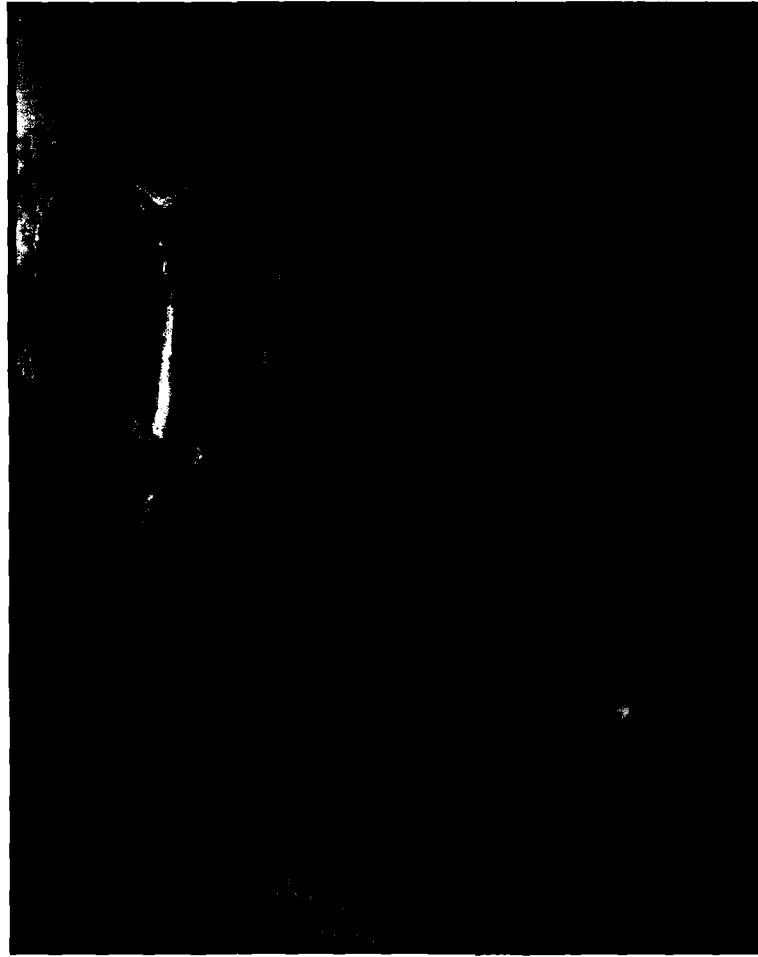
CHAPTER 3

Does direct human eye contact function as a

warning cue for domestic sheep?

Results are presented in the style of the journal, and some of the Methods and Materials section is similar to that of Chapter 2.

3.1 Abstract

Direct eye contact may function as a warning cue during interspecific interactions, and human staring has been shown to influence the behaviour of many species. An arena test was used to assess whether human staring altered the behaviour of domestic sheep compared with no human eye contact. Sheep glanced at the staring human's face more often in the first two minutes of the test, indicating that they perceived a difference between the human stimuli. Staring also elicited more locomotor activity and urination than averted gaze. However, there were no differences in fear-related behaviours, suggesting that a staring human did not represent a greater immediate threat than a non-watching human. These results imply that human staring is a warning cue for domestic sheep, but no more. Without further reinforcement, sheep quickly habituated to the warning cue.

3.2 Introduction

What an animal does with its eyes may predict its behaviour, and can therefore be used as a signal or cue by other animals (Hampton, 1994; Emery, 2000). Direct eye contact, or staring, may indicate that some kind of behavioural interaction is about to take place (Kendrick, 1991), and is therefore likely to evoke aggressive or evasive action. In social species (e.g. wolves, domestic dogs, primates), staring is often used as a threatening signal (Van Hooff, 1967; Mech, 1970; Fox, 1971; Bradshaw and Nott, 1995; Askew, 1996; Coss et al., 2002).

Direct eye contact may also have a warning function during interspecific interactions. Two forward-facing eyes are a salient and invariant cue to impending danger (Coss, 1979; Topal and Csanyi, 1994) and indicate to the observer it is being watched (Coss, 1991). A cue is defined as a feature that can be used by other animals as a guide to future action (Hasson, 1994). As such, a behaviour does not have to be intentionally directed towards the other species to function as an interspecific cue (McConnell and Baylis, 1985). For example, hunting predators often stare at their prey prior to attack (Hampton, 1994), primarily to focus their hunting effort on the potential target (Curio, 1993).

There is evidence that prey species have evolved to use the behaviour of potential predators as a cue to the appropriate behavioural response. This ability allows prey animals to avoid wasting time and energy responding every time a predator is present (Walther, 1969). Cues such as predator posture and orientation, including head and eye direction, are used by wild ungulates to judge the intention of potential predators (Murie, 1944; Walther, 1969; Mech, 1970; Van Lawick-Goodall and Van Lawick, 1971). Direct eye contact from a predator could evolve as a warning cue if it was detectable by prey animals, and detection was consistently associated with a decreased risk of predation (Coss et al., 2005).

Humans share a feature in common with most other mammalian predators: two forward-facing eyes (Walls, 1942; Hughes, 1977; Coss, 1978), and visual cues may be generalized from one 'predatory' species to another (Blumstein et al., 2000; Griffin et al., 2001). Therefore, it is likely that many wild species respond to humans as predators, and may use direct human eye contact as a warning cue. The effects of human eye gaze on behaviour have been demonstrated in iguanas (Burger and Gochfeld, 1990), snakes (Burghardt and Greene, 1988), gulls (Burger and Gochfeld, 1981), sparrows (Hampton, 1994), chickens (Gallup et al., 1972; Scaife, 1976), and primates (Exline, 1969; Kalin et al., 1991). Although little information on the responses of wild ungulates to human eye contact could be found, the fact that humans share common features with other ungulate predators may be sufficient to elicit similar behavioural responses to a staring human.

Sheep are highly vigilant social animals, that primarily use visual cues to detect predators (Geist, 1971; Dwyer, 2004). They are prey to a variety of carnivores, including pack hunters such as wolves that use body posture and eye contact to move the prey animal towards other group members (Crisler, 1956; Mech, 1970; Schaller, 1977). Therefore, predator staring could have evolved as a cue to impending predation for domestic sheep. Testament to this is the fact that sheep can be worked so effectively with 'eye' dogs that control the flight response of sheep using direct, intense eye contact (McConnell and Baylis, 1985; Lynch et al., 1992).

The process of domestication has produced animals that are much less responsive to humans. However, as a side-effect of domestication, some species have developed the

44

ability to use human communicative cues, including human visual behaviour (Kaminski et al., 2005). Whereas domestic dogs were able to make use of the orientation of human head or eyes to find hidden objects (Miklosi et al., 1998; McKinley and Sambrook, 2000), domestic goats (*Capra hircus*) were unable to use these cues alone (Kaminski et al., 2005). These results are not entirely surprising, as the opportunity to associate human eye gaze with positive events such as hidden food rewards may not have occurred during the domestication of ungulates. In contrast, it is relatively easy to imagine how human eye contact could have evolved as a cue to an impending aversive interaction for domestic animals such as goats and sheep. Most contact between man and sheep in modern management systems is aversive to the sheep e.g. shearing or vaccination.

There is some evidence that the presence and gaze direction of human eyes do influence the behavioural responses of sheep to humans (Kendrick et al., 1995). Kendrick (1991) found that adding eyes to a drawing of a human head significantly increased the response of sheep brain cells involved in facial recognition, but adding additional facial features did not. Sheep presented with the front view of a human stayed further away than when they were presented with the back view (Erhard, 2003). In addition, a human in a quadrapedal stance could approach closer to a flock of sheep when avoiding eye contact than when direct eye contact was maintained (Kendrick, 1994).

The quality and intensity of the behavioural responses of prey animals are related to short-term changes in predation risk or other disturbing stimuli (e.g. humans), with responses being stronger when the perceived risk is higher (Frid and Dill, 2002). Therefore, the behavioural responses of domestic sheep to a staring human should be related to the perceived risk of the situation. The arena test is designed to create approach/avoidance motivational conflict, by presenting a stimulus between the individual test sheep and a group of flockmates. If human staring is perceived as strongly aversive or representative of imminent danger, we would expect to see an increase in fear- or aversion-related behaviour in the arena. Behavioural responses indicative of higher fear or aversion in sheep in the arena test include: staying further from the group sheep, which are situated behind the stimulus, more vigilance behaviour, especially directed at the stimulus, and less exploratory behaviour (Torres-Hernandez and Hohenboken, 1979; Erhard, 2003; Beausoleil et al., 2005).

If, on the other hand, human staring is merely a warning cue, and is not aversive in itself, we would expect to see less pronounced behavioural responses, for example, increased vigilance. Warning cues elicit increased vigilance or attention in the recipient, to monitor the intentions and subsequent behaviour of the sender. However, if a cue does not predict an increase in potential risk, the recipient is unlikely to mount a further, more costly response, such as flight (Frid and Dill, 2002). In fact, the recipient might even habituate to the presence of a non-reinforced or unchanging warning cue. Wild prey animals have been reported to habituate even to the presence of a stalking predator, if it appears to remain still e.g. very slow ambush (Rice, 1986).

The following hypothesis was examined: If direct human eye contact does function as a cue to an impending aversive interaction for sheep, it was predicted that sheep would respond differently to a staring human than to a human averting his gaze. For experimental study, it was hypothesized that sheep would show more behaviour related to fear or aversion in the presence of a human than with a control object (cardboard box). It was also hypothesized that individual sheep would exhibit more vigilance behaviour in the presence of a staring human than with a non-watching human. If human staring was significantly aversive in itself (without reinforcement), sheep were expected to stay further away from the group sheep and exhibit less exploratory behaviour in the presence of the staring human.

3.3 Methods and Materials

Animals

Sixty 16-month-old female New Zealand Romney sheep were used in this study. They had been raised together since weaning on a Massey University farm and had regular contact with the shepherd and his dogs during normal husbandry procedures. Ten female East Friesen sheep, kept with the Romneys since weaning, were used as group sheep (Beausoleil et al., 2005). Sheep were randomly assigned to a test day and treatment, based on presentation order in the race. The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (protocol no. 01/84).

Experimental setup

Behavioural responses to the stimuli were tested using a modified arena test (Kilgour and Szantar-Coddington, 1995; Erhard, 2003; Beausoleil et al., 2005). All tests took place in November 2001 and were conducted between one and three pm. The arena measured 8 m x 3.5 m and a grid of squares (approximately 1 m x 1 m) was painted on the slatted wooden floor (Figure 3.1). Sheep waiting to be tested, and those which had already been tested that day were not visible during testing, however, they were still within the audible range of the test animal in the arena. The stimulus was placed in square 2 with a wooden cabinet unit (approximately 1 m wide x 1 m tall) behind it, to create a visual barrier between the stimulus and the group sheep.

The stimuli were:

Box: a plain cardboard box, placed on end to stand about 0.5 m tall, used as a novel object with no biological significance to the sheep.

Human not watching: the human directed his gaze at the floor near his feet for the duration of the test, regardless of the test sheep's behaviour. Therefore, the sheep would have been presented with an averted (downcast) head as well as eyes e.g. the head was not directly facing the sheep.

Human watching: the human followed the test sheep with his head and eyes for the duration of the test. Therefore, his head and eyes were always directly facing the sheep. If the test sheep made eye contact, it was maintained until the sheep looked away.

The same human (male, wearing the same blue overalls) was used for both treatments, and remained standing immobile during the test except for occasional small movements e.g. weight shifts. This person was not familiar to the test sheep but assisted each afternoon in moving the sheep from the paddock to the test facility.

Pre-test Pen
(15m away)

Start
Box

1m

Observer	Group Sheep	3	6	9	12	15	18	21	24
		X 2	5	8	11	14	17	20	23
3.5m		1	4	7	10	13	16	19	22

Post-Test Pen
(10 m away)

1.5m

8m

Exit
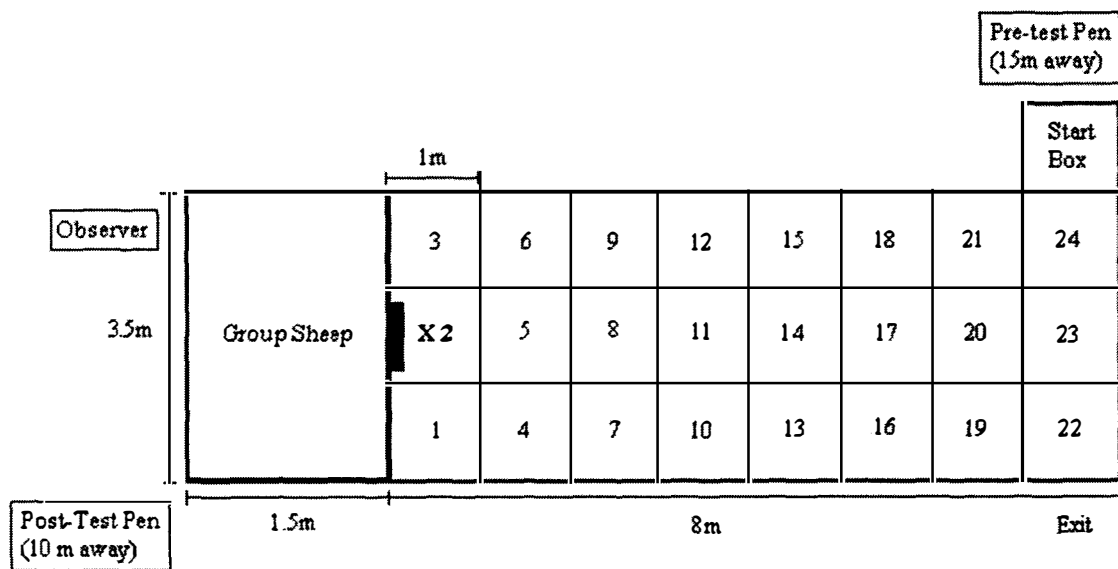
Figure 3.1 Test arena dimensions. X marks the position of the stimulus. The cabinet is represented as the black rectangle behind the stimulus in square 2. The sides of the arena were covered in shade cloth.

Procedure

The sheep were familiarized with the yards, races and test arena before testing, to reduce the confounding effects of novelty on their behavioural responses. Twice during the week prior to testing, the sheep were moved through the facility, and groups of about 20 sheep were left in the arena for half an hour.

On the day of testing, the test sheep were separated from the flock. Five of the East Friesen sheep were placed into the group sheep pen, which was separated from the arena by a metal farm gate (Figure 3.1). Test sheep had visual (and limited physical) access to group sheep at all times during the test. Each stimulus was presented twice on each afternoon, with the order of presentation being randomized for each day (block randomized). Twenty sheep were tested with each stimulus; each sheep was tested once, being exposed to only one of the three stimuli. When the stimulus and observer were in place, an individual sheep was pushed gently into the arena by a handler. Once the sheep entered the arena, the observer began the 10-minute observation period. When this was over, the sheep was moved out of the arena and into a post-test pen.

Behaviour measurement

The observer sat adjacent to the arena behind a window blocked with shade cloth, except for a small viewing slot. All behaviours were recorded continuously on a tape recorder during the test period. In addition, every 15 seconds, the square occupied by the sheep's left front foot was recorded. If the foot was on a grid line, the position of the right front foot was used, as this would best represent the position of the sheep's body. Table 3.1 presents the behaviours measured, and when necessary, gives an explanation of how the behaviour was defined or measured in this experiment.

Table 3.1 also shows 11 behaviours included in the synthesis of a fearfulness score, and their relationships to fear (Romeyer and Bouissou, 1992). Fearfulness scores were calculated for each sheep as outlined by Beausoleil et al. (2005). Briefly, the score was calculated by finding the mean of the ranks obtained by each sheep for the 11 behaviours. For example, in a group of 60 sheep, the animal showing the highest number of escape attempts (sign of fear) is given a rank of 60, while the sheep showing the lowest number is ranked 1 (less fear) for this behaviour. Conversely, a sheep sniffing the stimulus many times (absence of fear) is given a rank of 1, while the sheep sniffing the stimulus least is given a rank of 60 for this behaviour. These ranks are summed for each sheep and the results divided by the number of behavioural items. The higher the score, the more fearful the behaviour exhibited in this arena test.

Statistical analysis

None of the residuals of the counts data were normally distributed, and they could not be transformed to approximate normality. Therefore, the frequencies and latencies of these items were compared between the treatments using analysis of variance (ANOVA) on ranked data followed by Bonferroni post hoc tests to determine differences between individual treatments (Conover and Iman, 1981). The continuous variables were analyzed using one-way ANOVA on raw (mean distance from group sheep) or log transformed (minimum distance) data followed by Least Squares (LS) Means post hoc tests; however non-parametric tests yielded the same results for these parameters. Because the fearfulness scores were compiled using ranked data, they were analyzed using parametric ANOVA.

49

Table 3.1 Parameters of sheep behaviour measured in response to stimuli presented in an arena test.

Parameter of Behaviour	Relationship to fear	Definition of behaviour
Mean distance to group sheep [ab]	+	The mean distance test sheep maintained from group sheep over 10 minutes. Calculated by multiplying the distance from the group pen by the proportion of time spent there
Immobilization frequency [a]	+	Sheep stood still with no movement of any body part (including ears) for three seconds or more
Trot frequency [a]	+	Sheep broke out of a walk in any direction. Trots into the arena at the beginning of the test were discounted, as these were related to the handler, not the stimulus
Escape attempts [a]	+	Any attempt to escape from the arena, including jumping or rearing against the walls
Baulks [ab]	+	A sharp, sudden movement while remaining with all feet in the same spot ('flinch' from Romeyer and Bouissou, 1992)
Glances at stimulus [a]	+	Sheep looked towards the stimulus for less than three seconds before re-directing its gaze
Stares at stimulus [ab]	+	Sheep maintained its gaze at the stimulus for 3 seconds or more
Sniff stimulus [a]	-	Sheep brought its nose very close to stimulus and sniffed
Latency to sniff stimulus [a]	+	The 15-second interval in which the sheep sniffed the stimulus for the first time
Sniff in Square 2 [ab]	-	Sheep sniffed the ground in square 2 where the stimulus was located
Sniff cabinet (in square 2) [ab]	-	Sheep sniffed the cabinet located in square 2 behind the stimulus
Bleats	?	Only bleats that could be heard by the observer in the next room were recorded (high-pitched bleats)
Foot stamps	?	A front foot (or feet) was brought up off the ground in a controlled manner, and brought down with force to produce a sound on the ground
Lip licks	?	Sheep's tongue came out of its mouth in a licking motion. Each lick was counted as a separate event
Departures	?	The sheep turned its body to face away from the stimulus and moved away
Minimum distance to group sheep	+	Minimum distance between test sheep and group sheep during 10 minutes
Glances at group sheep	?	Glances directed towards group sheep
Sniff ground	?	
Sniff side of arena	?	
Urinations	?	
Advances	?	Forward movement in the direction of the stimulus
Sniff group sheep	?	
Latency to bleat	?	The 15-second interval in which the sheep bleated for the first time
Defecations	?	
Chew side of arena	?	
Duration chewing cud	?	Duration spent chewing cud in 10 minutes
Duration lying down	?	Duration spent lying down in 10 minutes
Duration spent in start gate	?	Duration spent in the start gate (out of sight of the stimulus) in 10 minutes

+ presence of fear for a high value of the parameter; – absence of fear for a high value of the parameter

[a] behaviour included in synthesis of Fearfulness Score

[b] derived behaviours; not explicitly related to fear by Romeyer and Bouissou, 1992.

The frequencies of behaviour in the entire 10-minute test, and the first and last two minutes of the test were compared between treatments. In addition, the differences in frequency between the first and last two minutes of the test were calculated and then compared to zero using a sign test or Wilcoxon signed-ranks tests (depending on distribution of the residuals), to assess whether the changes in behaviour over the duration of the test were statistically significant.

A multivariate canonical discriminant analysis (CDA) was performed on the raw data from the entire 10-minute test to determine which behaviours or groups of behaviours explained the variation in response between the treatment groups. One-tailed probabilities are given for variables that were hypothesized to change in a particular direction with different stimuli: mean and minimum distance, glances and stares at the stimulus, glances and stares at the human's face, glances at the group sheep, and sniffs of the stimulus, cabinet, group sheep, ground, and side. Two-tailed probabilities are given for other behavioural variables, for which the probable direction of change was unknown.

3.4   Results

Effect of human presence on behaviour

The presence of the human, whether watching or not, elicited more fearful behaviour than the box in the arena test. This was true over the entire 10-minute duration of the test (mean $\pm$ SE fearfulness score: box 18.57 ± 0.96; human not watching 36.68 ± 0.86; human watching 36.25 ± 0.70, ANOVA $F(2, 57) = 149.03$, $P < 0.0001$), and also in the first two minutes (box 21.07 ± 0.87; human not watching 34.65 ± 0.90; human watching 35.78 ± 0.52, $F(2, 57) = 110.22$, $P < 0.0001$) and last two minutes of the test (box 25.39 ± 0.79; human not watching 33.38 ± 0.74; human watching 32.74 ± 0.75, $F(2, 57) = 33.74$, $P < 0.0001$).

A number of individual behaviours differed significantly in frequency between the box and human treatments over the 10-minute duration of the test (Table 3.2 and Table 3.3). Test sheep stayed further from the group sheep when the human was present than with the box, and glanced and stared at the human more often. They also glanced at the group sheep, trotted and licked their lips more frequently in the presence of the human than with the box. In contrast, exploratory behaviours, such as sniffing the stimulus itself, square 2, the ground, the cabinet and the group sheep occurred less frequently in the presence of the human than with the box. Sheep also sniffed the box much more quickly than they sniffed the human.

In the first two minutes of the test, sheep stayed further from the group sheep and trotted more often when the human was present, and glanced and stared at the human and group sheep more often than at the box (Table 3.2 and Table 3.3). Exploratory behaviour was less frequent in the presence of the human than with the box during this period. During the last 2 minutes of the test period, fewer behaviours varied between treatment groups (Table 3.2 and Table 3.3). Sheep still stayed further from the group sheep, and glanced and stared at the human more often than the box. In addition, sheep still explored the areas surrounding the human less frequently.

Over the course of the 10-minute test there was a general decrease in behavioural activity (Table 3.4). The frequency of glances at the stimulus and group sheep declined in all groups. Sniffing the group sheep decreased with the box, but increased slightly over the test with both humans. Exploration of the stimulus and cabinet decreased only with the box, which probably reflects the high levels of such behaviour occurring at the beginning of the box test.

Effect of human eye contact on behaviour

There were no statistically significant differences in fearfulness scores (see above), frequencies of behaviour (Table 3.2 and Table 3.3), or the number of sheep performing each behaviour (not shown) between the two human treatments for the entire 10-minute test. The same was true for the last 2 minutes of the test. However, in the first two minutes, sheep glanced at the face of the watching human more often than at the face of

the non-watching human (Table 3.2 and Table 3.3). All but one sheep in this study specifically looked at the face of the human during the test.

The change in behaviour over the course of the 10-minute test differed, depending on whether the human was watching or not (Table 3.4). Sheep stared at the watching human less, and glanced at his face less often at the end, compared with the beginning of the test. However, they continued to stare at the non-watching human at the same frequency throughout the test. In the last two minutes of the test, sheep stayed further from the non-watching human than from the box; however, there was no statistical difference in the distance from the box and the staring human (Table 3.2). It is interesting to note that less than half of the total glances at the human (watching and non-watching) were directed towards his face. In contrast, the majority of stares at the human (about 80%) were focussed on his face.

Canonical discriminant analysis

The results of the CDA are shown in Table 3.5. Two canonical dimensions described all of the variation between the treatment groups. The first dimension, explaining 83.7% of the variation, was dominated by mean distance to the group sheep and latency to sniff the stimulus, and as such could be labelled 'avoidance of the stimulus'. This dimension clearly separated the box from the two human stimuli (Figure 3.2), with sheep presented with either human staying further away from the group sheep, and taking longer to sniff the stimulus than sheep presented with the box.

The second dimension, explaining the other 16.3% of the variation between groups, was primarily described by locomotor activity (squares entered) and frequency of urination. This dimension clearly separated the watching and non-watching human (Figure 3.2). Sheep presented with the watching human were more active and urinated more often during the 10-minute test than sheep presented with the non-watching human. The interpretation of this dimension is unclear; however, it does indicate that sheep did respond differently when the human was staring.

Table 3.2 Mean frequency per minute or latency of behaviours performed by individual sheep in response to the presence of a Box (B), Non-watching Human (HNW), or Watching Human (HW) measured over the entire 10-minute arena test, and during the first and last 2 minutes of the test. Raw data means and 95% confidence limits (in brackets) presented. Only behaviours found to be significantly different between at least two treatments using one-way ANOVA on ranked data (Kruskal-Wallis test) followed by Bonferroni adjustment for multiple comparisons are presented. Means with different letters are significantly different at $P < 0.01$, or if superscript is in brackets at $P < 0.05$. *Table continued on overleaf.*

Behaviour	10-minute test			First 2 minutes			Last 2 minutes		
	B	*HNW*	*HW*	*B*	*HNW*	*HW*	*B*	*HNW*	*HW*
Mean distance from group sheep (m)	2.19[a] (1.82, 2.56)	4.41[b] (3.63, 5.19)	3.96[b] (3.17, 4.76)	1.71[a] (1.28, 2.15)	3.87[b] (3.14, 4.60)	3.94[b] (3.35, 4.53)	2.45[a] (1.70, 3.20)	4.34[b] (3.33, 5.35)	3.28[ab] (2.31, 4.25)
Minimum distance from group sheep (m)	1.0[a] (1.0, 1.0)	1.95[b] (1.39, 2.51)	1.70[ab] (1.24, 2.16)	1.10[a] (0.89, 1.31)	2.60[b] (1.87, 3.33)	2.45[b] (1.80, 3.10)	1.25[a] (0.82, 1.68)	3.25[b] (2.21, 4.29)	2.60[ab] (1.71, 3.49)
Glances at stimulus	0.56[a] (0.33, 0.79)	3.40[b] (2.81, 3.99)	3.75[b] (2.69, 4.80)	1.25[a] (0.69, 1.81)	4.78[b] (3.60, 5.95)	5.50[b] (3.77, 7.23)	0.23[a] (0.06, 0.39)	3.00[b] (1.97, 4.03)	3.75[b] (2.44, 5.06)
Stares at stimulus	0[a] (0, 0)	0.35[b] (0.24, 0.47)	0.34[b] (0.22, 0.46)	0[a] (0, 0)	0.43[b] (0.21, 0.64)	0.58[b] (0.32, 0.83)	0[a] (0, 0)	0.45[b] (0.21, 0.69)	0.23[ab] (0.05, 0.40)
Glances at human face	-	1.45 (0.95, 1.94)	1.49 (1.15, 1.83)	-	1.80[a] (0.84, 2.76)	2.75[b] (1.90, 3.60)	-	1.35 (0.82, 1.88)	1.38 (0.82, 1.93)
Glances at group sheep	2.51[a] (2.04, 2.97)	4.52[b] (3.63, 5.41)	4.06[b] (3.23, 4.88)	3.15[a] (2.49, 3.81)	5.60[b] (4.22, 6.98)	5.33[b] (4.38, 6.27)	2.38 (1.52, 3.23)	4.15 (2.95, 5.35)	3.13 (2.28, 3.97)
Trots	0.02[a] (0, 0.03)	0.11[ab] (0.02, 0.19)	0.12[b] (0.05, 0.18)	0.05[a] (0, 0.12)	0.38[ab] (0, 0.75)	0.35[b] (0.16, 0.54)	0 (0, 0)	0.03 (0, 0.08)	0.08 (0, 0.19)

Behaviour	10-minute test			First 2 minutes			Last 2 minutes		
	B	*HNW*	*HW*	*B*	*HNW*	*HW*	*B*	*HNW*	*HW*
Liplicks	0.21[a] (0.13, 0.29)	0.54[b] (0.37, 0.70)	0.71[b] (0.36, 1.05)	0.50 (0.30, 0.70)	0.43 (0.11, 0.74)	0.80 (0.28, 1.32)	0.18 (0.02, 0.33)	0.35 (0.11, 0.59)	0.50 (0.06, 0.94)
Sniffs stimulus	0.34[a] (0.23, 0.44)	0.01[b] (0, 0.02)	0.02[b] (0, 0.05)	0.75[a] (0.52, 0.98)	0[b] (0, 0)	0[b] (0, 0)	0.15 (0, 0.32)	0 (0, 0)	0 (0, 0)
Sniffs square 2	0.17[a] (0.09, 0.25)	0.01[b] (0, 0.03)	0.01[b] (0, 0.02)	0.25[a] (0.09, 0.41)	0[b] (0, 0)	0[b] (0, 0)	0.15[a] (0, 0.30)	0[b] (0, 0)	0[b] (0, 0)
Sniffs cabinet	0.31[a] (0.20, 0.41)	0.01[b] (0, 0.02)	0.03[b] (0, 0.07)	0.48[a] (0.28, 0.67)	0[b] (0, 0)	0[b] (0, 0)	0.20[a] (0.04, 0.36)	0[b] (0, 0)	0[b] (0, 0)
Sniffs group sheep	0.31[a] (0.18, 0.44)	0.07[b] (0, 0.14)	0.11[b] (0.04, 0.18)	0.58[a] (0.29, 0.86)	0.03[b] (0. 0.08)	0.03[b] (0, 0.08)	0.23 (0.06, 0.39)	0.08 (0, 0.19)	0.13 (0.02, 0.23)
Sniffs ground	0.99[(a)] (0.73, 1.24)	0.57[(b)] (0.37, 0.77)	0.71[(ab)] (0.52, 0.90)	1.15 (0.63, 1.67)	0.78 (0.47, 1.08)	0.65 (0.36, 0.94)	0.48 (0.20, 0.75)	0.43 (0.16, 0.69)	0.38 (0.18, 0.57)
Sniffs side	0.63 (0.45, 0.81)	0.42 (0.27, 0.57)	0.48 (0.34, 0.62)	0.58[(a)] (0.32, 0.83)	0.30[(ab)] (0.08, 0.52)	0.20[(b)] (0.02, 0.38)	0.70 (0.37, 1.03)	0.40 (0.13, 0.67)	0.53 (0.27, 0.78)
Latency to sniff stimulus (mins)*	2.24[a] (0.63, 3.84)	9.73[b] (9.15, 10)	9.55[b] (8.89, 10)	1.16[a] (0.83, 1.50)	2.0[b] (2.0, 2.0)	2.0[b] (2.0, 2.0)	-	-	-

* Upper confidence limits cannot be larger than 10 minutes for latency to sniff stimulus.

Table 3.3 ANOVA statistics and effect sizes between treatment groups for behaviours performed by individual sheep over the entire 10-minute arena test, and during the first and last 2 minutes of the test. Glances at human face $F(1, 38)$, all other behaviours $F(2, 57)$. Positive effect sizes (Cohen's d statistic) indicate that the mean difference between treatment groups was in the predicted direction. Negative effect sizes indicate that the mean difference between groups was in the direction opposite to that predicted. The direction of change was not predicted for trots or lip licks. *Table continued on overleaf.*

	10-minute test				First 2 minutes				Last 2 minutes			
	ANOVA Statistics		Cohen's d		ANOVA Statistics		Cohen's d		ANOVA Statistics		Cohen's d	
Behaviour	F	*P*	B vs H	HNW vs HW	F	*P*	B vs H	HNW vs HW	F	*P*	B vs H	HNW vs HW
Mean distance	13.95	*<0.0001*	1.52	-0.27	22.37	*<0.0001*	1.85	0.05	4.43	*0.008*	0.71	-0.50
Minimum distance	7.81	*0.001*	1.09	-0.23	10.28	*0.001*	1.32	-0.10	8.0	*0.001*	1.05	-0.31
Glances at stimulus	50.96	*<0.0001*	2.27	0.19	32.71	*<0.0001*	1.64	0.23	42.03	*<0.0001*	1.75	0.30
Stares at stimulus	50.61	*<0.0001*	2.00	-0.06	15.09	*<0.0001*	1.40	0.30	9.53	*0.001*	1.05	-0.50
Glances at human face	0.07	*0.395*	-	0.05	5.99	*0.010*	-	0.49	0	*0.478*	-	0.02
Glances at group sheep	9.38	*0.001*	1.22	-0.25	7.05	*0.001*	1.14	-0.11	3.03	*0.026*	0.62	-0.46
Trots	6.14	*0.004*	0.83	0.06	4.93	*0.011*	0.69	-0.04	1.05	*0.355*	0.37	0.26
Lip licks	8.44	*0.001*	0.96	0.29	0.75	*0.476*	0.16	0.41	1.13	*0.330*	0.43	0.20
Sniffs stimulus	65.49	*<0.0001*	1.98	-0.32	93.38	*<0.0001*	2.12	0	3.35	*0.021*	0.58	0
Sniffs square 2	32.43	*<0.0001*	1.33	0.14	12.60	*<0.0001*	1.02	0	4.74	*0.006*	0.64	0

| Behaviour | 10-minute test | | | | First 2 minutes | | | | Last 2 minutes | | | |
| | ANOVA Statistics | | Cohen's d | | ANOVA Statistics | | Cohen's d | | ANOVA Statistics | | Cohen's d | |
	F	P	B vs H	HNW vs HW	F	P	B vs H	HNW vs HW	F	P	B vs H	HNW vs HW
Sniffs cabinet	83.92	<0.0001	1.79	-0.43	42.72	<0.0001	1.62	0	8.12	0.001	0.83	0
Sniffs group sheep	11.74	<0.0001	0.96	-0.29	22.05	<0.0001	1.24	0	1.73	0.093	0.43	-0.21
Sniffs ground	3.72	0.015	0.71	-0.33	1.43	0.124	0.48	0.20	0.05	0.474	0.14	0.10
Sniffs side	1.77	0.090	0.51	-0.19	3.33	0.021	0.67	0.24	1.18	0.156	0.37	-0.22
Latency to sniff stimulus	57.38	<0.0001	2.85	-0.13	33.88	<0.0001	1.65	0	-	-	-	-

Table 3.4 Mean difference in frequency of behaviours performed by individual sheep between the first and last 2 minutes of the arena test with a Box, Non-watching Human or Watching Human. Raw mean differences and 95% confidence limits (in brackets) for the differences are presented. Only behaviours found to differ significantly between 2-minute periods within at least one treatment group are presented. Negative values of mean difference and Cohen's d (effect size) indicate a decrease in frequency over the duration of the test. Sign test or Wilcoxon signed-ranks test statistics indicate whether the change in frequency is significantly different from zero.

Behaviour	Box				Human Not Watching				Human Watching			
	Mean Difference	Test statistic	P	Cohen's d	Mean Difference	Test statistic	P	Cohen's d	Mean Difference	Test statistic	P	Cohen's d
Advances	-0.50 (-1.31, 0.31)	-27.5	0.250	-0.34	-0.95 (-1.85, -0.05)	-42	0.065	-0.71	-1.15 (-1.94, -0.36)	-49.5	0.009	-1.04
Glances at stimulus	-2.05 (-3.16, 0.94)	-65.5	0.001	-1.16	-3.55 (-6.36, -0.74)	-60	0.023	-0.75	-3.50 (-6.29, -0.71)	-57	0.020	-0.53
Stares at stimulus	0 (0, 0)	-	-	-	0.05 (-0.51, 0.61)	3	0.959	0.05	-0.70 (-1.36, -0.04)	-19.5	0.049	-0.74
Glances at human face					-0.90 (-2.27, 0.47)	-11.5	0.395	-0.27	-2.75 (-4.66, -0.84)	-59	0.008	-0.90
Glances at group sheep	-1.55 (-3.65, 0.55)	-33.5	0.185	-0.47	-2.90 (-5.20, -0.60)	-51	0.024	-0.52	-4.40 (-6.08, -2.72)	-89.5	<0.0001	-1.15
Trots	-0.10 (-0.24, 0.04)	-1	0.500	-0.46	-0.70 (-1.48, 0.08)	-3	0.070	-0.61	-0.55 (-0.97, -0.13)	-4	0.022	-0.83
Urinations	-0.75 (-1.01, -0.49)	-52.5	0.001	-1.93	-0.60 (-0.88, -0.32)	-33	0.001	-1.35	-0.85 (-1.16, -0.54)	-52.5	0.001	-1.60
Sniffs stimulus	-1.20 (-1.76, -0.64)	-6.5	0.001	-1.37	0 (0, 0)	-	-	-	0 (0, 0)	-	-	-
Sniffs cabinet	-0.55 (-1.04, -0.06)	-4	0.039	-0.72	0 (0, 0)	-	-	-	0 (0, 0)	-	-	-
Sniffs group sheep	-0.70 (-1.27, -0.13)	-19.5	0.023	-0.70	0.10 (-0.16, 0.36)	1.5	0.750	0.26	0.20 (0.01, 0.39)	5	0.125	0.57
Sniffs ground	-1.35 (-2.55, -0.15)	-5	0.021	-0.76	-0.70 (-1.48, 0.08)	-3	0.210	-0.57	-0.55 (-1.13, 0.03)	-2.5	0.267	-0.52

Table 3.5 Results of the canonical discriminant analysis. All behaviours listed in Table 3.1 were included in the analysis. Only behaviours with high positive or negative loadings for each canonical dimension are listed in this table.

	Canonical Dimension 1		Canonical Dimension 2	
Eigenvalue	8.52		1.66	
Variation explained (%)	83.7		16.3	
	Behaviour	**Canonical Coefficient**	**Behaviour**	**Canonical Coefficient**
	Mean distance	1.03	Squares entered	-3.30
	Latency to sniff stimulus	0.70	Urinations	-1.28
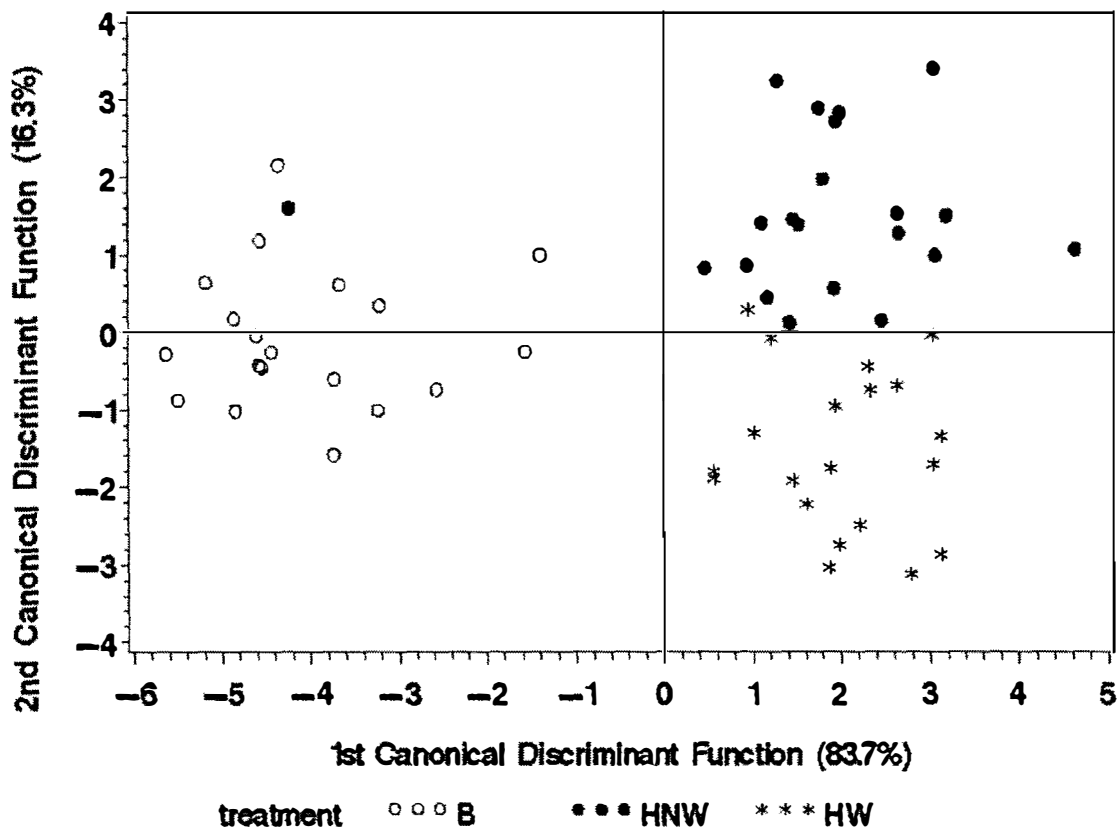
Figure 3.2 Plot of canonical scores for individual sheep, identified by the stimulus presented, Box (B), Non-watching human (HNW) and Watching human (HW).

3.5 Discussion

Effects of human presence on behaviour

As hypothesized, individual sheep showed more fear- or aversion-related behaviour in the presence of a human than with a cardboard box. Multivariate analysis suggested that the most important behaviours in differentiating the box from the human stimuli were those relating to avoidance of the stimulus (mean distance and latency to sniff stimulus). Mean distance from the group sheep was also found to be the most important behaviour in separating the responses of individual sheep to stimuli differing greatly in aversiveness (box, human, or dog), with sheep staying further away with the more aversive stimuli (Beausoleil et al., 2005).

These results show that the presence of a human does elicit a behavioural response from individual sheep, which is distinct from their response to the arena testing procedure. Human presence was also found to elicit more fear-related behaviour from individual sheep than a box, and less fear-related behaviour than a dog in a previous arena test (Beausoleil et al., 2005). It is concluded that the arena test was effective in differentiating behavioural responses of individual sheep to aversive and non-aversive stimuli. The results of the multivariate analysis highlighted this difference; most of the variation between treatment groups (84%) was due to differences in the sheep's behavioural responses to the box and to the human. By presenting stimuli differing only slightly (watching versus non-watching human), I move on to examine whether the arena test can also detect smaller differences in stimulus aversiveness.

Effects of human eye contact on behaviour

It was hypothesized that if direct human eye contact functions as a cue to an aversive event, sheep would respond differently to a staring and to a non-watching human. Specifically, it was hypothesized that sheep in an arena test would be more vigilant, and possibly stay further from the group sheep and exhibit less exploratory behaviour in the presence of a staring human than with a non-watching human.

In accordance with my hypothesis, univariate analysis revealed a difference in the frequency of vigilance behaviour, depending on whether the human was staring or not watching. Sheep were found to glance at the face of the staring human more often during the first two minutes of the test. These data suggest two things. Firstly, that the sheep were able to perceive a difference between the two human stimuli. The ability of domestic sheep to detect such subtle differences in the behaviour of other species is supported by the effective use of 'eye dogs' to move livestock (McConnell and Baylis, 1985). All but one sheep in this study specifically looked at the face of the human. In addition, the majority of stares at the human were focussed specifically on his face. This scrutiny suggests that the sheep were aware of the significance of the face in interpreting human intention, and were using facial behaviour to assess the potential risk presented by the human.

The sheep's perception of the difference between stimuli was likely enhanced by the fact that not only eye gaze direction, but also the orientation of the head differed between the two treatments. The watching human oriented his head and eyes directly at the sheep, whereas the non-watching human always presented a downcast (averted) head and eyes. Therefore, the sheep may have been responding to the orientation of the human's head, as well as to eye gaze direction. Sparrows were found to respond to head posture, rather than eye position (Hampton, 1994). A human head directed towards the bird elicited more flights than an averted head, regardless of whether the eyes were directed at the bird or averted. In contrast, the direction of human eye gaze alone influenced the duration of tonic immobility after manual restraint in domestic chickens (Gallup et al., 1972). Birds stayed immobile longer after exposure to a direct head orientation with direct eye contact, compared with a direct head posture but averted gaze (45 degrees to left). Further study is required to separate the effects of eye contact and head orientation on sheep behaviour.

Secondly, the increase in face-directed vigilance suggests that human eye contact does function as a warning cue for domestic sheep. That is, it has some biological relevance to the sheep, and warrants a change in their subsequent behaviour. Multivariate analysis also showed that the staring human elicited more locomotor activity and more frequent urination than the non-watching human. The relationship of these behaviours to fear in sheep in the arena test is uncertain, making interpretation of this result difficult (see below). However, it does indicate that staring affected the behavioural response of the sheep, supporting the idea that human eye contact functions as a warning cue.

An increase in vigilance is the logical behavioural response upon detection of a warning cue. Sheep were observed to be more vigilant in the presence of a potential predator (dog) than with a human, goat or cardboard box in a previous arena test (Beausoleil et al., 2005). Wild prey animals that are aware of the presence of a predator will monitor its behaviour, and some ungulates will even approach potential predators to keep them in view (Rice, 1986). Increased vigilance would prime the animal to react to secondary cues (e.g. movement), which could provide additional information on the risk of predation, thereby allowing the most efficient and cost-effective behavioural response to the situation (Brown et al., 2004).

Upon detecting human staring, the sheep became more attentive to the only feature that differed between the human stimuli: the face. More frequent eye contact suggests that the observed animal is checking for any change in the state of eye gaze of the human (Thomsen, 1974), which could provide additional information on the relative risk presented. The sheep may have been checking to see if the staring human was going to reinforce the warning cue by approaching them.

Did human eye contact increase fear of the human?

Contrary to the second part of my hypothesis, there were no differences in distance to the group sheep or frequency of exploratory behaviours in response to the staring and non-watching human. The lack of difference suggests that sheep were not more fearful of, or averse to, the staring human compared with the non-watching human. The addition of human eye contact did not increase the perceived risk sufficiently to warrant re-allocation of behaviour from other activities such as exploration of the arena (Brown et al., 2004).

There are several possible reasons why a staring human did not elicit behavioural responses indicative of increased fear or aversion. Firstly, human staring may function as a warning cue, and no more. Human eye contact did not appear to be aversive, in and of itself. As such, staring would elicit heightened attention from the sheep, but would not increase the expression of fear-related behaviour. To elicit an increase in fear or aversion, the warning cue would have to be reinforced, to increase the risk perceived by the sheep. Had the staring human reinforced the warning cue, for example, by approaching or attempting to catch the test sheep, the differences in behavioural response to the watching and non-watching human would likely have been much more pronounced.

Kendrick (1994) showed that when a human approached a flock of sheep in a quadrapedal stance, altering gaze direction did affect sheep behaviour (flight distance). Human approach with direct eye contact has also been shown to increase behavioural responses relative to approach with averted gaze in a number of wild species, including iguanas (Burger and Gochfeld, 1990) and herring gulls (Burger and Gochfeld, 1981). Infant rhesus monkeys approached by a gaze-averting human showed behavioural

responses which would typically reduce the chance of detection (freezing, decreased vocalization), whereas those approached by a staring human responded with hostile and submissive behaviours, presumably to threaten or placate a human aware of their presence (Kalin et al., 1991).

In these cases, the primary warning cue was reinforced by the approach of the human. An approaching predator focussed on the prey animal would represent a higher risk than a predator approaching but averting its gaze. In the present study, neither human stimulus was reinforced, therefore, the difference in eye gaze behaviour had limited functional relevance for the sheep. In this case, a staring human who did not move, change, or reinforce the warning cue in some way did not represent a greater immediate threat than a non-watching human.

Secondly, it is possible that these particular sheep have learned during rearing and management that human staring is not a reliable cue to aversive events such as handling. The present study showed that although sheep did perceive and attend to the difference between the watching and non-watching human, the difference in their behavioural responses was relatively minor. Although it is likely that the wild ancestors of domestic sheep perceived human staring as a warning cue, domestication has altered the relationship between humans and sheep. Domestic sheep in New Zealand are kept in large flocks, and it is unlikely that individual sheep would be consistently exposed to human eye contact before a handling event. In addition, in modern systems, sheep are often captured from behind, where they would be unable to see their captor's face and eyes.

Erhard (2003) showed that sheep do respond differently to the back or front of a human presented in an arena test, staying further from a forward-facing human than from one facing away. This makes sense, as the difference in outcome would be reinforced every time the sheep are handled (i.e. always presented with a front view before handling). In the present study, a front view was offered to both groups, effectively representing the same risk of handling from the sheep's perspective. In this case, the difference in head and eye orientation apparently offered very little in the way of additional information on the risk associated with the human presented.

Thirdly, habituation to the non-reinforced warning cue could have happened so quickly that it was not possible to detect the differences between the human stimuli, even within the first two minutes of the test. Habituation is broadly defined as the weakening of responses that are inadequate or unnecessary to cope with the stimulus or situation (Shulgina, 2005). Wild prey animals have been reported to habituate even to the presence of a stalking predator, if it appears to remain still, e.g. during the very slow ambush of feline predators (Rice, 1986). Sheep in the present study did habituate to the testing environment and to the stimuli over the course of the 10-minute test. In addition, the differences in face-directed vigilance were transient, which suggests that the sheep also habituated to the human warning cue very quickly.

Because human eye contact did not predict an aversive event during the test, it was quickly regarded as inconsequential by the sheep. When the staring human did not change, move, or further increase the risk perceived by the sheep, their responses became similar to those in the presence of the non-watching human. Similarly, Erhard (2003) found that when sheep were presented with the back or front view of a human, the initially observed difference in mean distance to the group sheep disappeared after the first two trials. As in the present trial, neither stimulus was reinforced, causing extinction of the behavioural responses to occur very quickly.

It is interesting to note that sheep in the present study did not habituate to the non-watching human to the same extent as they did to the staring human. Sheep stayed further from the non-watching human and continued to stare at him throughout the test. The rate and extent of behavioural habituation to predators or predator models have been shown to depend on a number of factors, including the behaviour of the predator (Herzog et al., 1989).

In the present study, the difference in habituation may have related to the ease of interpreting the human's behaviour. The message sent by a staring 'predator' is relatively unambiguous: I am watching you. After an initial period of increased vigilance, the sheep appeared to habituate to the unchanging stare of the watching human. In contrast, a human with downcast head and eyes may have provided little information on the relative risk of the situation, making it difficult for the sheep to interpret his intentions. Staring at the non-watching human may have represented a

65

sustained attempt to judge his intent. Wild Bonnet macaques were found to make longer visual assessments of a model of a less common form of a predator than a model of the common form (Coss and Ramakrishnan, 2000). At a proximity of less than 8 meters, a human averting his gaze may have represented a latent threat, worthy of watching.

Fourthly, the lack of difference in fear-related behaviour may have occurred because the arena test was not sensitive enough to detect small differences in fear-related behaviour to stimuli similar in aversiveness. However, multivariate analysis of arena behaviour did reveal differences in the sheep's responses, depending on the behaviour of the human stimulus. This suggests that the test was sensitive enough to detect differences in behaviour, but that differences in fear-related behaviours such as mean distance and exploration did not exist between the human treatments. However, the CDA also showed that the differences in response to the two human stimuli were relatively minor (only 16% of variation between treatment groups).

It is difficult to interpret the significance of the second canonical dimension, dominated by locomotor activity and urination. Using multivariate factor analysis, Vierin and Bouissou (2003) found two separate factors describing lamb behaviour in response to a human. The first described the degree of fear or anxiety, whereas the second, independent factor related to 'general locomotor activity'. Locomotor activity measured in the test was positively correlated with both factors. Bouissou and Vandenheede (1995) also found that a live human elicited more locomotor activity from individual sheep than a human-like model.

In the present study, the second canonical dimension may reflect increased fear of the staring human, or may simply relate to an increase in activity in his presence. It is possible that increased locomotion in the arena test is the physical manifestation of the animal's frustrated desire to join its flockmates while avoiding the stimulus (vacillation). In the presence of a staring 'predator', a single sheep would be strongly motivated to join other sheep, to dilute the focus on itself. In contrast, in the presence of a human directing his attention elsewhere, a single sheep would not feel as strongly motivated to join other sheep. The difference in social motivation could explain why the sheep did not stay further from the staring human than the non-watching human. In fact, in the last two minutes, there was a trend towards sheep coming and staying closer to

the staring human, which may have reflected their increased motivation to blend in with the flock.

Finally, individual variation in the sheep's perception of the risk associated with the stimulus, and therefore in their behavioural responses, could have obscured small but biologically relevant differences between the treatment groups. To reduce the confounding effects of individual variation on behavioural responses to the different stimuli, it would be useful to conduct a similar study in which each test animal acted as its own control. This could be achieved by presenting each animal with a watching and non-watching human alternately, and examining changes in the frequency of behaviour with changing human behaviour.

3.6 Conclusions

This study showed that domestic sheep are able to perceive differences in human visual behaviour. In addition, human eye contact did function as a warning cue for these domestic sheep. Sheep were more vigilant and active in the presence of a staring human. However, the staring human did not appear to represent a greater immediate threat than the non-watching human. This probably reflects the fact that for domestic sheep, human staring is not a reliable cue to subsequent aversive events, such as handling. In addition, because staring was not reinforced by further aversive action such as approach, sheep quickly habituated to the cue during the test. The confounding effects of individual variation should be reduced by using each animal as its own control in a similar arena test.

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CHAPTER 4

Are sheep selected for high or low activity in the presence of a human really more nervous or calm than unselected sheep?

4.1 Abstract

Merino sheep at the University of Western Australia have been selectively bred for their behavioural responses to social isolation and the presence of a human, resulting in the creation of two divergent lines. In previous studies, the more behaviourally active flock (MA) was labelled 'nervous' and the less active flock (LA) was labelled 'calm'. The aim of this study was to explore the idea that the flocks were differentially selected for fearfulness, as implied by the labels used in previous studies. Factor analysis of behavioural responses, and changes in plasma cortisol concentration were used to evaluate the fear responses of individual sheep to the presence of a human in an arena test. While MA sheep expressed high levels and LA sheep expressed low levels of behavioural activity, there was no behavioural or cortisol evidence to support the notion that the flocks were selected for differences in 'fearfulness' in the presence of a human in the arena test. Contrary to previous studies, increased locomotion and vocalization were interpreted as context-specific behavioural responses to social isolation (social motivation). Rather than selection based on fearfulness, the flocks may have been co-selected for variation in two separate motivational systems: sociality, and fear/aversion of a human. Therefore, the high locomotor activity of MA sheep may reflect both high social motivation and lower levels of fear (higher boldness) in the presence of a human. For the present, 'More Active' and 'Less Active' should be used to describe the flocks rather than labels which imply knowledge of the bearer's subjective experience.

4.2 Introduction

Merino sheep at the University of Western Australia (UWA) have been selectively bred since 1990 for putative differences in 'temperament' or 'emotional reactivity', based on their behavioural responses to the presence of a human in an arena test, and to social isolation (Murphy, 1999). Two divergent lines of sheep were created by selecting for differential expression of locomotor activity in an arena test with a human, and agitation (movement and vocalization) when socially isolated. However, whether selection has actually been based on a difference in 'fearfulness' or 'emotional reactivity' between the flocks, as has been contended by previous authors, is unknown. This study aims to expand the behavioural characterization of the different flocks, and to speculate on the

nature of the trait underlying those differences in manifest behaviour for which the flocks have been differentially selected.

Introduction to the study of animal temperament

Temperament is defined as 'the individual character of one's physical constitution permanently affecting the manner of acting, feeling and thinking' (Sykes, 1982). Personality or temperament traits may be used to explain patterns of behaviour, and to describe the tendency of individuals to behave in a particular way compared to other members of the same species (Kilgour, 1975; Buss and Craik, 1983). In humans, at least five factors of personality have been identified (review by Digman, 1990).

Variation in personality or temperament traits also appears to exist across a wide range of non-human animal taxa (Gosling and John, 1999), and a number of major axes of variation in animal temperament traits have been repeatedly identified. These include: activity, shyness-boldness, reactivity, and aggression (Sinn and Moltschaniwskyj, 2005). Activity refers to the frequency and duration of movements undertaken by the individual. Boldness relates to an individual's propensity to take risks, with bolder animals more likely to take risks than shyer animals. Reactivity refers to differences in the magnitude of responses between individuals, and may be a reflection of internal arousal states (Stifter and Fox, 1990). Reactivity may be analogous to 'fearfulness', a term used to describe the responses of animals to events or situations considered likely to elicit fear.

Another way of conceptualizing individual variation in behavioural responses is the idea of different 'coping strategies'. Active copers respond to challenge with high frequencies of active behaviour, such as locomotion, vocalization or attempts to remove themselves or the object causing the stress. Passive or reactive copers respond with low frequencies of active behaviour, instead tending to freeze, withdraw or disregard the source of challenge (Koolhaas et al., 1999; Groothuis and Carere, 2005). Active and passive behavioural responses are often correlated with specific patterns of physiological response to challenge; active copers often have lower adrenocortical reactivity, but higher activation of the sympathetic nervous system in response to acute

76

challenge, than do passive copers (Syme and Elphick, 1982; Fokkema et al., 1988; Koolhaas and Oortmerssen, 1989; Hessing et al., 1994; Jones and Saterlee, 1996).

In studies of domestic animals, 'temperament' is often used synonymously with terms such as 'fearfulness', 'emotional reactivity' and 'emotionality' (Boissy and Bouissou, 1995; Grandin and Deesing, 1998; Erhard et al., 2004). The diversity of terms arising in the ethological literature reflects the complexity of the concepts fundamental to the study of temperament and personality. It also highlights the conceptual difficulty associated with using observations of behavioural and physiological variables to characterize intangible phenomena such as temperament, motivation or emotional state.

The following two chapters represent the author's endeavour to untangle subtle differences between concepts such as fear, fearfulness and temperament, based on the responses of sheep selected for differences in behaviour. In doing so, I attempt to clarify the relationships between the behavioural and physiological responses of sheep, expressed in a given situation, and the emotional state underlying these manifest expressions.

Because these studies focus on the negative emotional states associated with situations considered to be fear-eliciting or challenging for domestic sheep, I consistently use the term 'fearfulness' when alluding to differences in the responses of sheep to such stimulation. Boissy and Bouissou (1995) define fearfulness as "a basic psychological characteristic of the individual that predisposes it to perceive and react in a similar manner to a wide range of potentially frightening events". However, it is important to point out the difference between the concepts 'fear' and 'fearfulness'.

Fear is a state an individual experiences at a given moment, and is the product of the interaction between the individual and the specific environment (context-specific). In contrast, fearfulness is a personality or temperament trait, which is the property of the individual alone, and may be stable over a range of environments (Zuckerman, 1983). It is possible, therefore, for an individual to express a high level of fear in a specific situation, but not be predisposed to react fearfully in general.

77

In the present chapter I examine the notion, put forward by previous researchers, that the UWA flocks have been differentially selected, in part, based on their propensity to react fearfully in the presence of a human in the arena test. Because the sheep were tested in the same environment in which selection took place, it was not possible to distinguish between 'fear' and 'fearfulness' in this part of the study. In other words, based on this analysis, it was not possible to ascertain whether inter-flock differences in the expression of fear-related behaviour were specific to the selection environment (fear), or representative of differences in the predisposition to react fearfully in a range of fear-eliciting situations (fearfulness).

However, as will be discussed in Chapter 5, the question of whether inter-flock differences in fear-related behaviour were consistently expressed in fear-eliciting situations other than the specific selection environment has also been addressed. In Chapter 5, I discuss whether differential selection at UWA has been based on fear, a context-specific temperament trait, or fearfulness, a domain-general temperament trait.

Measurement of temperament and fearfulness in sheep

Kilgour and Szantar-Coddington (1997) considered that an arena test could be used to measure differences in the temperament of individual sheep. The arena test is a behavioural test designed to create approach/avoidance motivational conflict, by presenting a stimulus between the individual test sheep and a group of flockmates.

Behavioural indicators of fear of, or aversion to, the stimulus in the arena test include increased avoidance and vigilance (Fell and Shutt, 1989; Chapman et al., 1994; Erhard, 2003; Beausoleil et al., 2005; Boissy et al., 2005; Paul et al., 2005). In addition, in domestic ungulates, longer latencies to contact, and less time spent in contact with novel objects and humans have been associated with higher adrenocortical activation in response to challenge (e.g. Lankin et al., 1979; 1980; Lyons et al., 1988a; Goddard et al., 2000; Van Reenen et al., 2005). Fear is also known to compete with other motivational systems, and high levels of fear may inhibit the expression of behaviours related to feeding, social interactions and exploration (e.g. Romeyer and Bouissou, 1992; Vandenheede and Bouissou, 1993b; Boissy, 1995; Beausoleil et al., 2005; Van Reenen et al., 2005).

Increased locomotor activity and vocalization in the arena test have also been interpreted as indicative of increased fearfulness in sheep (e.g. Murphy, 1999; Erhard and Rhind, 2004). Likewise, differences in behavioural responses to social isolation are also thought to reflect differences in the propensity of individual sheep to react fearfully (Romeyer and Bouissou, 1992; Vandenheede et al., 1998; Vierin and Bouissou, 2003). Accordingly, UWA sheep expressing high frequencies of locomotor and vocal behaviour in the two selection environments were classified as 'nervous' and sheep expressing low frequencies were classified as 'calm' (Murphy, 1999).

Dictionary definitions of 'nervous' include terms such as 'excitable', 'timid', 'easily agitated' and 'characterized by acute unease or apprehension' (Sykes, 1982). 'Apprehensive' is defined as 'fearful', while 'timid' is characterized as 'easily alarmed' or 'indicating fear' (Sykes, 1982). Although 'nervous' explicitly denotes an excitable or active behavioural character, it also undeniably refers to the increased level of fear associated with that behavioural activity. Therefore, the label 'nervous' implicitly refers to the negative emotional content of the bearer's experience of an event or stimulus. It implies that animals selected to have a 'nervous' temperament are expected to respond more fearfully than those selected for a 'calm' temperament, because they are predisposed to do so.

Although the flocks used in this study differ in the frequency of their locomotor and vocal activity in social isolation and human presence, the use of these behaviours to measure individual variation in the temperament trait 'fearfulness' in sheep has not been validated. Social isolation increases locomotor activity and vocalization in sheep, while also eliciting an increase in mean heart rate and plasma cortisol concentration (Romeyer and Bouissou, 1992; Lyons et al., 1993; Cockram et al., 1994). The concomitant physiological changes support the idea that increased locomotion and vocalization indicate an increase in fear or distress in socially isolated sheep. However, the relationship between locomotor activity and fear in an arena test with a human is uncertain, and changes in locomotor activity have been variously interpreted in studies of sheep fearfulness (e.g. Romeyer and Bouissou, 1992; Kilgour and Szantar-Coddington, 1995; 1997; Behrendt, 1998; Murphy, 1999; Degabriele and Fell, 2001; Erhard, 2003).

79

The relationship between behaviour and fear, stress or aversion may vary according to the context in which it is performed, and there is evidence that changes in locomotor activity are context-specific. While social isolation increases locomotor activity in sheep, the presence of a human or dog has been found to reduce the relative expression of locomotion (Torres-Hernandez and Hohenboken, 1979; Romeyer and Bouissou, 1992; Erhard, 2003). The fact that locomotor frequency changes in opposite directions with social isolation and predator presence, which are both assumed to represent negative experiences for sheep, suggests that the frequency of this behaviour alone cannot be considered a reliable indicator of the experience of a negative affective state, such as fear (Paul et al., 2005).

For the reasons outlined above, the meaning of quantitative changes in the expression of individual behaviours should be interpreted with caution. Factor analysis of a number of measured behaviours may aid in interpreting behavioural responses by revealing groups of behaviours which may relate to the same underlying motivational state, such as fear (De Passille et al., 1995; Ramos and Mormede, 1998; Vierin and Bouissou, 2003; Van Reenen et al., 2004).

Validation of proposed interpretations of behavioural responses to challenging situations should also include correlations with physiological stress indices, such as hypothalamic-pituitary-adrenal (HPA) activity (Broom and Johnson, 1993; Ramos and Mormede, 1998). Stress-induced activation of the HPA, as indicated by plasma corticosteroid concentrations, reflects the individual's capacity to cope with environmental challenge (Carere et al., 2003). As such, relative plasma cortisol responses to fear-eliciting stimulation may reflect the intensity of the internal emotional state associated with the stressful experience, e.g. fear (Ramos and Mormede, 1998; Mellor et al., 2000; Paul et al., 2005).

Situations designed to elicit fear or stress in domestic sheep, such as social isolation, novelty, physical restraint, human handling, and the presence of a dog produce transient increases in plasma cortisol concentration (Cronin, 1981; Fulkerson and Jamieson, 1982; Moberg and Wood, 1982; Parrott et al., 1987; Canny et al., 1990; Hargreaves and Hutson, 1990; Minton and Blecha, 1990; Minton et al., 1992; Lyons et al., 1993;

Cockram et al., 1994; Mears and Brown, 1997; Vierin and Bouissou, 2002; Cook, 2004). In addition, habituation of individual sheep to human presence and handling is associated not only with a decrease in fear-related behaviour, but also with a reduction in plasma cortisol responses to such procedures (Pearson and Mellor, 1976). The non-specificity of HPA responses adds credibility to its use to assess subjective experiences associated with stressful situations, as emotional state may be the variable common to a range of the different challenges, while behavioural responses may be more context-specific (Mellor et al., 2000).

High intensities of measured stress responses are generally taken to imply high levels of emotional activation (Ramos and Mormede, 1998), and larger corticosteroid responses to stimulation are often associated with larger increases in the expression of fear- or anxiety-related behaviours (e.g. Pellow et al., 1985; Handley and McBlanc, 1993; Landgraf et al., 1999). For example, sheep selected for strong avoidance of a human were found to have significantly higher adrenocortical responses to other psychological stressors, such as social isolation and transport, than sheep which did not avoid the human (Lankin et al., 1979; 1980).

Likewise, individual goats showing more fear-related behaviour in the presence of a human (aversion, avoidance) also had higher plasma cortisol responses (Lyons et al., 1988a). In addition, lambs which took a long time to move in the presence of a human (taken to indicate increased fear) had significantly higher plasma cortisol concentrations after the arena test than those which moved quickly (Goddard et al., 2000). Therefore, we might expect sheep expressing higher levels of locomotor activity in the arena with a human ('nervous') to also exhibit higher plasma cortisol concentrations than sheep expressing less locomotor activity ('calm'), in response to the presence of a human.

Thus far, neither multivariate analysis of behaviour, nor measurement of physiological indicators of stress have been employed to validate the use of such behaviours to measure individual variation in fearfulness in the UWA sheep. Therefore, I will refer to the flocks using simple descriptions of the behaviour for which they were differentially selected. Thus, the flock previously labelled 'nervous' will be referred to as More Active (MA), and the flock previously labelled 'calm' will be referred to as Less Active (LA). Sheep from the unselected base flock will be referred to as Reference (R) sheep.

The aim of this study is to explore the idea that MA and LA sheep have been selected for differences in fear in the presence of a human. In order to do this, I looked for differences in the behavioural and adrenocortical responses of MA and LA sheep to the presence of a human in the arena test. Factor analysis was used to explore the potential motivations underlying specific patterns of behaviour, and I also looked for relationships between behaviour patterns and adrenocortical responses to the stimuli. The experimental hypotheses were: 1. MA sheep would be more fearful, that is they would express more fear-related behaviour and mount higher adrenocortical responses to the presence of a human than LA and R sheep, and 2. LA sheep would be less fearful, that is they would express less fear-related behaviour and mount lower adrenocortical responses to the presence of a human than MA and R sheep, and 3. arena behaviours considered to be related to fear would be significantly correlated with plasma cortisol responses in the presence of the human.

The present observations represent a distinct aspect of a larger study which was directed towards evaluating whether variation in the behavioural responses of UWA sheep towards a human reflects selection based on a consistent underlying temperament dimension, such as fearfulness, which is stable in a range of challenging contexts.

4.3   Methods and Materials

Selection of flocks

Merino sheep at the University of Western Australia have been selectively bred for a trait called 'Temperament' since 1990. This selection has resulted in two divergent lines of animals, classified according to their locomotor activity in an arena test with an immobile human and a group of flockmates (zones crossed: TOTAL CROSS), as well as their responses to social isolation ('agitation', defined as high-pitched vocalization and movement while confined in a box: BOX), according to the selection index shown in Equation 4.1 (Murphy et al., 1994; Murphy, 1999).

$$\text{Selection index} = 100 + \frac{[BOX_i - BOX_x]}{BOX_{sd}} + \frac{[TOTAL\ CROSS_i - TOTAL\ CROSS_x]}{TOTAL\ CROSS_{sd}}$$

Equation 4.1 University of Western Australia 'Temperament' selection index, used to select sheep based on the amount of 'agitation' (high pitched vocalization and movement) expressed in social isolation (BOX) and on locomotor activity in an arena test with a human present (TOTAL CROSS); i = individual score, x = flock mean, sd = standard deviation of the mean (Murphy et al., 1994; Murphy, 1999).

These particular parameters were selected because they were found to be highly repeatable at different ages (CROSS correlation coefficient r = 0.57 – 0.65; BOX r = 0.73 – 0.77), moderately correlated with each other (r = 0.41), and moderately heritable (CROSS h^2 = 0.11; BOX h^2 = 0.22) (Murphy et al., 1994; Murphy, 1999). Heritability estimates for the selection index, and therefore the variable 'temperament', have not been published at this time.

The base flock was derived from a commercial line of Merino sheep. A number of animals from this unselected or base flock had been maintained (Reference or R sheep). The two divergent lines were generated using animals selected for 'temperament' from the two extremes of the base flock.

Briefly, lambs were tested in the arena and social isolation tests at weaning. The More Active (MA) flock was generated by selecting animals exhibiting high levels of locomotor activity in the arena test, and high levels of 'agitation' in social isolation (flock labelled 'Nervous' in previous publications (e.g. Murphy et al., 1994; Murphy, 1999; Gelez et al., 2003). The Less Active (LA) animals were selected for low levels of the two parameters (flock labelled 'Calm' in previous publications). In each generation, the 6 rams with the highest selection index scores (MA) and the 6 rams with the lowest scores (LA) were retained for breeding. MA ewes were bred only to the top-scoring MA rams, and LA ewes were bred only to bottom-scoring LA rams. However, all MA and LA ewes were retained for breeding, regardless of their weaning score.

Animals

Thirty female Merino hoggets from each of three lines were used in this experiment, which was carried out at Allandale Research Farm, Wundowie, Western Australia. The 30 LA sheep were chosen from among the 35 animals with the lowest selection index scores at weaning. The 30 MA sheep were chosen from among the 38 animals with the highest selection index scores at weaning. This was done in order to maximize the difference between the two selected flocks. R sheep were randomly selected from the base flock, and were matched to animals from the selected flocks for live bodyweight. In addition to the test sheep, three sheep were used as 'group sheep' and a number of others as companions, to provide company for the first and last test sheep in the pens. These sheep were never used as test sheep.

All three flocks had been raised on a University of Western Australia farm, with the two selected flocks (MA and LA) run together as one flock, and the R sheep run as part of a separate commercial flock. All MA and LA sheep used in this experiment had been tested once in the arena test with the human, and in the social isolation test at weaning (about three months prior to this experiment). R sheep had not been tested at any time. The sheep were all subject to normal husbandry procedures, including regular contact with the shepherd and his dogs e.g. moving sheep between paddocks. During the three weeks of the experiment, the test, group, and companion sheep were all run together as one flock. The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (Protocol 04/77) and University of Western Australia Animal Ethics Committee (Approval id. 04/100/318).

Experimental setup

Behavioural and physiological responses to the stimuli were tested using a modified arena test (Kilgour and Szantar-Coddington, 1995; Erhard, 2003; Beausoleil et al., 2005). All tests took place in January 2004 and were conducted between 8am and 2 pm. The arena was outdoors, but under cover, measured 7 m x 3.3 m and was divided into 5 zones on the concrete floor (Figure 4.1). The sides of the arena were 2 m high and covered in shade cloth to create a visual barrier. The stimulus was placed in zone 0, in front of a pen containing three group sheep, and test sheep had visual and limited physical access to the group sheep at all times.
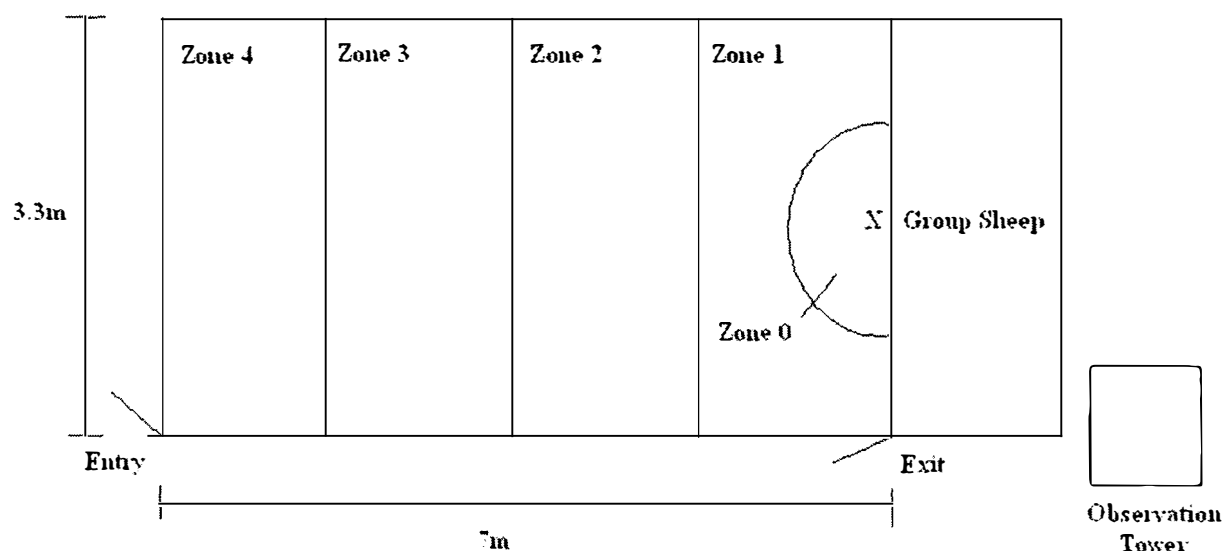
Figure 4.1 Test arena dimensions. X marks the position of the stimulus. The sides of the arena were 2 meters high and covered with shade cloth.

The same male human wearing the same green overalls for each test stood immobile in front of the group sheep, except for small movements such as weight shifts. This person was not familiar to the test sheep but assisted each morning and afternoon in moving the sheep from the paddock to the test facility.

Procedure

This procedure was part of a larger experimental design (see Chapter 5). Six sheep, two from each flock, were tested with the human each day, so that 90 tests were conducted over 15 test days (3 weeks). Sheep were randomly assigned to a test day, and the order of presentation into the arena was also randomized. Each morning, the flock was brought from the paddock into the testing facility. The sheep to be tested that day plus the group and companion sheep were drafted out of the flock, and the rest of the animals were returned to the paddock. The sheep were not familiarised with the yards, races and test arena before testing, however, the MA and LA sheep had been tested in the same arena once at weaning.

After drafting, the test sheep were left undisturbed for approximately one hour to reduce the effect of this procedure on pre-treatment plasma cortisol concentrations. Each sheep to be tested was caught by a handler and ushered into the pre-treatment pen, adjoining the arena. Here a pre-treatment blood sample was taken. The entry gate was then opened and the sheep was allowed to enter the arena. Once the sheep entered the arena, the observer began the 10-minute observation period. When the test was over, the sheep was quietly moved out of the arena and into a post-test pen, where the 10-minute blood sample was taken by the same handler (not the human stimulus). The sheep was then returned to a separate pen containing several companion sheep. At 20, 40 and 60 minutes after the start of the arena test, the handler entered this pen and took a blood sample from the test sheep. Water was available in all pens, except the testing arena, however, food was not offered in the testing facility.

Behaviour Measurement

The observer sat in a tower above the arena, located behind the group sheep pen. This position allowed the observer to see the test sheep at all times, and also to determine the focus of its attention e.g. stimulus or group sheep. The observer was not aware of which flock the test sheep was from. All behaviours were recorded continuously on a tape recorder during the test period. In addition, every 15 seconds, the zone occupied by the sheep's right front foot was recorded. If the foot was on a zone border line, the position of the left front foot was used, as this would better represent the position of the sheep's body.

Only one behaviour directly related to the selection of the different flocks was measured in this study: zones crossed (locomotor activity) in an arena test with an immobile human and flockmates. A number of non-selected behaviours were also measured. Table 4.1 presents the behaviours measured, and when necessary, gives an explanation of how the behaviour was defined or measured in this experiment.

Blood sample collection and hormone assay

Blood samples were collected into heparinized tubes by jugular venepuncture immediately before (0 mins) and then 10, 20, 40 and 60 minutes after the start of the arena test. Approximately 5mls of blood were collected each time, and placed on ice

until the sample could be centrifuged. After centrifugation, the plasma fraction was drawn off and stored at -20°C until assayed.

Samples were generally taken within 2 minutes of approaching the sheep. The time required, after the induction of a potential stressor, for the release and action of ACTH on the adrenal glands, and for the blood to circulate from the adrenal glands to the jugular vein where it was sampled, makes it unlikely that handling and venepuncture affected the plasma concentration of cortisol in the immediate sample (Broom and Johnson, 1993). However, it must be acknowledged that the handling and venepuncture required for each sample were likely to have evoked a rise in plasma cortisol concentration in subsequent samples (De Silva et al., 1986; Hargreaves and Hutson, 1990). Therefore, as well as reflecting adrenocortical responses to the testing procedure and stimulus, cortisol concentrations measured after the arena test will also reflect responses to previous sampling procedures.

A commercially available cortisol radioimmunoassay kit (Cortisol GammaCoat RIA kit CA-1549, DiaSorin Inc., Stillwater MN), designed for measurement of cortisol in human plasma, serum or urine was used. The kit was modified for use on ovine plasma by diluting the cortisol serum calibrators (to include an extra point of 13.8 nmol/L in the standard curve) and adding 25 µL of the plasma instead of 10 µL. These modifications were required to improve the sensitivity of the assay. The radioimmunoassay uses ^{125}I-labelled cortisol as a tracer, and the tubes are coated with a cortisol-specific antibody (rabbit). The cortisol in the sample competes with the ^{125}I-labelled cortisol for a limited number of antibody binding sites. The radioactive cortisol bound to the antibody is thus inversely proportion to the concentration of cortisol in the sample. The sensitivity of the cortisol assay was 3 nmol/L, and the mean inter-assay coefficient of variation was 6%.

Table 4.1 Parameters of individual sheep behaviour measured in response to a human presented in an arena test.

Behaviour	Abbreviation	Definition of behaviour
Zones crossed	CROSS	Number of times the sheep crossed from one zone of the arena into another, defined as moving the right front foot across a zone boundary
Mean distance to group sheep	MD	Mean distance (m) test sheep maintained from group sheep. The location of the sheep's right front foot was noted every 15 seconds during the test period, and mean distance was calculated by multiplying the distance from the stimulus by the proportion of time spent there
Minimum distance to group sheep	MIND	The closest distance (m) from the group sheep during the test period
Glances at stimulus	GLSTIM	Sheep looked towards the stimulus for less than three seconds before re-directing its gaze
Stares at stimulus	STSTIM	Sheep maintained its gaze at the stimulus for 3 seconds or more
Glances at group sheep	GLGS	Glances directed towards group sheep
Sniff stimulus	SNSTIM	Sheep brought its nose very close to stimulus and sniffed
Sniff group sheep	SNGS	
Sniff ground	SNGRND	
Sniff side of arena	SNSIDE	
Total bleats	BLT	All vocalizations performed
High-pitched bleats	HIBLT	Relatively loud vocalizations, made with an open mouth
Low pitched-bleats	LOBLT	Relatively quiet vocalizations (murmurs), made with a closed mouth
Foot stamps	STAMP	Front foot (or feet) were brought up off the ground and brought down with force to produce a sound on the ground
Lip licks	LIPLK	Sheep's tongue came out of its mouth in a licking motion. Each lick was counted as a separate event
Trot frequency	TROT	Sheep broke out of a walk in any direction. Trots into the arena at the beginning of the test were discounted, as these were related to the handler
Chew side of arena	CHWSD	
Urinations	URIN	
Defecations	DEF	
Latency to sniff stimulus	LSS	First interval in which test sheep sniffed the stimulus
Latency to bleat	BL	First interval in which test sheep bleated
Latency to high bleat	BLHI	First interval in which test sheep performed a high-pitched bleat
Latency to low bleat	BLLO	First interval in which test sheep performed a low-pitched bleat

Statistical Analysis

In order to examine patterns of behaviour expressed in the arena test, multivariate Principal Factor Analysis was performed on the data. Twenty-one behaviours were included; the only measured behaviour that was not included was total bleats (as this was the sum of high and low pitched bleats). Prior to factor analysis, the variables were transformed to ensure that their distributions approximated multivariate normality. Counts variables were log transformed after the addition of a constant to accommodate 0 values ($c=2$), distances from the stimulus were log transformed, and latencies were transformed using $-1/sqrt(y)$. In the factor analysis, squared multiple correlations were used as estimates of communality, and three factors, with Eigenvalues greater than 1.0, were retained to explain 71.7% of the common variation. These factors were obliquely rotated (PROMAX), as there was no reason to believe that the patterns of behaviour (factors) would be uncorrelated (De Passille et al., 1995). Individual factor scores were then analyzed using ANOVA, with flock and week of testing as main factors in the model.

Plasma cortisol concentrations were analyzed statistically using repeated measures for sampling time (0, 10, 20, 40, 60 minutes after the start of testing), with flock and week of testing as main effects in the model. Pre-treatment cortisol concentration was also included in the model as a covariate to control for individual variation in cortisol concentrations before testing. There were no significant differences in plasma cortisol concentrations with the time of day that sampling began, therefore this variable was not included in the model.

Concentrations at each sampling time were also analyzed separately, in case any minor, but biologically relevant differences were obscured in the overall model. Concentrations at individual sampling times, the integrated cortisol response (area under concentration x time curve) and the peak concentration reached within one hour after the start of testing were analyzed, with flock and week of testing as main effects in the model. Pre-treatment cortisol concentration was also included in the model as a covariate for the 10, 20, 40 and 60 minute samples and for peak response to control for individual variation in cortisol concentrations before testing. All plasma cortisol variables were log

transformed to satisfy assumptions of homoscedasticity and normal distribution of the residuals.

Differences between the flocks were examined using Least Squares (LS) Means post hoc tests with Bonferroni correction for multiple comparisons. Differences are reported as significant at $P < 0.05$. Two-tailed probabilities are given, and all data are presented as LS means of the raw data and pooled standard errors. The relationships between individual factors, and between factors and adrenocortical responses were examined by calculating Spearman rank correlations.

4.4 Results

Factor analysis

Principal factor analysis revealed three factors that accounted for almost three quarters of the common variation in behaviour (Table 4.2). The first factor, which accounted for the largest portion of the variation, had high loadings for avoidance of the human (mean and minimum distance), vigilance, and latency to sniff the human, and high negative loadings for locomotion and exploration. This factor may reflect the sheep's fear of, or aversion to, the presence of the human, and as such, is labelled *'aversion to human'*. There was a significant flock effect on aversion to the human (ANOVA, $F(2, 77) = 8.50$, $P = 0.001$); MA sheep were significantly less averse to the human than were R sheep (Paired t-test, $t = 4.09$, $P < 0.001$) and LA sheep (Paired t-test, $t = 2.46$, $P < 0.05$) (Figure 4.2).

Factor 2 had moderate to high loadings for locomotion and high-pitched vocalization and a high negative loading for latency to perform the first high-pitched bleat. This factor represents those behaviours for which the flocks were differentially selected, and is conservatively labelled *'selected behaviours'*. There was a flock effect on the expression of selected behaviours (ANOVA, $F(2, 77) = 7.14$, $P = 0.002$). LA sheep performed less of the selected behaviours than MA sheep (Paired t-test, $t = 3.74$, $P < 0.01$), and tended to perform less than R sheep (Paired t-test, $t = 2.28$, $P < 0.10$) (Figure 4.2).

Factor 3 had a high negative loading for frequency of low-pitched bleating and a high positive loading for latency to perform the first low-pitched bleat. There were no differences between the flocks for factor 3 (ANOVA, $F_{(2, 77)} = 0.28$, $P = 0.756$) (Figure 4.2).

Adrenocortical responses

Plasma cortisol concentration was significantly elevated above pre-treatment concentrations at 10 and 20 minutes after the start of testing for all flocks, but had returned to pre-treatment concentrations within one hour after the start of testing (Sampling time effect: ANOVA, $F_{(4, 445)} = 627.83$, $P < 0.0001$) (Table 4.3). In fact, 60 minutes after the start of testing, concentrations were significantly lower than pre-treatment concentrations.

The flock effect on plasma cortisol response was not statistically significant (ANOVA, $F_{(2, 87)} = 2.77$, $P = 0.064$). However, there was a tendency for LA sheep to have higher cortisol responses than R sheep (Paired t-test, $t = 2.28$, $P < 0.10$). There was no flock x sampling time effect (ANOVA, $F_{(8, 1335)} = 0.89$, $P = 0.524$) in the overall model. Nor were there any significant differences between the flocks in plasma cortisol concentration at any individual sampling time, in peak concentration or in the integrated response (Table 4.3). However, at the end of the arena test (10-minute sample), there was a weak flock effect on plasma cortisol concentration (Table 4.3). MA sheep tended to have lower plasma cortisol concentrations than LA sheep at the end of the arena test (Paired-t test, $t = 2.19$, $P < 0.10$).

Correlations

There were no significant relationships between the three behavioural factors when the human was present (Table 4.4). Nor were there any significant relationships between the behavioural factors and plasma cortisol concentrations in response to the human (Table 4.5).

Table 4.2 Results of factor analysis on behaviours performed by individual sheep in an arena test with a human.

	Factor 1	Factor 2	Factor 3
Eigenvalue	4.86	1.71	1.31
% common variation	44.2	15.6	11.9
Mean distance	**0.794**	0.135	-0.114
Minimum distance	**0.751**	-0.129	-0.125
Zones crossed	**-0.563**	**0.430**	0.019
High-pitched bleats	0.068	**0.833**	-0.167
Low-pitched bleats	0.046	0.178	**-0.675**
Glances at stimulus	**0.645**	0.195	0.141
Stares at stimulus	**0.689**	-0.036	0.205
Glances at group sheep	**0.575**	0.345	0.146
Sniffs stimulus	**-0.832**	0.178	0.074
Sniffs group sheep	**-0.697**	-0.077	0.008
Sniffs side	**-0.593**	0.088	-0.070
Sniffs ground	**-0.609**	0.258	-0.148
Defecation	-0.077	0.118	-0.209
Urination	-0.151	0.140	-0.174
Trots	0.120	-0.036	-0.015
Lip licks	-0.113	0.243	-0.120
Foot stamps	0.283	0.022	0.271
Chews side	**-0.466**	-0.089	-0.070
Latency to sniff stimulus	**0.750**	-0.106	-0.085
Latency to high bleat	-0.140	**-0.811**	-0.018
Latency to low bleat	0.008	0.006	**0.750**

Figure 4.2 Factor scores for the three flocks in the presence of the human. Columns with different letters indicate significant differences between flocks at P < 0.05. LS means and pooled standard errors are presented. R = Reference flock, LA = Less Active flock, MA = More Active flock.

Table 4.3 Plasma cortisol concentrations (nmol/L) of sheep from More Active (MA), Less Active (LA) and Reference (R) flocks, presented with a human in a 10-minute arena test. Blood samples were taken immediately before (0) and 10, 20, 40, and 60 minutes after the start of the test. Peak response up to one hour after the start of testing, and integrated response (area under concentration x time curve nmol/L*mins) are also shown. Rows with different superscripts refer to differences between sampling times, not flocks. Differences are significant at $P < 0.05$. LS means and pooled standard errors are presented.

Flock	R	LA	MA	Pooled SE	Flock effect	
					$F (2, 87)$	P
0 mins	19.1^a	20.9^a	21.3^a	2.6	0.14	*0.874*
10 mins	57.0^b	62.6^b	53.5^b	3.7	2.42	*0.096*
20 mins	48.9^b	52.2^b	44.3^b	3.6	2.09	*0.131*
40 mins	20.8^a	22.5^a	22.1^a	2.0	0.65	*0.526*
60 mins	13.4^d	15.8^d	20.4^d	3.0	2.05	*0.135*
Peak concentration	58.6	64.1	59.7	3.7	1.59	*0.210*
Integrated response	1933	2132	1959	132	1.36	*0.263*

Table 4.4 Spearman rank correlations between factors describing sheep behaviour expressed in the presence of the human. The significance level associated with each correlation is shown in italics below.

	Factor 1	Factor 2	Factor 3
Factor 1	1.0	-0.211	-0.101
		0.060	*0.375*
Factor 2		1.0	-0.164
			0.147
Factor 3			1.0

4.5 Discussion

Behavioural responses

Factor analysis was used to address the main aim of this study: to examine whether the flocks had been selected for differences in fearfulness in the presence of a human. As the original labels 'nervous' and 'calm' imply, MA sheep were expected to be more fearful, and LA were expected to be less fearful in the presence of the human.

In the present study, the behaviour of the Reference sheep suggested that they were most fearful of the human. However, this effect can be explained by a difference in recent experience between the flocks. Unbeknownst to the author at the time of the experiment, there were significant differences in the prior experience of the selected and Reference flocks. While the R sheep had never experienced the arena testing procedure before the present experiment, the selected flocks had been exposed to this situation once, about three months earlier. Therefore, the testing procedure was relatively more novel and potentially more frightening to R sheep. In addition, differences in the frequency of husbandry procedures between the two selected flocks and the Reference flock may have contributed to this effect. Therefore, any comparisons between the Reference and selected flocks have been made with this limitation in mind, and my discussion will concentrate on the differences between the two selected flocks.

Table 4.5 Spearman rank correlations between factor scores and plasma cortisol concentrations (time in minutes since start of testing) when the human was present. Peak response up to one hour after the start of testing, and integrated response (area under concentration x time curve nmol/L*mins) are also shown. The significance level associated with each correlation is shown in italics below.

	0	10	20	40	60	Peak	Integrated response
Factor 1	-0.125	0.080	0.124	-0.032	-0.171	-0.003	-0.024
	0.268	*0.482*	*0.275*	*0.777*	*0.131*	*0.976*	*0.836*
Factor 2	0.001	-0.133	-0.160	-0.020	0.044	-0.047	-0.075
	0.995	*0.239*	*0.156*	*0.862*	*0.696*	*0.680*	*0.511*
Factor 3	-0.004	-0.091	-0.044	0.088	0.178	-0.036	0.056
	0.973	*0.420*	*0.696*	*0.437*	*0.114*	*0.753*	*0.625*

Interestingly, while R sheep exhibited behavioural responses suggestive of increased fear of the human, their adrenocortical responses to the human were no higher than those of the other flocks.

The largest proportion of the common variation in behaviour was accounted for by behaviour specifically relating to the presence of the human, including avoidance and vigilance. A smaller proportion of the variation was accounted for by those behaviours upon which differential selection of the flocks was based: locomotion and high-pitched vocalization (Table 4.2). Consistent with the selection process, MA sheep expressed high levels, and LA sheep expressed low levels of these selected behaviours in the presence of the human (Figure 4.2).

However, contrary to the main hypothesis, the behaviour of MA sheep suggests that they were less fearful of, or averse to, the presence of the human in the arena than were the other flocks. MA sheep approached and interacted with the human more quickly and more often, were more active and explored the arena more, and were less vigilant. Lower levels of avoidance and vigilance are considered to indicate lower fear of, or aversion to, the stimulus in the arena test (Fell and Shutt, 1989; Chapman et al., 1994; Erhard, 2003; Beausoleil et al., 2005; Paul et al., 2005). In addition, higher levels of active behaviour may also be related to lower levels of fear in the arena test, as the presence of fear-eliciting stimuli, such as predators and humans, has been shown to suppress the expression of locomotion and exploration in sheep (Torres-Hernandez and Hohenboken, 1979; Erhard, 2003; Beausoleil et al., 2005).

There is some suggestion that selection of MA sheep for increased behavioural activity in social isolation and in the presence of a human has also resulted in lower levels of aversion to a human. However, we must consider the possibility that the increase in time spent near the stimulus was simply a consequence of the increased activity of the MA sheep, as suggested by Murphy (1999). Likewise, the decrease in vigilant behaviour may simply have related to the sheep's otherwise engagement in active behaviours, such as locomotion and exploration, rather than reflecting a lower level of fear of the human per se. However, the results presented in Chapter 5 show that while MA sheep were still more active and vocal in the presence of a predator (dog) or novel object (box) in the arena test, there were no differences in the expression of stimulus-

related aversion or fear between the three flocks with these stimuli. Therefore, the decrease in fear-related behaviour in MA sheep appears to be independent of the selected active behaviours, and specific to the presence of the human.

Adrenocortical responses

While the differences in adrenocortical responses between the flocks were not statistically significant, MA sheep tended to have lower plasma cortisol concentrations at the end of the test (10-minute sample) than did LA sheep, rather than having higher adrenocortical responses as predicted. This is a logical sampling time to expect a minor, but potentially biologically relevant difference between the flocks, as this sample would most accurately reflect the individual's subjective experience of the stimulus presented in the arena test. In addition, LA sheep tended to have higher plasma cortisol responses than R sheep overall. These results suggest that MA sheep were not more fearful or nervous, and that LA sheep were not more calm (less fearful) than the other flocks in the presence of a human.

The magnitudes of the adrenocortical responses elicited in all three flocks by the presence of a human were only moderate compared to responses reported for young and adult sheep subjected to a range of physical and psychological stressors (e.g. Coppinger et al., 1991; Lester et al., 1991; Niezgoda et al., 1993; Cockram et al., 1994; Lester et al., 1996; Dinnis et al., 1997; Kilgour and Szantar-Coddington, 1997; Cook, 2004). In addition, sheep habituated quickly to handling and blood sampling, as indicated by the return of cortisol concentrations to pre-treatment concentrations or below within one hour of the start of testing. The moderate magnitude and duration of the cortisol responses in this study indicate that none of the sheep were excessively stressed by the testing procedure or the presence of the human. As concentrations were found to be in the lower, more sensitive part of the range of adrenocortical responses, any minor differences between stimuli or flocks are likely to be definitive (Mellor et al., 2000).

Correlations

Behaviours considered indicative of fear or aversion to the human and the adrenocortical responses were not significantly correlated in this study. Previous studies have also reported that physiological changes may correlate poorly with how strongly

an animal avoids an aversive stimulus (Rushen, 1996; Dawkins, 2001). The absence of relationships between behavioural and adrenocortical responses suggests that behavioural responses may not directly reflect the level of fear or stress experienced by the animal. While plasma cortisol response may represent the individual's subjective experience of an event e.g. fear or stress (Mellor et al., 2000), it seems more likely that behavioural responses are a method of dealing with a specific problem, rather than reflecting an emotional state (Rushen, 1990). Thus, individuals may employ different behavioural strategies for dealing with the same stimulus or event, while experiencing similar levels of emotional activation (see discussion of coping strategies below).

Selection for differences in fearfulness?

The lack of statistically significant differences in adrenocortical responses between the flocks (Table 4.3) suggests that their negative subjective experiences of the presence of a human did not differ, despite significant differences in the expression of fear- or aversion-related behaviour and the selected behaviours (Figure 4.2). Kilgour and Szantar-Coddington (1997) also found no differences in the adrenocortical responses to an ACTH challenge or to a barking dog between a Merino flock selected for improved lamb survival and an unselected flock, even though the flocks differed in their behavioural responses in an arena test with a human. Similar to LA sheep in the present study, ewes selected for improved maternal ability were found to stay further away from a human, travel less far and bleat less often than unselected ewes (Kilgour and Szantar-Coddington, 1995; 1997). The lack of difference in adrenocortical responses supports the notion that the flocks used both in the present study and that of Kilgour and Szantar-Coddington have not been selected based on differences in some consistent predisposition to react fearfully in a range of contexts (e.g. fearfulness or nervousness), but instead on some context-specific behavioural response.

In previous studies on the flocks used in this experiment, increased locomotion and vocalization in the arena test with a human was interpreted as a sign of increased fearfulness, emotional reactivity or 'nervousness' (Murphy, 1999; Gelez et al., 2003). However, other studies suggest that there may be a negative, rather than positive, relationship between physiological indicators of fear or stress and the expression of active behaviour. Behrendt (1998) found that immunizing sheep against ACTH reduced

circulating cortisol concentrations and altered arena test behaviour. Immunized sheep, with lower plasma cortisol concentrations, stayed further from the human, but also had increased locomotor activity and exploratory behaviour, relative to non-immunized animals. Similarly, treatment of silver foxes (*Vulpes vulpes*) with chloditane, which inhibits adrenal cortex function, resulted in lower baseline plasma cortisol concentrations, as well as increased locomotion and exploration in a novel environment (Plyusnina et al., 1991).

Behrendt (1998) noted that the change in arena behaviour in sheep was not associated with increased fearfulness, as the immunized sheep were less vigilant in the arena test, exhibited a lower flocking instinct when tested in groups, and were easier to handle. In addition, chloditane-treated foxes showed significantly weaker behavioural fear responses in the presence of a human than did controls (Plyusnina et al., 1991). In the present study, there is weak evidence for a similar relationship; MA sheep were more active than LA sheep, and tended to have lower plasma cortisol concentrations at the end of the arena test. These results suggest that increased locomotion and exploration may relate to lower, rather than higher levels of fearfulness in the presence of a human.

Selection for differences in motivational states other than fearfulness?

Locomotor activity has also been found to correlate with motivational systems other than anxiety or fear, e.g. exploratory motivation, boldness, or simply the motivation to be physically active (Maier et al., 1988; Markel et al., 1989; Cruz et al., 1994). Boldness refers to the propensity of the individual to take risks, particularly in novel environments (Kagan et al., 1988; Wilson et al., 1994; Coleman and Wilson, 1998). For sheep, both locomotion and high-pitched vocalization increase the risk of detection by predators, and could be considered risky behaviours in the presence of a human (Dwyer, 2004). Boldness appears to be at least partially heritable (Wilson et al., 1994), and may represent the temperament dimension for which the flocks were differentially selected, with MA sheep being bolder than LA sheep.

Variation on the boldness/shyness continuum may be related to variation in the activity of the hypothalamic-pituitary-adrenal axis (Wilson et al., 1994). For example, Lyons et al. (1988b) found that shy goat kids, which avoided a human, also mounted significant

100

plasma cortisol responses to the human's presence. In contrast, in bold kids, which approached and interacted with the human, the increase in plasma cortisol concentration above baseline was not significant. Similarly, children described as 'inhibited' showed greater adrenocortical responsiveness (Kagan et al., 1988), and basal salivary cortisol was found to be negatively correlated with 'sensation seeking' in male humans (Rosenblitt et al., 2001).

These results suggest that bolder animals have lower HPA reactivity, and may experience less fear or stress in challenging or novel situations, than do shyer animals. The results of the present study concur with this idea; MA sheep, which approached and interacted with the human more often and more quickly, and which expressed higher frequencies of locomotor and vocal activity, also tended to have lower plasma cortisol concentrations than did LA sheep at the end of the arena test. Therefore, MA sheep may have been selected for boldness, and LA sheep for shyness.

Selection based on more than one motivational state?

Locomotor activity may also correlate with more than one separate underlying factor, indicating that a single behaviour can be affected by variation in a number of different motivational states (De Passille et al., 1995; Vierin and Bouissou, 2003). In the present study, locomotion loaded on two separate factors; negatively on *'aversion to the human'*, and positively on *'selected behaviours'*. Therefore, the level of locomotor activity in the arena test may reflect the simultaneous influence of two separate motivational systems.

As noted above, higher expression of active behaviour in the presence of an aversive stimulus in the arena test may reflect lower levels of fear experienced by individual sheep. In contrast, social isolation is known to increase the frequency of locomotion and vocalization in sheep (Romeyer and Bouissou, 1992; Lyons et al., 1993; Cockram et al., 1994), and high frequencies of these behaviours in the arena test have been interpreted as indicative of high levels of social motivation (Boissy et al., 2005). In fact, many of the studies relating increased locomotion and vocalization to fearfulness in sheep have involved testing animals in social isolation (e.g. Torres-Hernandez and Hohenboken,

1979; Romeyer and Bouissou, 1992; Vandenheede and Bouissou, 1993b; 1993a; Vandenheede et al., 1998; Goddard et al., 2000; Vierin and Bouissou, 2002; 2003).

Because the UWA flocks were selected not only for behavioural response to a human, but also for responses to social isolation, the higher locomotor activity of MA sheep in the arena may reflect co-selection for two context-specific temperament traits: higher social motivation (response to social isolation), and lower fear of a human (or higher boldness). The arena test involves not only the presence of a human, but also physical separation from conspecifics, so that both motivational systems could be influencing the behaviour of the test animal. Contrary to previous studies using the UWA flocks, the level of expression of the 'selected behaviours' is here interpreted as the individual's context-specific behavioural response to the event of social isolation, rather than reflecting a consistent predisposition to respond similarly in a range of fear-eliciting situations e.g. fearfulness. It is suggested that differences in the expression of active behaviour between the selected flocks would diminish if sheep were tested in groups.

The potential for two separate motivational states to simultaneously influence a single behavioural response was illustrated by Mills and Faure (1991). Four genetically distinct lines of Japanese quail (*Coturnix coturnix japonica*) were created, by selecting for either high or low social reinstatement (SR, the tendency to rejoin flockmates) or for high or low fear (TI), as indicated by the duration of tonic immobility elicited by human restraint. Selection was based on differential expression of these two motivational states because both can influence the behavioural responses of animals in fear-eliciting situations (Faure and Mills, 1998).

Chicks from all four lines were tested in a 10-minute open field test. While fear is known to inhibit active behaviour in birds in the open field test, high levels of social motivation increase activity, as the individual searches for conspecifics (Gallup and Suarez, 1980). In the open field test, both chicks selected for low fear (short TI) and those selected for high SR expressed high levels of active behaviour. In contrast, chicks selected for high fear (long TI) and low SR expressed lower levels of activity (Launay, 1993). The results of this study illustrate how different motivational systems can influence behaviour in a similar manner, while the emotional experience of the animals may differ significantly e.g. high fear or low social motivation. Therefore, caution is

required when relating similar patterns of behaviour expressed in different contexts to a single underlying temperament trait or motivational state, such as fearfulness.

Co-selection for two separate temperament traits (sociality, aversion to human) might explain the lack of statistically significant differences in emotional activation, as indicated by adrenocortical responses, between the MA and LA flocks. Each trait may have a different relationship with the level of fear or stress experienced in the testing environment. In other words, physical separation from conspecifics in the arena may have elicited higher adrenocortical responses in the highly social MA sheep, while the presence of the human may have elicited higher responses in the human-averse LA sheep.

Selection for different coping strategies?

Another way of interpreting the results of the present study is in terms of different coping strategies. The behavioural and physiological responses of MA sheep were consistent with the use of an active coping strategy, while LA sheep appeared to employ a passive or reactive strategy (Syme and Elphick, 1982; Fokkema et al., 1988; Lyons et al., 1988a; Koolhaas and Oortmerssen, 1989; Hessing et al., 1994; Jones and Saterlee, 1996). In accordance with the characteristics generally associated with a passive coping style, LA sheep showed less active behaviour and tended to have higher adrenocortical responses to the presence of the human than did MA sheep.

One problem with ascribing differences between the flocks to different coping styles is that this approach only allows for the discussion of one axis of behavioural variation, or one temperament trait (Van Reenen et al., 2004). Individuals may vary in the extent to which they show active or passive behaviours, depending on the specific challenge presented (Broom and Johnson, 1993). This means that active and passive coping strategies employed in different contexts may relate to different dimensions of temperament or different motivational states (e.g. fear of a specific stimulus, boldness, social motivation), rather than relating to one consistent predisposing factor.

A related problem is that differences in coping strategy may not be equivalent to differences in emotional reactivity or fearfulness (Steimer et al., 1997). Steimer et al.

103

(1997) found two independent dimensions underlying rat behaviour, reflecting emotional reactivity and coping strategy. Grouping by emotional reactivity led to different sets of individuals than did grouping by coping strategy. Therefore, while individuals may employ different behavioural strategies for dealing with the same stimulus or event, they may experience similar levels of emotional activation.

In the present study, the absence of a relationship between the expression of the 'selected behaviours' and adrenocortical responsiveness suggests that coping strategy, as characterized by activity in the arena, was not directly analogous to fearfulness. Inter-flock differences in active behaviour may therefore relate primarily to differences in some other neuroendocrine system, for example, sympathetic nervous system reactivity in response to challenge.

4.6 Conclusions

Based on the results of this study, it may be appropriate to say that MA and LA sheep have been selected for different 'temperaments' on the basis of differential expression of locomotion and vocalization in the presence of a human in an arena test. Consistent with the selection process, MA sheep displayed a more active behavioural character than LA sheep in the presence of a human. However, this study provided no behavioural or cortisol evidence to suggest that selection has been based on some underlying predisposition to react fearfully e.g. fearfulness, nervousness or emotional reactivity.

In contrast to previous studies, increased locomotor and vocal activity in the arena test are interpreted as a specific behavioural response to social isolation (social motivation). Rather than selection based on fearfulness, it is likely that the flocks have been co-selected for variation in two separate motivational systems: sociality, and fear/aversion of a human. Therefore, the high locomotor activity of MA sheep may have reflected both high social motivation and lower levels of fear (higher boldness) in the presence of a human.

No support was found for the hypothesis that MA sheep were more fearful or nervous than the other flocks. In fact, there was some evidence that MA sheep experienced less fear due to the presence of the human. Therefore, the use of labels such as 'nervous' and

'calm', which imply knowledge of the bearer's subjective experience of the emotional challenge (e.g. fear or stress), is, at the present time, inappropriate. For the time being, labels which simply describe the measurable differences in behaviour, on which selection of the flocks has been based, should be retained: More Active and Less Active.

While this distinction may seem pedantic, precision in terminology is vital to the understanding of concepts such as individual temperament, personality, coping strategies, and to the selection of domestic animals based on parameters such as behaviour and physiology. Without solid biological evidence to support them, such labels may unduly prejudice the interpretation of behavioural and physiological responses of the animals under study.

Future studies should look for differences in sympathetic nervous system activation between the flocks, as well as other neuroendocrine differences. In addition, the flocks should be tested in groups in order to separate the effects of social motivation and fear of a human on behavioural and adrenocortical responses.

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111

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CHAPTER 5

Selection for lower fear in domestic sheep: a context-specific or domain-general temperament trait?

5.1 Abstract

Merino sheep at the University of Western Australia have been selectively bred for their behavioural responses to social isolation and the presence of a stationary human, resulting in the creation of two divergent lines of animals: more active (MA) and less active (LA). The results presented in Chapter 4 suggested MA sheep experienced less fear or aversion in the presence of a stationary human than did LA sheep, as indicated by lower levels of fear-related behaviour and a non-significant tendency towards lower plasma cortisol concentrations at the end of the arena test (10-min sample). The aim of the present study was to determine whether the lower levels of fear exhibited by MA sheep in the presence of a human reflected selection based on a context-specific temperament trait, or selection for lower levels of general fearfulness (domain-general trait). Factor analysis of behavioural responses, and changes in plasma cortisol concentration were used to evaluate the fear responses of individual sheep to the presence of a human, a novel object (cardboard box), or a predator (dog) in an arena test. Inter-flock differences in fear-related behaviour and plasma cortisol concentration at the end of the test existed only in the presence of a human; there were no such differences between the flocks with the box or dog. Therefore, selection of MA sheep for lower fear responses to the presence of a human in the arena test appears to have been based on a context-specific trait, rather than a domain-general trait (fearfulness). Behavioural aversion appeared to be context-specific, while adrenocortical responses were more consistent for individuals across the three tests. In accordance with this, behavioural and adrenocortical responses were not significantly correlated. The individual consistency in adrenocortical responses to the three stimuli may reflect consistent levels of emotional activation (fearfulness), or consistent responses to some feature common to all three tests, e.g. physical separation from conspecifics. There is also evidence that divergent selection of the MA and LA flocks has altered the individual consistency of behavioural and adrenocortical responses across a range of contexts. There are both management and animal welfare implications associated with the potential selection of domestic sheep based on a context-specific trait, such as lower fear in the presence of a human.

5.2 Introduction

Excessive fear in domestic animals can negatively affect productivity, product quality and animal welfare (Sefton and Crober, 1976; Hutson, 1980; Craig et al., 1983; Mills and Faure, 1990; Jones, 1996; Grandin, 1997; Jones, 1997; Jones et al., 1997; Voisinet, 1997; Hemsworth and Coleman, 1998; Murphy, 1999). However, certain husbandry practices remain necessary, even though they may provoke fear or stress in domestic sheep (e.g. Hargreaves and Hutson, 1990b; 1990c; 1990d; 1990a).

The trend in modern management systems towards increased group sizes and less frequent contact between individual animals and their human handlers may also increase the stress experienced by the animals due to the presence of humans during routine procedures (Le Neindre et al., 1998). Habituation of individual sheep to human presence and handling is associated with a decrease in fear-related behaviour, as well as a reduction in plasma cortisol responses to such procedures, suggesting that tamed sheep experience less fear in the presence of humans (Pearson and Mellor, 1976). However, taming and training is a laborious and time-consuming process, and is not practical in commercial sheep husbandry.

One way to potentially decrease the fear or stress experienced by domestic animals during essential husbandry procedures is to genetically select for animals which perceive and experience such situations as less fear-provoking (Faure and Mills, 1998; Boissy et al., 2005b). Genetic selection has been successfully used to improve production-related variables such as muscle growth, fibre and milk production. While there is much evidence of individual and breed variation in fear-related behaviour, at present, there is insufficient information about behavioural genetics in domestic ruminants to develop effective selection programmes for lower levels of fear. Research into inter-individual and group variation in the perception and experience of fear-eliciting events is needed to improve our fundamental understanding of the basis and expression of fear, and to facilitate the future use of genetic tools to reduce fear levels in domestic livestock species (Boissy et al., 2005b).

Temperament and fearfulness

A significant impediment to the study of animal temperament and fear has been the inconsistency in terminology and ambiguity in the definition and measurement of behavioural and physiological indicators of temperament traits. In studies of domestic animals, 'temperament' is often used synonymously with terms such as 'fearfulness', 'emotional reactivity' and 'emotionality' (Boissy and Bouissou, 1995; Grandin and Deesing, 1998; Erhard et al., 2004). However, different problems in the evolutionary history of a species have likely resulted in a range of context-specific psychological solutions, which may be conceived as separate dimensions of personality or temperament (Cosmides and Tooby, 1987; Buss, 1991), and fearfulness is likely to reflect only one dimension of animal temperament (Boissy and Bouissou, 1995).

Fearfulness is defined as the propensity of the individual animal to perceive and react similarly to a range of potentially frightening events (Boissy, 1998). Most of the studies on domestic animal temperament involve measuring individual variation in the behavioural and physiological responses of animals to fear-inducing situations (e.g. Boivin et al., 1992; Romeyer and Bouissou, 1992; Murphy et al., 1994; Grignard, 2001; Daniewski and Jezierski, 2003). As such, the term 'fearfulness' is a more appropriate term than 'temperament' for describing variation in these responses.

However, some of the experimental tests designed to elicit fear in domestic animals may actually be measuring variation in different temperament traits (Visser et al., 2001). For example, tests may measure variation in traits such as sociality (motivation to seek conspecifics), boldness (propensity to take risks), tameness/docility (ability to cope with the presence of humans) or activity, rather than fearfulness per se. In addition, some behavioural responses expressed in fear-eliciting situations are influenced by more than one motivational state, and their expression may reflect variation in a number of temperament traits e.g. fearfulness and sociality (Mills and Faure, 1991). Therefore, concise definition of terms and careful design of experimental procedures are required to facilitate the study of temperament traits such as fear in domestic animals (Boissy et al., 2005b).

119

Temperament traits - domain general or context specific?

Another factor to consider in the study of animal temperament is whether temperament traits are domain-general or context-specific. In other words, a trait may influence individual responses similarly in a range of situations (domain-general), or may vary in its influence from context to context (context-specific) (Wilson et al., 1994). Another way to describe the difference between domain-general and context-specific temperament dimensions is in terms of 'states' and 'traits'. For example, fear is a state experienced by an individual at a given moment, and is the product of the interaction between the individual and the specific environment (context-specific). In contrast, fearfulness is a personality or temperament trait, which is the property of the individual alone, and may be stable over a range of environments (domain-general) (Zuckerman, 1983).

Studies of non-human animals have reported examples of both domain-general (Lyons et al., 1988a; Hessing et al., 1993; Boissy and Bouissou, 1995; Capitanio, 1999; Malmkvist and Hansen, 2002) and context-specific personality or temperament traits (Coleman and Wilson, 1998; Reale et al., 2000; Seaman et al., 2002; Sinn and Moltschaniwskyj, 2005). However, in many studies it has proven difficult to find consistency in individual behavioural responses to a variety of challenging or fear-eliciting contexts, some of which include social challenge or isolation, novelty, and human presence (e.g. Lawrence et al., 1991; Boivin et al., 1992; Wilson et al., 1994; Coleman and Wilson, 1998; Reale et al., 2000; Ruis et al., 2000; Seaman et al., 2002; Sinn and Moltschaniwskyj, 2005; Wilson and Stevens, 2005).

Individual variation in temperament traits is underpinned by variability in neuroendocrine, physiological and hormonal systems (Boissy, 1995). For example, individual variation in behavioural indicators of fear or boldness/shyness is reported to be associated with variation in hypothalamic-pituitary-adrenocortical (HPA) reactivity in response to challenge (e.g. Kagan et al., 1988; Lyons et al., 1988a; Lyons et al., 1988b; Sapolsky, 1990; Van Reenen et al., 2005). When faced with the same challenge, more fearful or shy animals show more pronounced adrenocortical responses (quicker response, greater magnitude, longer duration) than bolder or less fearful animals (Van Reenen et al., 2005).

This association between behavioural and physiological responses to challenging events suggests that both may be regulated by a common central mechanism (Boissy, 1995). As such, thresholds for behavioural and physiological responsiveness could be expected to increase or decrease as a unit, rather than thresholds changing independently in response to each specific stimulus or context. Therefore, temperament traits may be domain-general, not because of some evolutionarily adaptive advantage, but because of physiological and hormonal constraints (Wilson et al., 1994).

However, strong behavioural and physiological responses to every novel stimulus or situation would be energetically expensive and time-consuming when such responses are required only occasionally (Price, 2002). In addition, from an evolutionary perspective, individual differences that are adaptive in one context may not be adaptive in other contexts. For example, boldness, or the propensity to take risks, may be adaptive during exploration of a novel environment, investigation of new food sources, or during social interactions. However, boldness is likely to be less adaptive in the presence of a predator (Coleman and Wilson, 1998).

Finally, there is evidence that individual differences in physiological responsiveness to fear-eliciting situations are mediated at a high level of the central nervous system, and reflect differences in individual perception and appraisal of potential threat, rather than differences in thresholds for adrenal response (Kilgour and Szantar-Coddington, 1997; Van Reenen et al., 2005). In support, there is evidence that habituation of physiological responses to one fear-eliciting event does not necessarily affect responses to other stressful events (Hall et al., 1998). Therefore, temperament traits may influence behavioural and physiological responses in a variable manner, according to context.

Sheep selection at the University of Western Australia

Merino sheep at the University of Western Australia (UWA) have been selectively bred for differences in their behavioural responses to two potentially fear-eliciting situations: confinement in social isolation, and the presence of a stationary human in an arena test (Murphy, 1999). This selection has resulted in two divergent lines of animals: the More Active (MA) flock and the Less Active (LA) flock. The original aim of using a selection regime involving more than one potentially challenging situation was to differentiate

121

sheep based on 'fearfulness', a domain general temperament trait predisposing the individual to react similarly in a range of situations, rather than on 'fear', a context specific response (Eysenck, 1967; Zuckerman, 1983).

In previous studies using the MA and LA flocks, the higher levels of locomotor activity and vocalization expressed by MA sheep in an arena test with a stationary human were interpreted as indicative of higher fearfulness (e.g. Murphy, 1999; Gelez et al., 2003). However, in Chapter 4, I found that locomotion in the arena test with a human loaded on two separate behavioural factors; negatively on *aversion to the human* and positively on *selected behaviours*. The latter, describing variation in the expression of locomotion and high-pitched vocalization, has been interpreted as indicative of an individual sheep's level of social motivation (Boissy et al., 2005a). In addition to being more behaviourally active in the arena, MA sheep also showed lower levels of fear- or aversion-related behaviour towards the human, and tended to have lower plasma cortisol concentrations at the end of the arena test than did LA sheep.

Based on the results discussed in Chapter 4, I postulate that rather than selection based on one underlying temperament trait (fearfulness), the flocks may have been co-selected for variation in two separate traits: social motivation and fear/aversion in the presence of a human in the arena test. Therefore, the higher locomotor activity of MA sheep in the arena may simultaneously reflect higher social motivation (response to social isolation), and lower fear of a human (higher boldness or tameness).

It is important to note that, in this case, the level of response to a human (tameness) refers to the genetically mediated responses, not to tameness acquired over the animal's lifetime due to habituation or positive associative conditioning to the presence of humans (Price, 2002). None of the flocks had been 'tamed or trained' to any degree during their lifetime.

Objectives of this study

The aim of the present study was to determine whether the lower levels of fear expressed by MA sheep in the presence of a human (higher tameness or boldness), indicated by lower levels of fear-related behaviour and a non-significant tendency

towards lower adrenocortical responses at the end of the arena test (10-min sample), reflected selection based on a context-specific temperament trait, or selection for lower levels of general fearfulness (domain general trait).

If selection was based on a domain-general temperament trait, such as fearfulness, I would expect to see consistent differences in fear-related behaviour and adrenocortical responses between the flocks in a range of fear-eliciting contexts. In addition, I would expect individual animals to show consistent relative levels of fear-related behaviour and adrenocortical responses to different challenges. In contrast, if selection was based on a context-specific temperament trait, such as tameness (low levels of fear in the presence of a human), I would not expect to see any inter-flock differences in fear-eliciting situations other than the presence of a human. Nor would I expect to see individual consistency in fear-related behavioural or adrenocortical responses to different challenges.

The experimental hypotheses were: 1. MA sheep will express lower levels of fear- or aversion-related behaviour, and exhibit lower adrenocortical responses than LA or unselected Reference (R) sheep in the presence of a novel object (box) or predator (dog) in an arena test, 2. behavioural and adrenocortical indicators of fear or aversion will be correlated, 3. the relative behavioural and adrenocortical responses of individual sheep will be consistent in response to the presence of a human, novel object and predator.

The results relating to the presentation of the human stimulus have been separately discussed in Chapter 4, but are also included here to allow comparison of intra-flock responses to a range of different stimuli.

5.3   Methods and Materials

The methods used to select the UWA flocks, the animals used in this study, experimental setup, behaviour measurement, arena testing and blood sampling procedures and hormone assays were described in Chapter 4. The testing procedure described below includes those tests in which the human stimulus was presented to the sheep, which were also discussed separately in Chapter 4. The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (protocol

123

04/77) and University of Western Australia Animal Ethics Committee (approval id. 04/100/318).

Stimuli

Box: a plain cardboard box, about 0.5 m tall, used as a novel object with no biological significance to the sheep.

Human: the same male human wearing the same green overalls for each test stood immobile in front of the group sheep, except for small movements such as weight shifts. This person was not familiar to the test sheep but assisted each morning and afternoon in moving the sheep from the paddock to the test facility.

Dog: an adult female Red Heeler, experienced in working with sheep. This particular dog was not familiar to the test sheep, and spent most of the time lying down. The dog's behaviour was mostly passive (very little attention paid to the sheep), although she did bark and snap when aggressively and repeatedly confronted by one sheep. This particular test had to be stopped less than 2 minutes after it began.

Procedure

Each individual sheep was tested once per week for three consecutive weeks, each time being presented with a different stimulus. Sheep from each flock were randomly assigned to a test day, and each animal was tested on the same day each week, and in the same order. In the first week, sheep order was randomized, as was stimulus presentation. In subsequent weeks, the order of stimulus presentation was fixed for each sheep, according to a randomized block design so that treatment order was balanced over the three flocks (e.g. if the box was presented in first week, the sheep was then presented with the human or dog in the second week, and the remaining stimulus in the third week).

Eighteen sheep were tested each day, so that 270 tests could be conducted over 15 test days (3 weeks). The time of testing for each sheep was kept as similar as possible between weeks (± 1 hour 45 mins) to reduce potentially confounding effects of circadian fluctuations in plasma cortisol concentration (De Silva et al., 1986; Ingram et

124

al., 1999). Each stimulus was presented to two sheep from each flock every day, for a total of six presentations of that stimulus per testing day.

Statistical Analysis

Principal factor analysis was used to elucidate patterns of behaviour expressed by sheep in the arena, as described in Chapter 4. Individual factor scores were analyzed using repeated measures for stimulus, with the effect of flock tested using sheep(flock) as a random effect, and week of testing as a further main effect in the model.

Plasma cortisol concentrations were analyzed statistically using doubly repeated measures for sampling time (0, 10, 20, 40, 60 minutes after the start of testing) and stimulus, with the effect of flock tested using sheep(flock) as a random effect, and week of testing as a further main effect in the model. Pre-treatment cortisol concentration was also included in the model as a covariate to control for individual variation in cortisol concentrations before testing. There were no significant differences in plasma cortisol concentrations with the time of day that sampling began, therefore this variable was not included in the model.

Concentrations at each sampling time were also analyzed separately, in case any minor, but biologically relevant differences were obscured in the overall model. Concentrations at individual sampling times, the integrated cortisol response (area under concentration x time curve) and the peak concentration reached within one hour after the start of testing were analyzed using repeated measures for stimulus, with the effect of flock tested using sheep(flock) as a random effect, and week of testing as a main effect in the model. Pre-treatment cortisol concentration was also included in the model as a covariate for the 10, 20, 40 and 60 minute samples and for peak response to control for individual variation in cortisol concentrations before testing. All plasma cortisol variables were log transformed to satisfy assumptions of homoscedasticity and normal distribution of the residuals.

Differences between flocks with each stimulus were examined using Least Squares (LS) Means post hoc tests with Bonferroni correction for multiple comparisons. Differences are significant at P < 0.05 unless otherwise stated. Tendencies are reported at P < 0.10.

125

Two-tailed probabilities are given, and all data are presented as LS means of the raw data and pooled standard errors.

The relationships between individual behavioural factors, and between factors and adrenocortical responses were examined by calculating Spearman rank correlations. Significance levels were adjusted for multiple correlations and are therefore given as $P < 0.05$, $P < 0.10$ or non-significant (NS). Correlations were calculated including responses to all three stimuli (overall), and also separately for responses in the presence of the box, human and dog. Correlations were also calculated with all flocks pooled, and separately for each flock, as selection could have altered the relationships between behavioural factors, or between behavioural and adrenocortical responses in MA and LA sheep, relative to unselected (R) sheep.

Individual consistency in behavioural and adrenocortical response to the different stimuli was examined by calculating Spearman rank correlations between individual responses to pairs of stimuli (box: human, box: dog, human: dog). Correlations were calculated for all sheep (all flocks pooled), and also separately for each flock. In order to reveal inter-flock differences in individual consistency, analysis of covariance (ANCOVA) on ranked data was used to test the homogeneity of the flock slopes from linear regressions between pairs of stimuli.

5.4 Results

Factor analysis

Principal factor analysis revealed three factors that accounted for almost three quarters of the common variation in behaviour (Table 5.1). The first factor, which accounted for the largest portion of the variation, had high loadings for avoidance of the stimulus (mean and minimum distance), vigilance, and latency to sniff the stimulus, and high negative loadings for locomotion and exploration. This factor may reflect the sheep's fear of, or aversion to, the presence of the stimulus, and as such, is labelled *'aversion to the stimulus'*.

126

There was a weak flock effect on aversion to the stimulus (Table 5.2); overall MA sheep showed less aversion to the stimulus than did LA sheep (Paired t-test: $t_{161} = 3.09$, $P < 0.01$) and R sheep ($t_{161} = 2.74$, $P < 0.05$). Overall, sheep exhibited the most aversion to the dog, intermediate levels to the human, and the least with the box (Dog versus Box, $t_{163} = 22.37$, $P < 0.0001$; Dog versus Human $t_{160} = 18.55$, $P < 0.0001$; Box versus Human $t_{161} = 3.57$, $P = 0.0015$). There was also a significant flock x stimulus interaction effect on aversion to the stimulus (Table 5.2). While there were no significant inter-flock differences in aversion to the box or to the dog, MA sheep were significantly less averse to the human than were LA sheep ($t_{52} = 3.13$, $P < 0.05$) and R sheep ($t_{51} = 4.91$, $P < 0.001$) (Figure 5.1). All flocks were more averse to the two live stimuli than to the box. However, whereas LA and R sheep showed no difference in aversion to the two live stimuli, MA sheep showed less aversion to the human than to the dog (all Ps < 0.001, Figure 5.2).

Factor 2 had moderate to high loadings for locomotion and high-pitched vocalization and a high negative loading for latency to perform the first high-pitched bleat (Table 5.1). This factor represents those behaviours for which the flocks were differentially selected, and as such is labelled 'selected behaviours'. There was a significant flock effect on the expression of selected behaviours, but no significant flock x stimulus interaction (Table 5.2). Overall MA sheep performed more selected behaviour than R sheep, which performed more than LA sheep (all Ps < 0.001, Figure 5.1). All flocks performed the selected behaviours most frequently with the box, significantly less often with the human, and least often with the dog (all Ps < 0.001, Figure 5.2).

Factor 3 had a high negative loading for frequency of low-pitched bleating and a high positive loading for latency to perform the first low-pitched bleat (Table 5.1). As such, it is labelled 'lack of low-pitched bleating'. There was no significant flock effect on this factor, nor any significant flock x stimulus interaction effect (Table 5.2). In all flocks, the presence of the dog significantly reduced the performance of low-pitched bleating relative to the other stimuli (Table 5.2, Figure 5.2, all Ps < 0.001).

127

Table 5.1 Results of factor analysis on behaviours performed by individual sheep in an arena test with a box, human or dog. For detailed descriptions of each behaviour see Table 4.1.

	Factor 1	Factor 2	Factor 3
Eigenvalue	4.86	1.71	1.31
% common variation	44.2	15.6	11.9
Mean distance	**0.794**	0.135	-0.114
Minimum distance	**0.751**	-0.129	-0.125
Zones crossed	**-0.563**	**0.430**	0.019
High-pitched bleats	0.068	**0.833**	-0.167
Low-pitched bleats	0.046	0.178	**-0.675**
Glances at stimulus	**0.645**	0.195	0.141
Stares at stimulus	**0.689**	-0.036	0.205
Glances at group sheep	**0.575**	0.345	0.146
Sniffs stimulus	**-0.832**	0.178	0.074
Sniffs group sheep	**-0.697**	-0.077	0.008
Sniffs side	**-0.593**	0.088	-0.070
Sniffs ground	**-0.609**	0.258	-0.148
Defecation	-0.077	0.118	-0.209
Urination	-0.151	0.140	-0.174
Trots	0.120	-0.036	-0.015
Lip licks	-0.113	0.243	-0.120
Foot stamps	0.283	0.022	0.271
Chews side	**-0.466**	-0.089	-0.070
Latency to sniff stimulus	**0.750**	-0.106	-0.085
Latency to high bleat	-0.140	**-0.811**	-0.018
Latency to low bleat	0.008	0.006	**0.750**

Table 5.2 Main effects and first-order interactions from ANOVA on individual factor scores. Degrees of freedom: main effects F (2, 243); first order interactions F (4, 236).

	Factor 1		Factor 2		Factor 3	
	F	*P*	F	*P*	F	*P*
Model	7.99	*<0.0001*	5.92	*<0.0001*	2.60	*<0.0001*
Flock	3.46	*0.036*	12.53	*<0.0001*	0.93	*0.400*
Stimulus	287.87	*<0.0001*	34.10	*<0.0001*	24.64	*<0.0001*
Flock x Stimulus	4.74	*0.001*	0.76	*0.550*	0.97	*0.427*

Correlations between behavioural factors

Overall, there was a moderate negative relationship between aversion to the stimulus and the expression of selected behaviours, and this was true for all flocks; sheep that showed less aversion to the stimulus were also more active and vocal (Table 5.3). However, when the stimuli were examined separately, this relationship was significant only with the dog (Table 5.3). When the flocks were examined separately, those Reference sheep that were more averse to the dog also expressed less of the selected behaviours. Likewise, within the MA flock, those sheep that were more averse to the human were also less active (Table 5.3).

Aversion to the stimulus was not significantly related to the performance of low-pitched bleats. However, sheep expressing more locomotor activity and high-pitched vocalization also tended to perform more low pitched bleats (negative relationship between factors 2 and 3). This was particularly so for MA sheep, and the relationship was strongest in the presence of the dog (Table 5.3).
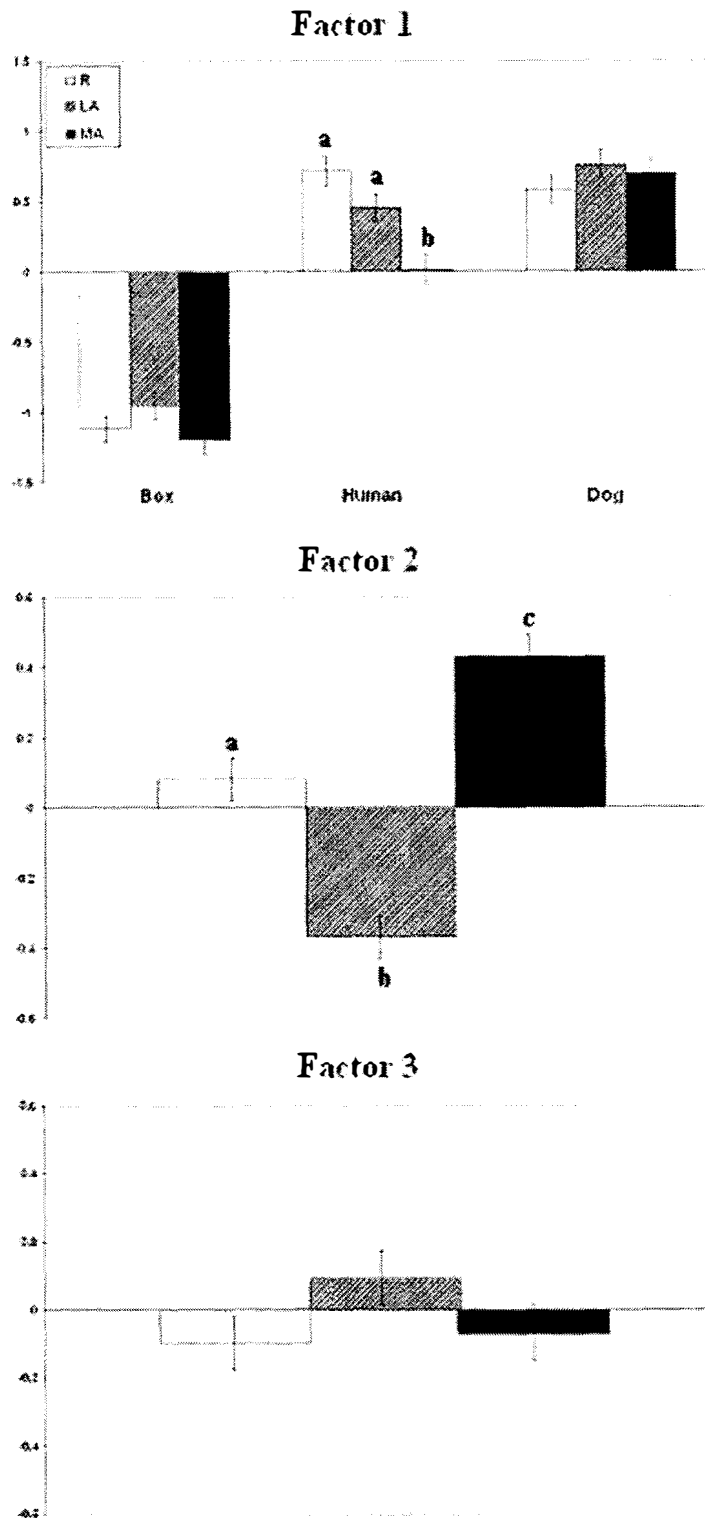
Figure 5.1 Factor scores for the three flocks by stimulus. Flock x stimulus effect was not significant for factors 2 or 3; overall flock effects are shown. Columns with different letters indicate significant differences between flocks at P < 0.05. LS means and pooled standard errors are presented. R = Reference flock; LA = Less Active flock; MA = More Active flock.

130
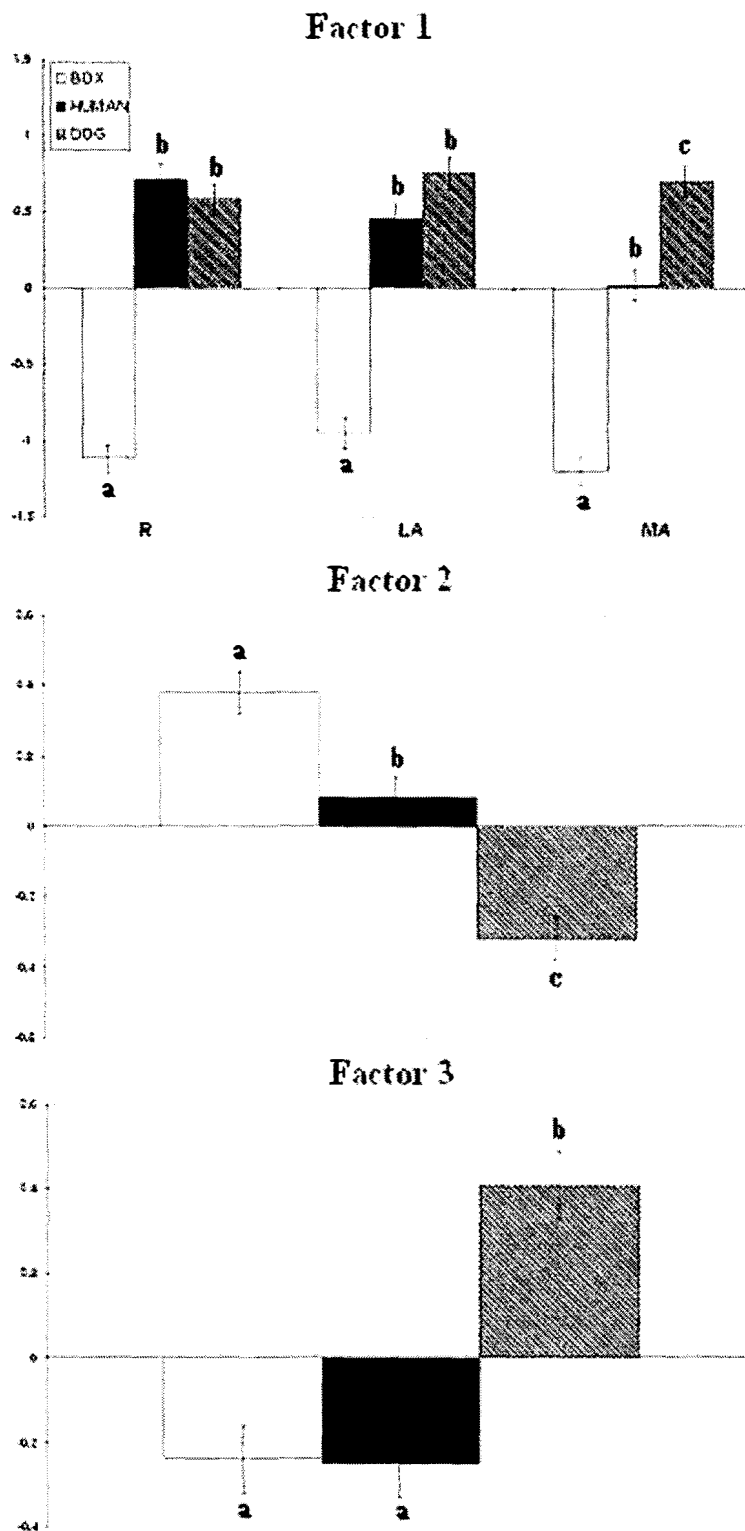
Figure 5.2 Factor scores of each flock in response to the three stimuli. Flock x stimulus effect was not significant for factors 2 or 3; overall stimulus effects are shown. Columns with different letters indicate significant differences between stimuli at P < 0.001. LS means and pooled standard errors are presented. R = Reference flock; LA = Less Active flock; MA = More Active flock.

131

Table 5.3 Spearman rank correlation coefficients (r_s) between behavioural factors calculated for all flocks pooled, and separately for each flock: Reference (R); Less Active (LA); More Active (MA), and for each stimulus. Significance levels corrected for multiple correlations are presented in italics beneath each coefficient.

	Pooled	R	LA	MA
Factor 1: Factor 2				
Overall	**-0.417**	**-0.332**	**-0.402**	**-0.503**
	<0.001	*<0.01*	*<0.001*	*<0.001*
Box	-0.165	0.211	-0.310	-0.011
	NS	*NS*	*NS*	*NS*
Human	-0.211	-0.191	0.074	**-0.491**
	NS	*NS*	*NS*	*<0.05*
Dog	**-0.383**	**-0.497**	-0.289	-0.321
	<0.01	*<0.05*	*NS*	*NS*
Factor 1: Factor 3				
Overall	**0.160**	0.206	0.056	0.192
	<0.05	*NS*	*NS*	*NS*
Box	-0.024	0.033	-0.031	-0.218
	NS	*NS*	*NS*	*NS*
Human	-0.100	-0.027	-0.275	0.103
	NS	*NS*	*NS*	*NS*
Dog	0.051	0.058	-0.035	0.030
	NS	*NS*	*NS*	*NS*
Factor 2: Factor 3				
Overall	**-0.256**	-0.194	-0.209	**-0.298**
	<0.001	*NS*	*NS*	*<0.05*
Box	**-0.234**	-0.100	-0.195	-0.106
	<0.10	*NS*	*NS*	*NS*
Human	-0.163	-0.207	-0.248	-0.162
	NS	*NS*	*NS*	*NS*
Dog	-0.089	0.182	0.120	**-0.407**
	NS	*NS*	*NS*	*<0.10*

Adrenocortical responses

Pre-treatment plasma cortisol concentrations were not affected by flock, stimulus or week of testing. Concentrations were significantly elevated above pre-treatment concentrations at 10, 20 and 40 minutes after the start of testing with all stimuli and for all flocks, but had returned to pre-treatment levels within one hour after the start of testing (Sampling time effect, Table 5.4, Table 5.5). In fact, 60 minutes after the start of testing, concentrations were significantly lower than pre-treatment concentrations. Peak responses were about 2.5-3.5 times the pre-treatment concentrations.

In the overall model, the presence of the dog elicited significantly higher adrenocortical responses than the box (Table 5.5, Paired t-test: $t_{178} = 3.79$, $P < 0.001$) and human ($t_{178} = 2.83$, $P < 0.05$). There was no significant difference in response to the box and human ($t_{178} = 0.96$, NS). Integrated and peak cortisol responses were also higher with the dog than with the box (Table 5.5, Paired t-tests: integrated response, $t_{178} = 3.63$, $P < 0.01$; peak response, $t_{178} = 4.08$, $P < 0.001$), and higher with the dog than with the human (integrated response, $t_{178} = 2.88$, $P < 0.05$; peak response, $t_{178} = 3.14$, $P < 0.01$).

There was no significant flock effect on plasma cortisol concentration in the overall model, however there was a weak flock x stimulus interaction effect (Table 5.5). While there were no differences between the flocks in response to the box or dog, LA sheep tended to have higher adrenocortical responses than R sheep to the presence of the human (Paired t-test: $t_{58} = 2.90$, $P < 0.10$). In addition, while there were no significant differences in the adrenocortical responses of MA and LA sheep to the three stimuli, R sheep had higher responses to the dog than to the human (Paired t-test: $t_{58} = 3.41$, $P < 0.05$).

Table 5.4 Plasma cortisol concentrations (nmol/L) of sheep from Reference (R), Less Active (LA) and More Active (MA) flocks, presented with a cardboard box, human or dog in a 10-minute arena test. Blood samples were taken immediately before (0) and 10, 20, 40, and 60 minutes after the start of the test. Peak response up to one hour after the start of testing, and integrated response (area under concentration x time curve nmol/L*mins) are also shown. Rows with different superscript letters refer to significant differences between the flocks in response to the human. Rows with different subscript letters refer to significant differences in the responses of MA sheep to the three stimuli. Differences are significant at $P < 0.05$. LS means and pooled standard errors are presented.

Stimulus	Flock	0 min	10 min	20 min	40 min	60 min	Peak	Integrated response
Box	R	19.1	50.2	50.8	21.2	12.7	56.6	1915
	LA	19.7	59.1	50.0	19.3	11.0	59.9	1936
	MA	16.1	$_{xy}58.0$	46.6	21.3	13.5	58.8	1939
Human	R	19.1	56.7^{ab}	48.5	20.7	13.3	58.3	1940
	LA	20.9	62.6^{a}	52.5	22.6	15.8	64.2	2121
	MA	21.3	$_{x}53.2^{b}$	44.5	22.1	20.3	59.6	1943
Dog	R	24.2	60.7	55.8	24.8	15.8	65.4	2197
	LA	18.1	63.5	54.7	22.0	13.4	66.4	2128
	MA	19.9	$_{y}65.1$	58.9	25.1	14.4	69.5	2280
Pooled SE		**2.0**	**2.4**	**2.7**	**1.6**	**2.2**	**2.6**	**89**

Table 5.5 Main effects and selected first-order interactions from ANOVA on plasma cortisol responses of sheep, before and after a 10-minute arena test. Degrees of freedom are listed below each effect. Time parameters refer to the time of sampling after the start of the arena test. Effects on peak response up to one hour after the start of testing, and the integrated response (area under concentration x time curve nmol/L*mins) are also shown.

Parameter	Flock $F_{(2, 267)}$	P	Stimulus $F_{(2, 267)}$	P	Sampling time $F_{(4, 265)}$	P	Flock x Stimulus $F_{(4, 261)}$	P
Overall response	0.12	0.885	7.74	0.001	627.83	<0.0001	2.14	0.074
0 min	0.11	0.898	0.80	0.500			0.68	0.608
10 min	1.44	0.242	6.16	0.003			2.48	0.046
20 min	0.91	0.406	5.20	0.006			1.00	0.411
40 min	0.01	0.992	2.96	0.055			1.07	0.373
60 min	1.16	0.317	0.77	0.465			1.32	0.264
Peak response	0.73	0.484	9.12	0.001			0.93	0.449
Integrated response	0.18	0.834	7.35	0.001			1.28	0.280

There was no significant flock effect on integrated response or peak concentration nor on plasma cortisol concentration at any of the individual sampling times (Table 5.5). However, at the end of the arena test (10-minute sample) there was a weak flock x stimulus effect on concentration. While there were no differences between the flocks in cortisol concentration at the end of the test in response to the box or dog, MA sheep had significantly lower concentrations than LA sheep in response to the human (Table 5.4, Paired t-test: $t_{58} = 3.21$, P < 0.05). At this sampling time, R sheep tended to have higher concentrations in response to the dog than to the box (Paired t-test: $t_{58} = 2.82$, P < 0.10). Whereas both LA and R sheep had similar concentrations in response to the two live stimuli (Paired t-tests: LA $t_{58} = 0.11$, NS; R $t_{58} = 1.22$, NS), MA sheep had significantly lower plasma cortisol concentrations at the end of the test (10-minute sample) in response to the human than to the dog (Table 5.4, Paired t-test: $t_{58} = 3.62$, P < 0.01).

Correlations between behavioural factors and adrenocortical responses

There were no significant correlations between the behavioural factors and any of the plasma cortisol variables. This was true overall (Table 5.6), and when the relationships were examined separately for each stimulus (data not shown). Nor were there any significant correlations when the flocks were examined separately, either overall, or with each stimulus separately (data not shown).

Individual consistency in behavioural responses

Table 5.7 shows measures of consistency in the behavioural responses of individual sheep to the novel object, human and dog. When the flocks were combined, behavioural aversion to the stimulus (factor 1) was moderately consistent, but only in response to the human and dog (Table 5.7). However, there was a tendency toward inter-flock differences in this relationship (ANCOVA for testing homogeneity of slopes: $F(5, 66) = 2.52$, P = 0.080, Figure 5.3a). When the correlations were examined separately for each flock, individual R sheep showed highly consistent levels of aversion to the human and dog; R sheep that were more averse to the human were also more averse to the dog. In LA sheep, there was a weak, but non-significant relationship between aversion to the dog and to the human, but there was no such relationship for MA sheep (Figure 5.3a).

136

Table 5.6 Spearman rank correlation coefficients (r_s) between factor scores and plasma cortisol concentrations (time in minutes since start of testing) over all three stimuli. Significance levels corrected for multiple correlations are presented in italics beneath the coefficients (P values are recorded as Not Significant (NS) if corrected P > 0.10). Peak is the peak response reached up to one hour after the start of testing. Integrated response is the area under concentration x time curve (nmol/L*mins).

Cortisol Concentration	0 min	10 min	20 min	40 min	60 min	Peak	Integrated response
Factor 1	0.091	0.144	0.063	0.001	-0.007	0.106	0.066
	NS	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Factor 2	-0.130	-0.123	-0.071	-0.022	0.049	-0.070	-0.071
	NS	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*
Factor 3	0.069	0.103	0.054	-0.005	0.048	0.106	0.076
	NS	*NS*	*NS*	*NS*	*NS*	*NS*	*NS*

The expression of selected behaviours (factor 2) was moderately consistent across all stimulus tests when the flocks were combined (Table 5.7). However, there was a non-significant tendency toward inter-flock differences in the relationship between expression with the human and with the dog (ANCOVA: $F_{(5, 66)} = 2.18$, $P = 0.121$, Figure 5.3b). When the flocks were examined separately, individual R sheep expressed consistent levels of the selected behaviours with all three stimuli, whereas in the MA and LA flocks, this relationship was not significant (Figure 5.3b). There was no individual consistency in the performance of low bleating (factor 3).

Individual consistency in adrenocortical responses

When the flocks were combined, individual adrenocortical responses were moderately to highly consistent across all stimulus tests, with the exception of concentrations at 60 minutes after the start of testing (Table 5.8). There was a tendency toward inter-flock differences in the consistency of plasma cortisol concentration at the end of the arena test (10-minute sample) in response to the human and to the dog (ANCOVA for testing homogeneity of slopes: $F_{(5, 84)} = 2.26$, $P = 0.110$, Figure 5.4a). When the flocks were examined separately, plasma cortisol concentration at the end of the human and dog tests was moderately to highly consistent for individuals from R and LA flocks, but this relationship was weaker for MA sheep (Figure 5.4a).

Peak and integrated adrenocortical responses to the human and dog were also moderately consistent when all flocks were combined. There were no inter-flock differences in individual consistency for these variables (ANCOVA: peak response, $F_{(5, 84)} = 1.18$, $P = 0.311$; integrated response $F_{(5, 84)} = 0.03$, $P = 0.972$; Figure 5.4b and c). Likewise, peak and integrated responses were moderately consistent between the other stimulus tests. However, the relationship between peak response to the box and to the human tended to differ according to flock (ANCOVA: $F_{(5, 84)} = 2.72$, $P = 0.072$). While MA sheep had consistent peak responses to the human and box, R sheep did not.

Table 5.7 Spearman rank correlation coefficients (r_s) between individual factor scores in response to a novel object (box), human or dog, calculated separately for each flock: Reference (R); Less Active (LA); More Active (MA) (N=26 per comparison), and for all flocks combined (Pooled; N=80 for comparisons between human and other stimuli, and N=82 for dog: box comparisons). Significance levels corrected for multiple correlations are presented in italics beneath the coefficients (P values are recorded as Not Significant (NS) if corrected P > 0.10).

Stimulus Pair	Flock	Factor 1	Factor 2	Factor 3
Human: Dog				
	Pooled	**0.308** *<0.05*	**0.362** *<0.01*	0.233 *NS*
	R	**0.722** *<0.001*	**0.653** *<0.01*	0.403 *NS*
	LA	0.310 *NS*	0.203 *NS*	0.121 *NS*
	MA	0.008 *NS*	0.045 *NS*	0.248 *NS*
Human: Box				
	Pooled	0.014 *NS*	**0.457** *<0.001*	0.078 *NS*
	R	-0.200 *NS*	**0.626** *<0.01*	-0.036 *NS*
	LA	-0.060 *NS*	0.122 *NS*	-0.198 *NS*
	MA	0.219 *NS*	0.214 *NS*	0.376 *NS*
Box: Dog				
	Pooled	0.019 *NS*	**0.389** *<0.01*	0.108 *NS*
	R	0.091 *NS*	0.481 *NS*	0.218 *NS*
	LA	-0.014 *NS*	0.102 *NS*	0.125 *NS*
	MA	-0.108 *NS*	0.162 *NS*	0.012 *NS*

a.
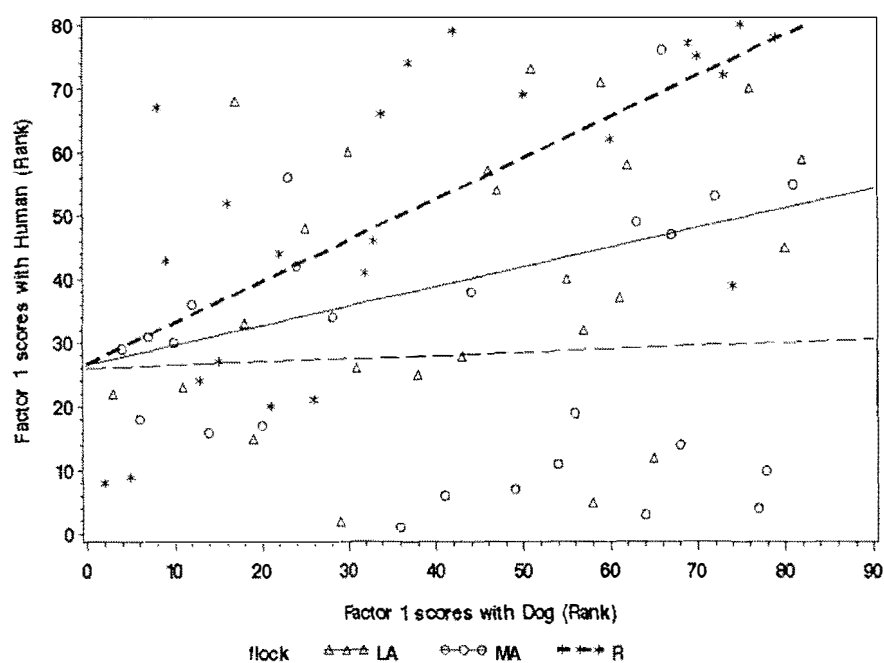
b.
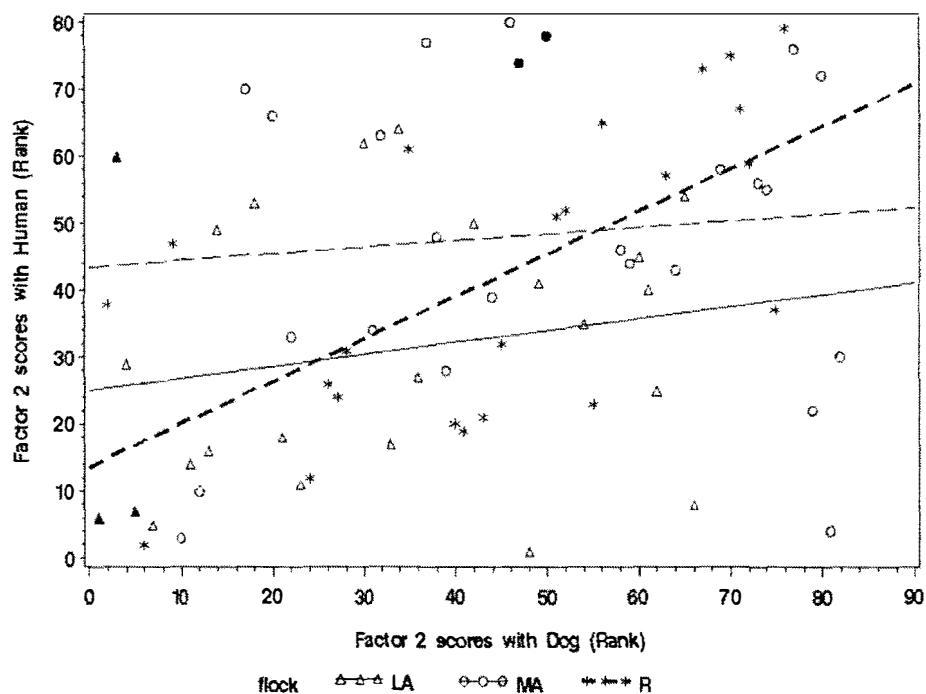
Figure 5.3 Relationships between behavioural factor scores (ranks) in response to a Human and to a Dog for the three flocks (R = Reference flock, LA = Less Active flock, MA = More Active flock), **a.** Factor 1 scores, **b.** Factor 2 scores. Each point represents an individual sheep, and l ines represent separate linear regressions for each flock

140

a.
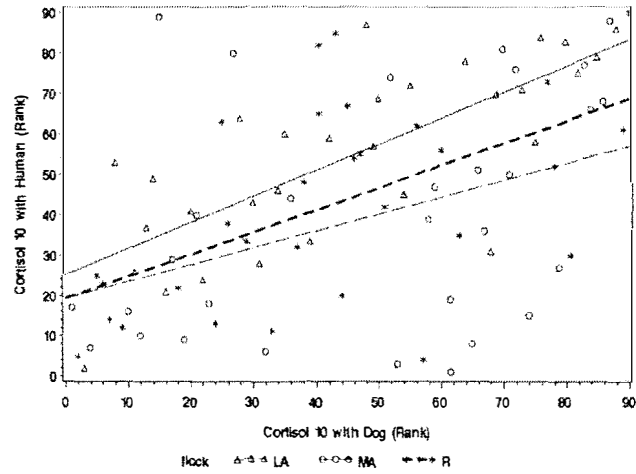
b.
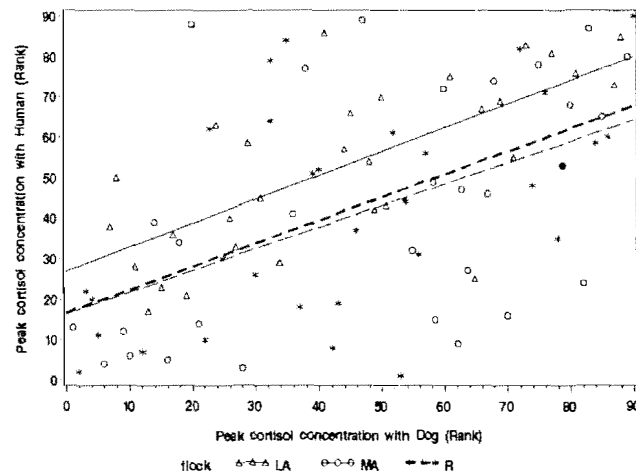
c.
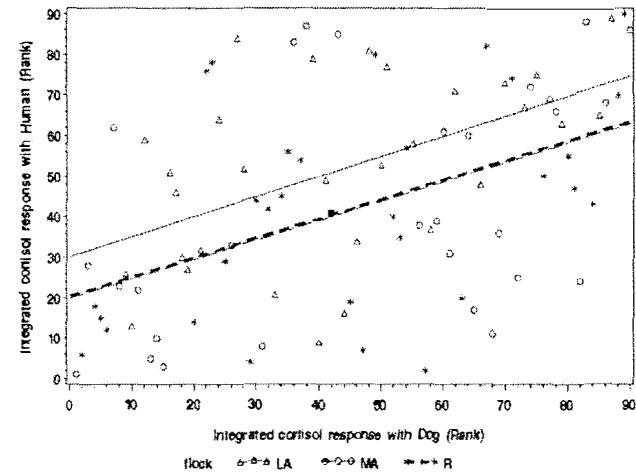
Figure 5.4 Relationships between plasma cortisol responses (ranks) to a Human and to a Dog for the three flocks (R = Reference, LA = Less Active, MA = More Active), **a.** Plasma cortisol concentration at the end of arena test (10-min sample), **b.** Peak cortisol concentration reached within one hour of the start of testing, **c.** Integrated cortisol response (area under concentration x time curve (nmol/L*mins)). Each point represents an individual sheep, and lines represent separate linear regressions for each flock

141

Table 5.8 Spearman rank correlation coefficients (r_s) and significance levels (in italics, corrected for multiple correlations) between plasma cortisol concentration in response to a novel object (box), human or dog, calculated separately for each flock: Reference (R); Less Active (LA); More Active (MA) (N=30 per comparison), and for all flocks combined (Pooled; N=90). 0-60 min refer to plasma cortisol concentrations at 0 – 60 mins after the start of the arena test. Also shown are peak concentration reached within one hour of the start of the arena test, and the integrated cortisol response (area under Concentration x Time curve (nmol/L*mins)). *Table continued on overleaf.*

Stimulus Pair	Flock	0 min	10 min	20 min	40 min	60 min	Peak	Integrated response
Human: Dog								
	Pooled	**0.339** *<0.01*	**0.519** *<0.001*	**0.441** *<0.001*	**0.292** *<0.05*	0.145 *NS*	**0.550** *<0.001*	**0.470** *<0.001*
	R	0.340 *NS*	**0.515** *<0.10*	0.343 *NS*	0.405 *NS*	0.103 *NS*	0.481 *NS*	0.465 *NS*
	LA	0.466 *NS*	**0.744** *<0.001*	0.392 *NS*	0.200 *NS*	**0.553** *<0.05*	**0.698** *<0.001*	**0.520** *<0.10*
	MA	0.145 *NS*	0.403 *NS*	**0.569** *<0.05*	0.408 *NS*	-0.154 *NS*	**0.533** *<0.05*	**0.499** *<0.10*
Human: Box								
	Pooled	**0.370** *<0.01*	**0.502** *<0.001*	**0.417** *<0.001*	**0.301** *<0.05*	0.220 *NS*	**0.456** *<0.001*	**0.442** *<0.001*
	R	**0.545** *<0.05*	0.283 *NS*	0.233 *NS*	0.275 *NS*	0.141 *NS*	0.182 *NS*	0.372 *NS*
	LA	0.329 *NS*	**0.503** *<0.10*	0.438 *NS*	0.287 *NS*	0.300 *NS*	0.475 *NS*	0.457 *NS*
	MA	0.283 *NS*	**0.606** *<0.01*	0.489 *NS*	0.347 *NS*	0.232 *NS*	**0.621** *<0.01*	**0.532** *<0.10*

Stimulus Pair	Flock	0 min	10 min	20 min	40 min	60 min	Peak	Integrated response
Box: Dog								
	Pooled	0.227 *NS*	**0.511** *<0.001*	**0.406** *<0.001*	**0.370** *<0.01*	0.254 *NS*	**0.488** *<0.001*	**0.522** *<0.001*
	R	0.385 *NS*	0.394 *NS*	0.381 *NS*	0.391 *NS*	0.363 *NS*	0.417 *NS*	**0.515** *<0.10*
	LA	0.171 *NS*	**0.508** *<0.10*	0.242 *NS*	0.360 *NS*	0.166 *NS*	0.452 *NS*	0.468 *NS*
	MA	0.156 *NS*	**0.514** *<0.10*	**0.548** *<0.05*	0.301 NS	0.166 NS	**0.554** *<0.05*	**0.545** *<0.05*

5.5 Discussion

The aim of the present study was to determine whether lower levels of fear expressed behaviourally and physiologically by MA sheep in the presence of a stationary human reflected selection based on some consistent underlying predisposition of the individual to respond similarly in a range of fear-eliciting situations (domain-general temperament trait, fearfulness); alternatively, selection was based on a context-specific temperament trait operating only in the presence of a stationary human in the arena test.

Flock responses to the human

To briefly reiterate what was presented in Chapter 4, MA sheep showed less fear- or aversion-related behaviour than the other flocks in the presence of the human, and had lower plasma cortisol concentrations at the end of the arena test (10-minute sample) than did LA sheep. MA sheep expressed higher levels of the selected behaviours than the other flocks.

While statistically significant inter-flock differences in the 10-minute plasma cortisol concentrations suggest that MA sheep experienced lower levels of fear or stress than the other flocks due to the presence of a stationary human in the arena test, the lack of significant differences in the integrated and peak responses imply that the flocks were not particularly different in terms of their adrenocortical reactivity to challenge. The integrated response may have reflected the individual's emotional activation (fear or stress) due to the entire testing procedure, including holding in the yards, repeated capture and handling by an unfamiliar human, and repeated venepuncture, as well as the experience of the human in the 10-minute arena test.

If MA sheep had been selected to be less fearful of humans in general (more tame), I would have expected to see lower adrenocortical responses to the overall procedure which included handling by humans, which was not the case. The inter-flock differences in adrenocortical responsiveness to a human appear to have existed only within the context of the arena test, and it is unlikely that the flocks have been selected for differences in general fearfulness. Therefore, the present discussion on the inter-flock differences in fearfulness will be limited to the context of the arena test.

Flock responses to the novel object and predator

Contrary to the first hypothesis, the behaviour of MA sheep did not indicate lower levels of fear or aversion in the presence of the novel object (box) or predator (dog). There were no significant differences in behaviour indicative of '*aversion to the stimulus*' between the flocks in the presence of the box or dog. Nor were there any significant differences in the adrenocortical responses of the flocks to the presence of these stimuli, supporting the idea that the levels of fear or stress experienced by the flocks with the box or dog were not different. These results suggest that lower levels of fear in MA sheep in the arena test were specific to the presence of a human. Therefore, selection of MA sheep appears to have been based on a context-specific trait expressed in the arena test, rather than for a consistent predisposition to react similarly in a range of fear-eliciting situations e.g. fearfulness (domain-general).

Although MA sheep appeared to be less fearful in the presence of the human, it is possible that the lower apparent avoidance of the human was related to the higher levels of social motivation of MA sheep. As discussed in Chapter 4, the UWA flocks may have been co-selected for two separate traits, sociality and aversion to the human, both of which may influence the expression of behaviour in the arena test. However, selection for higher sociality alone may be sufficient to explain the higher propensity of MA sheep to come and stay close to the front of the arena near their conspecifics, giving the erroneous impression of lower aversion to the human.

While this is possible, the flocks did not differ in their behavioural aversion to the novel object or to the predator, even though MA sheep were more active and vocal (indicating higher social motivation) than the other flocks in the presence of all three stimuli. Likewise, the lower plasma cortisol concentrations at the end of the arena test suggest that MA sheep did, in fact, experience lower levels of fear than did LA sheep in the presence of a stationary human in the arena. Therefore, the decrease in fear-related behaviour in MA sheep appears to be independent of the expression of socially motivated behaviours, and specific to the presence of the human.

In the present study, the consistent inter-flock differences in the expression of the selected behaviours (locomotion and vocalization) may be due to 'behavioural

carryover'. This term refers to the consistent relative expression of behaviour in different situations, which may result in inappropriate expression of behaviour in an isolated context (Sih et al., 2003). For example, while all individuals may decrease their activity levels in the presence of a predator, some individuals or genotypes may consistently show more active behaviour across various situations, even those in which its performance may be maladaptive e.g. predator presence (Sih et al., 2003).

In the present study, MA sheep expressed more active behaviour (locomotion and vocalization) than LA sheep, even in the presence of the predator. Previous authors have interpreted this result as relating to inter-flock differences in a domain-general temperament trait such as anxiety or fearfulness (Murphy, 1999; Gelez et al., 2003). However, I believe that these behaviours relate specifically to differential selection for responses to social isolation, and that there may have been some carryover of this pattern of behaviour to the other contexts, including the presence of a human or dog, in which such behaviour is risky for individual sheep (Dwyer, 2004).

Interpretation of adrenocortical responses

In this study, the presence of the human did not elicit plasma cortisol responses that were higher than those elicited by the presence of the box in the arena. This suggests that the presence of a stationary human in the arena did not add to the stress experienced by the sheep due to the total arena testing procedure. In contrast, the presence of the dog elicited higher concentrations at the end of the arena test (10-minute sample), and higher overall and peak responses than either the human or box, suggesting that the presence of the dog itself elicited significantly greater stress in all flocks.

Peak plasma cortisol concentrations in response to the dog were around 3 times higher than pre-treatment concentrations, but even these were only moderate compared to peak concentrations reported for young and adult sheep subjected to a range of psychological stressors (e.g. Harlow et al., 1987; Coppinger et al., 1991; Niezgoda et al., 1993; Cockram et al., 1994; Apple et al., 1995). Other studies exposing adult sheep to a barking dog report peak plasma cortisol concentrations 4-8 times above pre-treatment concentrations (Harlow et al., 1987; Canny et al., 1990; Kilgour and Szantar-Coddington, 1997; Cook, 2004). In addition, the duration of the responses was

relatively short (returned to pre-treatment concentrations within 60 minutes) compared to responses to physical stressors such as tissue removals (e.g. Lester et al., 1991; Lester et al., 1996; Dinnis et al., 1997).

The moderate magnitude and duration of the cortisol responses in this study indicate that although all tests produced a significant rise about pre-treatment concentration, the sheep were not excessively stressed by the testing procedure, or by the presence of any of the stimuli. As concentrations were found to be in the lower, more sensitive part of the range of adrenocortical responses, any minor differences between stimuli or flocks are likely to be definitive (Mellor et al., 2000). The plasma cortisol concentration immediately after the end of the test (10-minute sample) would most accurately reflect the individual's subjective experience of the stimulus presented in the arena. As such, this the most logical point to expect minor, but potentially biologically relevant differences between the flocks in response to the stimulus in the arena.

Relative responses to the three stimuli

In addition to the inter-flock differences in the presence of the human, there were also differences in the relative responses of the flocks to the three stimuli. While LA and R sheep expressed similar levels of behavioural aversion to the two live stimuli, MA sheep showed less aversion to the human than to the dog. This pattern was reiterated in the plasma cortisol responses of the flocks to the different stimuli; while LA and R sheep had similar cortisol concentrations at the end of the test (10-minute sample) in response to the two live stimuli, MA sheep had significantly lower concentrations in response to the human than to the dog.

These results suggest that the primary effect of the UWA selection regime has been to specifically reduce the level of fear experienced by MA sheep in the presence of a human in the arena test. The level of fear experienced by LA sheep appears to have been similar to that of the unselected (R) sheep, regardless of the stimulus presented. However, it is important to remember that, unlike the selected flocks, R sheep had not previously been exposed to the arena testing procedure. Therefore, as discussed in Chapter 4, my ability to compare the selected and unselected flocks is limited. In any

147

case, MA sheep appear to have specifically experienced lower levels of fear in the presence of the human, while LA sheep responded similarly to both live stimuli.

Correlations between behaviour and adrenocortical responses

In the present study, behaviours considered indicative of fear or aversion of the stimulus and adrenocortical responses were not significantly related, either overall, or when examined separately for each stimulus. As discussed in Chapter 4, one explanation for the absence of relationships between behavioural and adrenocortical responses is that behaviour may not directly reflect the level of fear or stress experienced by the animal. Whereas plasma cortisol response may reflect the individual's subjective experience of an event, e.g. fear or stress (Mellor et al., 2000), behavioural responses represent the animal's attempt to deal with a specific problem (Rushen, 1990). Therefore, individual animals may use different behavioural strategies to cope with the same challenge, while experiencing similar levels of emotional activation, fear or stress (Van Reenen et al., 2005).

Although behavioural and adrenocortical responses were not significantly correlated, the inter-flock differences in behaviour and plasma cortisol concentrations at the end of the test were coherent. MA sheep showed both behavioural and adrenocortical evidence of lower fear or aversion in the presence of the human in the arena test. In addition, MA sheep showed both more behavioural aversion and higher adrenocortical concentrations at the end of the test in response to the dog than to the human. Likewise, the absence of inter-flock differences in response to the box and dog was consistently expressed both behaviourally and physiologically. The coherence of the behavioural and adrenocortical results lends credibility to the conclusion that MA sheep experienced lower levels of fear than did the other flocks in the presence of the human in the arena test.

Context-specific temperament traits? – evidence from group comparisons

Other studies on domestic sheep provide evidence that their fear related responses vary according to context. Although the behavioural responses of a Merino flock selected for improved lamb survival differed from an unselected flock in an arena test with a human, there were no inter-flock differences in adrenocortical responses to an ACTH challenge or to the presence of a barking dog (Kilgour and Szantar-Coddington, 1997). Likewise,

piglets showing more social aggression and resistance to restraint had no higher adrenocortical responses to ACTH challenge than did non-aggressive, non-resistant piglets (Hessing et al., 1994).

The lack of difference in adrenocortical response to ACTH challenge is perhaps not surprising. This challenge is purely physiological, and does not necessarily involve differences in emotional experience (e.g. fear) of the event. Therefore, individual variation in fear responses may be mediated at a higher level of the central nervous system, and involve differences in perception and appraisal of potential threat, rather than differences in peripheral physiological responsiveness (Van Reenen et al., 2005). This supports the idea that selection may be based on an intervening variable, such as fear, which influences the behavioural and adrenocortical responses of individuals, but it also suggests that it is possible for a temperament trait such as fearfulness to influence behavioural and physiological responses differently according to context.

The absence of inter-flock differences in adrenocortical response to the presence of a dog, both in the study of Kilgour and Szantar-Coddington (1997) and in the present study, supports the idea that selection has not been based on a difference in the predisposition to react fearfully in a range of contexts (e.g. fearfulness), but instead on some context-specific behavioural response i.e. response to a human in an arena test. It is also possible that the flocks in both studies were exhibiting differential responses to the novelty of the testing environment and procedure, rather than to the presence of the human per se. This would explain the absence of inter-flock differences in adrenocortical responses to a predator when testing occurred in the familiar home pens in the study of Kilgour and Szantar-Coddington (1997), but does not explain the absence of a difference in response to the dog in the present study.

Studies on the behavioural responses of different sheep breeds also support the existence of context-specific fear responses in domestic sheep. Two different sheep breeds, reported to differ in their 'emotional reactivity' were subjected to four fear-eliciting tests (Romeyer and Bouissou, 1992). Whereas Romanov sheep appeared to be more disturbed by social isolation than Isle-de-France sheep, they showed less fear of novel objects. In addition, while dam-reared Romanov ewes showed more fear-related behaviour in social isolation, in the presence of a human and in a novel object test, they

showed less fear-related behaviour in an open-field test. As all tests were conducted in social isolation, the major difference between the breeds may have been in their specific responses to social isolation i.e. social motivation, rather than in some domain-general temperament trait such as fearfulness.

In contrast, other studies have shown that selection based on a specific behavioural response can also influence responsiveness to other challenges. Sheep selected for strong behavioural avoidance of a human also had significantly higher adrenocortical responses to other psychological stressors, such as social isolation and transport, than did sheep selected for low avoidance of a human (Lankin et al., 1979; 1980). Likewise, selection for lines of animals exhibiting high or low behavioural or adrenocortical indicators of fear in one context also resulted in consistent inter-group differences in fear-related responses to a range of other stressful events (Jones et al., 1991; Launay, 1993; Jones et al., 1994a; Jones et al., 1994b; Jones and Saterlee, 1996; Jones et al., 1997; Malmkvist and Hansen, 2002). These results support the existence of, and capacity to genetically select for, one underlying trait influencing the propensity of individuals to respond fearfully in a range of situations.

Context specific temperament traits? – individual consistency in behavioural responses

In the present study, aversion-related behaviour appears to have been largely stimulus-specific, with the moderate overall consistency in response to the human and dog likely reflecting the perception of both stimuli as predators. The absence of individual consistency in behavioural responses to the different stimuli may reflect the fact that behaviour is the individual's method of dealing with a specific problem. As such, the presence of either the human or dog in the arena may have warranted a similar behavioural response while a different response was appropriate in the presence of a novel object. However, behavioural responses may not directly reflect the level of fear or stress experienced by the animal (Rushen, 1990). Thus, the lack of consistency in behavioural aversion does not necessarily invalidate the notion of a domain-general trait influencing fear responses in the arena.

The results of the present study are consistent with other studies on both domestic and wild animals, which suggest that temperament traits, as indicated by behavioural responses, are context-specific (e.g. Lawrence et al., 1991; Boivin et al., 1992; Wilson et al., 1994; Coleman and Wilson, 1998; Reale et al., 2000; Ruis et al., 2000; Seaman et al., 2002; Sinn and Moltschaniwskyj, 2005; Wilson and Stevens, 2005). For instance, free-living wild pumpkinseed sunfish (*Lepomis gibbosus*) showed highly consistent individual responses to the same test over time (novel food test or response to a potentially threatening object), but no consistency was found in individual behavioural responses to the two qualitatively different tests (Coleman and Wilson, 1998).

In contrast, a number of studies on domestic livestock species have reported individual consistency in behavioural responses across a range of different fear-eliciting test situations (e.g. Lyons et al., 1988a; Hessing et al., 1993; Boissy and Bouissou, 1995; Van Reenen et al., 2005). Hessing et al. (1993) found a strong association between the individual responses of piglets in a social confrontation test and in a non-social restraint test. Piglets which showed high levels of social aggression also tended to show more active resistance to restraint than non-aggressive piglets. In addition, aggressive/resistant piglets vocalized less often, and were quicker than non-aggressive/non-resistant piglets to approach a novel object in an open-field test (Hessing et al., 1994), suggesting that they were less fearful in a range of challenging situations.

Context specific temperament traits? – individual consistency in adrenocortical responses

In contrast to the stimulus-specific behavioural responses found in the present study, there was a high degree of individual consistency in the adrenocortical responses to the three stimuli. Plasma cortisol responses may represent the individual's subjective experience of an event, e.g. fear or stress (Mellor et al., 2000). Therefore, the 'emotional activation', or the stress or fear experienced may have been consistent for individual sheep, suggesting that individuals may be predisposed to react similarly in a range of fear-eliciting situations.

On the contrary, habituating adult ewes to handling by humans resulted in a progressive reduction in salivary cortisol response to the handling event, but did not effect a general

decrease in cortisol response to other stressful events, such as transport (Hall et al., 1998). However, there was some evidence that individuals with greater responses to taming (e.g. greater reduction in cortisol response) were also less responsive to transport, which suggests a generalized decrease in adrenocortical responsiveness to stressful situations.

It is important, when concluding that correlations between behavioural and/or physiological responses to different test situations reflect individual consistency in fear responses, to carefully consider the features that may have been common to the different test situations. For example, while Van Reenen et al. (2005) concluded that calves showed consistent variation in 'fearfulness' based on their behavioural and physiological responses, both testing situations used novelty to elicit fear (novel environment and novel object placed in a novel environment). Likewise, while Romeyer and Bouissou (1992) reported some consistency in the expression of fear-related behaviour by sheep tested in different fear-eliciting situations, all of these situations involved testing animals in social isolation. Therefore, rather than reflecting individual consistency in fear responses over different contexts, these results may simply reflect individual consistency in responses to elements of a similar nature or quality e.g. novelty or isolation.

Similarly, boldness/shyness in individual rainbow trout (*Oncorhynchus mykiss*), as indicated by latency to feed, was consistent for individuals tested in four different contexts: in a novel environment, in the presence of a novel object and with two types of predators (Wilson and Stevens, 2005). However, when individuals were tested in a non-foraging novel environment test, there was no consistency in individual boldness/shyness, leading the authors to conclude that boldness/shyness is a context-specific temperament trait in trout. Likewise, whereas the behavioural responses of individual pigs were found to be moderately consistent in a number of handling tests, there was no individual consistency between responses to these non-social challenges and to a social group-feeding test (Lawrence et al., 1991). The consistency in individual behaviour in the various foraging or handling tests may be attributed to the inclusion of common features in all contexts, rather than to the influence of a domain-general temperament trait (Sih et al., 2003; Wilson and Stevens, 2005).

152

In the present study, the individual consistency in both the expression of selected behaviours (locomotion and vocalization) and in adrenocortical responsiveness may be attributable to the presence of common features in the testing environment. Individual sheep may simply have been exhibiting consistent behavioural and adrenocortical responses to novelty, or to physical separation from conspecifics during arena testing, rather than the consistency reflecting the influence of some domain-general temperament trait such as anxiety or emotional reactivity, as has been previously suggested (Murphy, 1999; Gelez et al., 2003). Because all three stimuli were presented in the same testing environment, the design of the present experiment does not allow differentiation between consistent responses to a common feature, and the influence of a domain-general trait on behavioural and physiological responses.

Inter-flock differences in individual consistency

In the present study there was evidence that the selection regime had altered the relationships between behaviour patterns expressed in the arena. For example, aversion to the stimulus (factor 1) and the expression of selected behaviours (factor 2) in the presence of the human were negatively related within the MA flock, but not significantly related within the R and LA flocks. This meant that MA individuals which were more active also tended to show less aversion to the human, but that no such relationship existed for LA or R sheep.

Other studies using divergently selected lines of animals have found dissociation between groups in the relationships between behavioural and adrenocortical responses to the same stimulus. For example, in Japanese quail (*Coturnix japonica*) selected for high or low bodyweight, the relationship between plasma corticosterone response to mechanical restraint and fear-related behaviour differed according to group (Jones et al., 1997). In the low bodyweight group, plasma corticosterone response was positively associated with latency to struggle and negatively related to number of struggling bouts, while the relationships were reversed in the unselected group. This supports the idea, put forward by Rushen (1991) that the relationship between behaviour and physiology is not always a straightforward one.

Individual consistency in behavioural and adrenocortical responses to the three stimuli also varied according to flock. Unselected (R) sheep showed consistent levels of the selected behaviours (locomotor and vocal activity) in all three tests, while there were no such relationships for the selected flocks (Figure 5.3b). This likely reflects the fact that MA and LA sheep were selected for uniform levels of these behaviours in the presence of a human: higher for all MA sheep and lower for LA sheep.

Likewise, whereas R sheep showed strong individual consistency in behavioural aversion to the human and dog, there was no such relationship within the MA flock. A similar, though less pronounced, pattern was seen in the plasma cortisol concentrations in response to the dog and human at the end of the test. This pattern may reflect the fact that MA sheep had been inadvertently selected for more uniform (and lower) levels of aversion to a human in the arena test, while selection had not altered behavioural or adrenocortical responsiveness to the dog. This supports the idea that MA sheep had been selected for a context-specific response, rather than a general decrease in fearfulness.

5.6 Conclusions

As discussed in Chapter 4, MA sheep showed less behavioural aversion to the presence of a human in the arena test than did R and LA sheep, and had lower plasma cortisol concentrations than LA sheep immediately after the human test. However, contrary to my hypothesis, there were no significant inter-flock differences in behavioural aversion or adrenocortical response to the presence of the novel object or predator. In addition, whereas R and LA sheep had similar behavioural and adrenocortical responses to the two live stimuli, MA sheep showed less behavioural aversion and lower plasma cortisol concentrations at the end of the test in response to a human than to a dog.

Therefore, selection of MA sheep for lower fear responses to the presence of a human in the arena test appears to have been based on a context-specific trait, rather than a domain-general trait (fearfulness). In support of this, the differences in plasma cortisol concentration were minor, being evident only immediately after the arena test. There were no inter-flock differences in the integrated adrenocortical responses, which likely reflected the experience of repeated handling by humans during the general testing

154

procedure. Therefore, inter-flock differences in response to a human are likely relevant only within the context of the arena test.

Behavioural aversion appeared to be context-specific, while adrenocortical responses were more consistent for individuals across the three tests. In accordance with this, behavioural and adrenocortical responses were not significantly correlated. The correlations between adrenocortical responses between the three tests may reflect individual consistency in emotional activation (fearfulness), or consistent responses to some feature common to all three tests, e.g. physical separation from conspecifics, novelty. Likewise, individual consistency in the expression of selected behaviours (locomotion and vocalization) may be due to differential responses to social isolation. The design of the present experiment does not allow differentiation between the two possibilities. There is also evidence that divergent selection has altered the individual consistency of behavioural and adrenocortical responses across a range of contexts within the LA and MA flocks, relative to the unselected flock.

Variation in fear responses is likely mediated at a high level of the central nervous system, and future experiments should continue to elucidate the behavioural, physiological and neuroendocrine differences between the flocks in a range of challenging situations. There are both management and animal welfare implications associated with the potential selection of domestic sheep based on a context-specific trait, such as lower fear in the presence of a human.

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CHAPTER 6

Which do sheep find more aversive – a barking dog or a human with rattle? Evaluation using Y maze preference tests and adrenocortical responses

6.1   Abstract

Dogs are one of the primary aids used to handle sheep on farms and prior to slaughter in meat processing premises in New Zealand. Although we know that sheep move away from dogs, it is not clear the amount of stress, if any, experienced by sheep under normal pre-slaughter conditions. Despite this uncertainty, there has been consumer pressure to remove dogs from meat processing premises. The common alternative is to use humans with rattles or other noise-making devices to move sheep. However, there is no scientific evidence to suggest that this practice is less stressful for the sheep. In this study, I used a series of Y maze preference tests to determine whether sheep preferred close confinement with a barking dog or a human with rattle. In addition, changes in the plasma concentrations of cortisol were measured after exposure to the barking dog or the human with rattle to determine the aversiveness of each treatment to the sheep. The results of this study appear to validate the use of the Y maze test for assessing relative preferences of sheep presented with treatments differing greatly in aversiveness. In addition, both the choice behaviour in the Y maze and the adrenocortical responses indicate that sheep found the barking dog more aversive than the noise-making human. Although sheep avoided both the dog and human, their adrenocortical responses to both were only moderate in magnitude and duration compared to other accepted sheep management practices (e.g. shearing, castration). Therefore, if the results of the present study are confirmed by experimental testing in a real pre-slaughter environment, handling either with dogs or by people with rattles should cause little concern on the basis of animal welfare. However, minimizing animal stress in all stages of production is advantageous, and this preliminary study provides some support for the replacement of barking dogs with people using noise-making devices before slaughter.

6.2   Introduction

Background

Dogs are one of the primary aids used to handle sheep on farms and prior to slaughter in meat processing premises in New Zealand. It is the fact that sheep avoid dogs that makes dogs so efficient for handling sheep. However, although we know that domestic

165

sheep move away from dogs, it is not clear the amount of stress, if any, experienced by sheep under normal farm and pre-slaughter conditions.

Despite this uncertainty, there has been consumer pressure to remove dogs from meat processing premises. Increasingly, consumers are concerned about the processes involved in meat production, and consider ethical treatment of production animals to be an important factor influencing their purchasing behaviour (Warris, 1995). Consumer preferences based on concerns about animal welfare have the potential to significantly influence animal management practices, regardless of whether consumer perceptions are accurate or not (McInerney, 1997; 2004). Scientific research is required to provide a rational basis for decision making about the continued use or discontinuation of management practices which are perceived to compromise animal welfare.

In addition to concerns about animal welfare, there is evidence that pre-slaughter stress can negatively affect meat quality. Both physical (exercise, transportation) and psychological (restraint, isolation) pre-slaughter stress in sheep have been related to reduced meat quality (Forrest et al., 1964; Shorthose, 1977; Schaefer et al., 1988; Schaefer et al., 1990; Apple et al., 1995; Ruiz-de-la-torre et al., 2001). Pre-slaughter stress may decrease meat quality by depleting muscle glycogen stores, resulting in an elevated ultimate pH, which can negatively affect meat colour, flavour and keeping quality e.g. dark-cutting meat (Geesink et al., 2001; Warner et al., 2005). Consumers are likely to discriminate against dark-cutting meat on the supermarket shelf due to the dark colour (Warner, 1989).

Additional concerns associated with dog use for moving sheep include increased prevalence of injuries, such as torn skin and bruises, which may compromise both animal welfare and meat quality. Such injuries can relate directly to the dog (e.g. bites) or to sheep behaviour associated with avoidance of close proximity with a predator (e.g. stumbling, smothering or contact with features of the handling facility) (Grandin, 1993; Gregory, 1996; Grandin, 2001).

Although pre-slaughter stress can compromise animal welfare and meat quality, the effect of dog presence on stress in sheep before slaughter has not been explicitly examined. However, the presence of dogs has been used to experimentally elicit

behavioural and physiological stress responses in sheep for comparison against other treatments (Torres-Hernandez and Hohenboken, 1979; Harlow et al., 1987; Canny et al., 1990; Kilgour and Szantar-Coddington, 1997; Hansen et al., 2001; Cook, 2004; Beausoleil et al., 2005). For example, herding with dogs combined with swim-washing before slaughter was found to increase the percentage of muscles in lamb meat with high ultimate pH associated with undesirable toughness and colour characteristics (Geesink et al., 2001).

The common alternative to dog-use in meat processing premises is to use humans with rattles or other noise-making devices to move sheep. However, there is no scientific evidence to suggest that this practice is less stressful for the sheep. The fact that such practices allow efficient handling of sheep suggests that noise-making humans are also aversive to sheep. In fact, Rushen (1990) stated with respect to sheep that, 'whether humans or dogs cause more distress has not been shown'. To date, apart from Beausoleil et al. (2005), there has been little progress in this field since this statement was made. In the present study I sought to progress this matter by using a Y maze preference test to determine whether sheep prefer close confinement with a barking dog or a human with rattle. In addition, physiological stress responses, as indicated by changes in plasma cortisol concentrations, were measured before and after exposure to the barking dog or the human plus rattle to complement the behavioural results of the Y maze test.

Y maze preference test

Y and T maze preference tests offer subject animals the choice between two maze arms, each of which is associated with a particular treatment. As implied by the name, individuals are fed up a single file race to a decision point at the crux of the Y or T. By selecting either the right or left arm of the maze, individuals can theoretically express their preferences for one treatment relative to the other (Fraser and Matthews, 1997).

The presumption underlying preference testing is that animals are more likely to voluntarily enter into, or remain within an environment that more closely agrees with their preference than one that is in opposition with it (Arnold, 2005). The history and scope of preference and motivation testing in domestic animals has been

comprehensively reviewed by Fraser and Matthews (1997). Therefore, only a brief general introduction to preference testing will be given in this chapter. Potential limitations related to the preference test methodology, as they pertain to the results of this study, will be considered in the Discussions section of this chapter.

Preference tests generally fall into two categories: those offering two permanently available alternatives, and those in which the choice of one alternative precludes access to the other. The first type of test is often used to assess animal preferences about the total environment on offer, and preferences are primarily measured by comparing the proportion of time spent in each of the alternative environments. Such tests have been used to assess the preferences of domestic animals for flooring or nesting materials, the social environment and space requirements (e.g. Hughes, 1976; Dawkins, 1977; Dawkins, 1982; Blom et al., 1995; Millman and Duncan, 2000; Mills et al., 2000; Kavaliers et al., 2003).

Measuring the proportion of time spent in each treatment area is a useful method of assessing preference. The collection of continuous data allows the use of parametric statistical analyses, and avoids the necessity of imposing an arbitrary criterion for 'preference' (e.g. proportion of choices), which may be biologically irrelevant. However, the nature of the test treatment may preclude the use of this method; for example, if preferences between short-lived or one-off treatments are of interest, or if the treatments are strongly aversive. In addition, for species with lower natural levels of exploratory motivation, such as domestic ungulates, this test may reveal less about preferences than would be the case for inherently inquisitive species such as rats and mice (Fraser and Matthews, 1997).

The other type of preference test requires that each animal be tested repeatedly, with each choice precluding access to the other option. This type of test is commonly used to assess animal preferences for specific features, stimuli or treatments, and often asks the question '*how aversive/attractive is this feature to the animal?*' (Fraser and Matthews, 1997). Such tests usually involve short periods of contact or confinement between the test animal and treatment, primarily because of the requirement for repeated trials for statistical confidence; the result of each trial represents one data point. Short treatments also ensure that animals do not rapidly habituate to aversive treatments, or become

satiated in feeding trials. In addition, in many cases, the aversiveness of occasional, short-lived, or one-off treatments is being tested, and it is the animal's initial response to the stimulus which is of interest e.g. shearing, restraint, electroimmobilization (Grandin et al., 1986; Rushen, 1986a; Hargreaves and Hutson, 1990b; 1990a).

Y and T tests are an example of this latter type of preference test, and are often used to assess relative preferences between attractive or aversive treatments. Maze tests have been used to assess preferences between attractive alternatives, such as types of food in goats, sheep and rabbits (Hosoi et al., 1995; Leslie et al., 2004), or access to different social partners in sheep, pigs, poultry, rodents, reptiles and crustacea (Dawkins, 1982; Kendrick et al., 1995; Millman and Duncan, 2000; Kavaliers et al., 2003; Diaz and Thiel, 2004; O'Donnell et al., 2004; McLeman et al., 2005).

More commonly, Y and T maze tests are used to assess relative preferences between stimuli or treatments considered to be aversive (avoid-avoid tests). 'Preferences' in these tests may reflect a lesser degree of aversion towards one treatment relative to the other. In other words, both treatments may be aversive, but one may be considered the lesser of two evils. On the other hand, neither treatment may be particularly aversive, and the non-preferred option may still be perfectly acceptable to the animal (Duncan, 1978). Avoid-avoid maze tests have been used to assess the aversion of domestic ungulates to different methods of handling or restraint (Grandin et al., 1986; Rushen, 1986a; Pollard et al., 1994; Pajor et al., 2003), different methods and frequencies of milking (Prescott et al., 1998), and different environmental features, such as flooring materials, light intensities and contrasts, and space allowances (Jensen, 1999; Phillips and Morris, 2001; Arnold, 2005).

Y and T maze tests have also been used to test the discriminative abilities of species or individuals. This process is similar to preference testing, except that one stimulus is designated as 'correct', and choice of this stimulus is reinforced, either using a food reward, or by granting access to the stimulus chosen (Kendrick et al., 1995; Rybarczyk, 2001; McLeman et al., 2005). In the absence of cues as to the location of the reinforcer, consistent choices of the correct stimulus imply that the test animal can differentiate between the two alternatives offered (McLeman et al., 2005).

Common measures of behaviour in Y or T maze tests include the proportion of trials resulting in choice of one treatment compared to the other, the proportion of animals in the test population reaching the criterion defining a significant 'preference' for one treatment, the time taken to choose a treatment (latency to enter the maze arm or treatment area), number of vacillations at the decision point (looks right and left), and other behavioural indicators of uncertainty or stress e.g. frequency of lifting feet in treatment room (Dawkins, 1977; Dawkins, 1982; Grandin et al., 1986; Rushen, 1986a; Kendrick et al., 1995; Prescott et al., 1998; Pajor et al., 2003; Diaz and Thiel, 2004; Arnold, 2005).

In the type of Y maze test used in the present study, the subject animals are unable to visually assess the two options before making each choice. Therefore, the animal has to remember the association between the side of the maze and treatment presented there. There is evidence that domestic ungulates resist changing their choice behaviour once they have learned to associate a specific treatment with a side of the maze (Grandin et al., 1994). Therefore, in this type of test, treatment-location associations must be kept constant for each individual, and the use of naïve animals for comparing each pair of treatments is recommended (Pajor et al., 2003).

The first objective of this study was to validate the use of the Y maze preference test for assessing the relative aversiveness of treatments to sheep. This was done by presenting sheep with a choice between an empty room (control) and a treatment expected to be moderately aversive. The two aversive treatments presented in experiments 6a and 6b were designed to represent commonly used methods of moving sheep in the yards and races of meat processing premises: dog plus recorded barking, and human plus rattle (noise maker). It was hypothesized that sheep would prefer the empty room to the aversive treatment in both cases.

The second aim was to use the Y maze test to assess the relative aversiveness of the two treatments when compared to each other. Sheep were offered choices between a real barking dog and a human plus rattle. Physiological stress responses to the two treatments were also compared by measuring plasma cortisol concentration before and after the initial exposure to the dog and human. It was hypothesized that both the choice

170

behaviour and the adrenocortical responses would indicate that sheep found a barking dog more aversive than a human plus rattle.

6.3   Methods and Materials

Animals

One hundred and eighty female mixed breed hoggets (Romney x Finn or Romney x Texel) were randomly divided into three groups of 60 animals. The hoggets had been raised together since weaning on a Massey University farm under normal New Zealand farm conditions, including handling by people and dogs. It is unlikely that these sheep had ever been exposed to the type of rattle used in this experiment. Each group of 60 was moved to the testing facility and maintained on nearby pasture for the two week duration of testing, before being returned to the farm. Individual sheep were identified using numbers sprayed on the back. The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (protocol 03/113).

Experimental setup

All Y maze preference tests and blood sampling for plasma cortisol concentration were conducted using the indoor facility shown in Figure 6.1. The three experiments were performed from November 2003 to January 2004, between 8am and 3pm under the natural light conditions in the building. The stimuli used are described separately for each experiment.

The maze was constructed of plywood, with walls 1.2 m high, except for the front and side walls of the treatment rooms, which were 2.4 m high. The single file race was 38cm wide, which was narrow enough to prevent most sheep from turning around. However, sheep could, and occasionally did turn around at the crux of the Y. During familiarization and training runs, a solid black gate was placed at the crux of the Y. This gate was hinged, and could be fixed to block one or other of the maze arms, thereby forcing the sheep to enter the other arm and treatment room (direction-forcing gate). This gate was removed for all free choice trails.
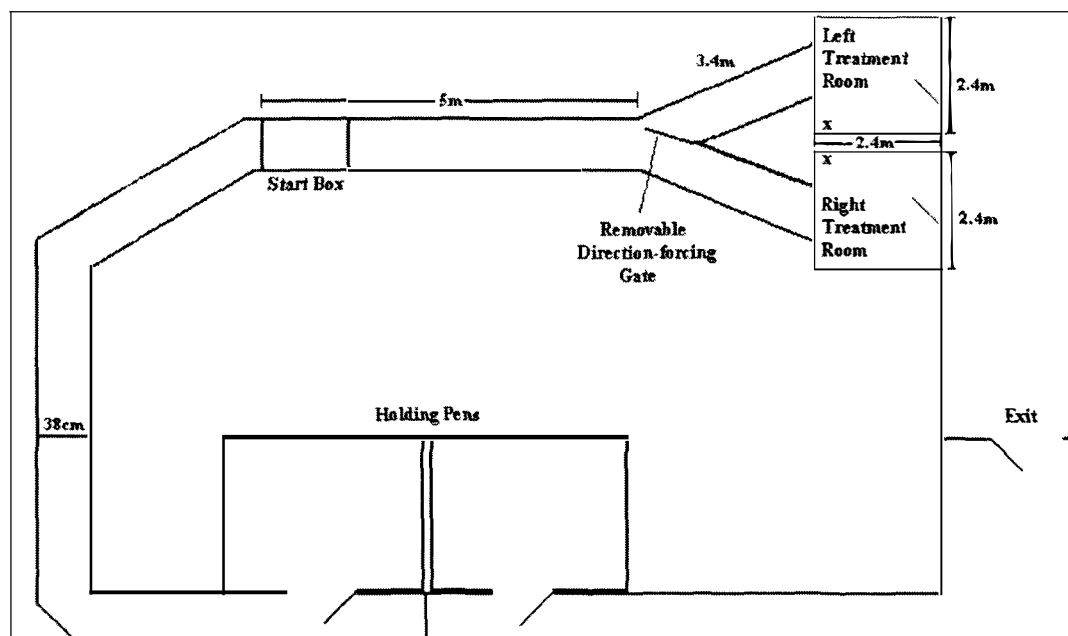
Figure 6.1 Y Maze facility. X marks the position of the stimulus in the treatment room.

The start box consisted of a visually solid back gate and a wire mesh front gate. This design was used to encourage the test sheep to enter the box voluntarily (as it could see ahead), and to stop the animal behind from viewing the behaviour of the test sheep, although there is no evidence that sheep use visual or olfactory cues from previous animals to direct their behaviour in a maze (Liddell, 1925; Franklin and Hutson, 1982a; Hosoi et al., 1995). The start gates were manually operated by the handler, while all other doors and gates were remotely operated using a pulley system. The exit gates from the treatment rooms were wire mesh to encourage the test sheep to move completely into the treatment room so that the guillotine door could be closed behind it. Except in the case of the real barking dog in experiment 3, the rattling/recorded barking was not applied until the sheep was fully inside the room and the door closed behind it.

The shape of the Y maze meant that the animal could not see into either treatment room from the decision point. Therefore, the sheep could not see which treatment it had chosen until it entered the treatment room. The stimulus was located in the front inside corner of the treatment room, so that it was not visible until the sheep's head and shoulders were inside the room (Figure 6.1). Each treatment room was 5.8m^2, and the maximum distance a sheep could retreat from the stimulus was 3.4m. Therefore,

172

entering the treatment room meant the sheep was confined in relatively close contact with the stimulus.

General procedure

Procedures common to each of the three experiments are outlined in this section, while specific procedures and schedules for each experiment are described separately below. Each sheep was tested with only one pair of treatments (Control/Dog, Control/Human or Human/Dog), thereby avoiding the confounding effects of an individual's memory of previous side/treatment associations (Rushen, 1986a). For each individual, the pair of treatments were presented on the same sides of the maze for all tests (e.g. Control on right, Human on left).

Each morning during the two weeks of testing, all 60 sheep were brought from the pasture into the testing facility. The sheep to be tested that day were drafted out, and the remainder were returned immediately to pasture. Because of the difficulty in switching the stimulus, particularly the dog, between the two sides of the maze, sheep were tested in groups of animals with the same stimulus-side association (two groups at a time). Training and testing runs were alternated between the two groups, to give each sheep the maximum amount of rest time between each trial. All sheep were also given regular breaks, usually after every 3-5 trials. The amount of time between each trial for an individual animal varied according to the time taken by other animals, but was always at least 15 minutes.

During training and testing runs, sheep were moved as a group out of the holding pen, and into the single file race. Each sheep was individually held in the start gate for at least 10 seconds, before being released into the maze. The sheep was then given 30 seconds to voluntarily leave the start gate, after which time it was gently encouraged to move forward using light pressure on the hindquarters. After the sheep left the start box, the gate was closed behind it. The next sheep was not permitted to enter the start box until the test sheep had returned to the holding pen. When the sheep had entered the treatment room, the door was closed behind it, and it was held for 10-30 seconds (see below), before being released to return to the holding pen. The next sheep was then released from the starting gate.

173

Familiarization and training

The sheep were familiarized with the empty testing facility (no stimuli) and procedures by moving them through the pens, race, maze arms and treatment rooms in groups of approximately 15 animals, and then as individuals. The direction of familiarization runs was balanced across groups. After familiarization, each sheep was tested for any lateral bias or side preference in the empty maze (no stimuli). Treatment/side pairings were assigned according to lateral bias expressed in the empty maze (see Tables 6.1, 6.2, and 6.3 below). For further details on lateral bias testing and side/treatment pairings see Chapter 7.

After lateral bias testing, the stimuli were placed in the appropriate sides of the maze, and each sheep received 2-4 training runs. The sheep were directed into first one, and then the other side of the maze. When the sheep had entered the treatment room, the door was closed behind it, and the treatment applied for 10 seconds, before it was released to return to the holding pen. There were two purposes of these training runs: to teach the sheep the association between stimulus and side of the maze, and also to ensure that each animal was aware of, and had experienced both options in the maze, as this has been shown to facilitate learning of the side-stimulus association (Pollard et al., 1994). Training was repeated on the second day of testing, to reinforce the association between side and stimulus, and to remind the sheep of both options. No data were recorded during training trials.

Preference testing

After the training runs, the direction-forcing gate was removed and the sheep were allowed to enter the arm and room of their own choosing. The treatment chosen by the sheep was applied for 30 seconds before the sheep was released. For the human plus rattle and dog plus recorded barking treatments, intermittent, rather than continuous noise was applied (e.g. 2 seconds of recorded barking or rattling every 10 seconds) to avoid rapid habituation over several days of testing (Ames and Arehart, 1972).

An observer on a platform above the Y maze recorded the side and stimulus chosen, or 'no choice' if the animal had not entered an arm or room within the time limit. Choice of a treatment arm/room was recorded when the animal's head and shoulders were

174

inside the arm/room. The sheep were given a maximum of 2 minutes to enter an arm of the maze, and a maximum of 5 minutes to enter a treatment room (protocol altered for experiment 6c, see below). If the sheep had not entered an arm/room within the maximum time allowed, 'no choice' was recorded.

When the time limit was exceeded, the animal was encouraged to move forward by the handler using a progression of interventions. Firstly, the handler placed a hand behind the sheep in the maze without touching it. If this was unsuccessful, light pressure was applied to the rump of the animal; if the animal still would not make a choice, upward pressure was applied under the tail (tail grab). Finally, for extremely uncooperative animals, the handler entered the race behind the animal and pushed it forward into the arm of its choice (e.g. animal still got to choose which arm it entered) where the treatment was applied as usual.

Experiment 6a: Dog plus recorded barking versus Control

Of the 60 sheep tested for lateral biases, 39 were subsequently tested for their preference between a dog plus recorded barking in the treatment room and an empty room, following the schedule shown in Table 6.1. Sheep R10 was removed from the trial after 5 free choice trials, because she refused to make a choice in any trial and became apparently agitated (n=38).

The dog used in this experiment was an 11 year old neutered male Border Collie (Tiger) experienced in working sheep. Tiger was tied up on a short leash in the inner front corner of the treatment room. His demeanour in the Y Maze was generally passive and he never barked, however when confronted by a sheep he would respond by eyeing the sheep, and if physically challenged (e.g. head butted), he would growl or snap. Due to the length of the testing days, Tiger spent some of the time asleep.

Table 6.1 Training and testing schedule for Dog plus recorded barking versus Control. The order of familiarization and training runs was balanced over the test population.

Day 1	**Familiarization*** 2 x group runs (15 sheep/group): R, L 2 x individual runs: R, L
Day 2	**Familiarization*** 2 x individual runs: R, L
Day 3	**Lateral bias testing*** 2 x individual runs: R, L 10 x free choices
Day 6	**Y Maze Preference Testing** Group 1 Day 1: Right bias group (n=15) 2 x training runs: Control, Dog 10 x free choices
Day 7	Group 1 Day 2: Right bias group (n=15) 2 x training runs: Dog, Control 10 x free choices
Day 8	Group 2 Day 1: Left bias group (n=15) 2 x training runs: Control, Dog 10 x free choices
Day 9	Group 2 Day 2: Left bias group (n=15) 2 x training runs: Dog, Control 10 x free choices
Day 10	Group 3 Day 1: No bias group (n=9) 4 x training runs: Control, Dog, Control, Dog 10 x free choices
Day 11	Group 3 Day 2: No bias group (n=9) 2 x training runs: Dog, Control 10 x free choices

*No stimuli present in maze during familiarization or lateral bias testing

Recorded barking was used in an attempt to standardize the treatment, and because of the difficulty in procuring a dog that would bark on cue. The taped barking was recorded from the same dog, and consisted of intermittent bursts of barking and whining upon instruction from his owner. No sheep were present during the recording of the barking, therefore, it is unlikely that the vocalizations were predatory/threatening. Thirty seconds of intermittent barking (approximately 2 seconds of barking in every 10 seconds) was broadcast during each treatment.

The recording was broadcast from a speaker placed directly above the dog in the treatment room, at a height of about 1m above ground level. The peak intensity of the playback was 85.6 dBa in the right room and 89 dBa in the left room, when measured

from the farthest point from the speaker inside the treatment room (3.4 m) (Bruel and Kjaer modular precision sound level meter, Bruel and Kjaer Sound and Vibration, DK-2850 Naerum, Denmark). The barking was broadcast at this volume to approximately match the peak intensity of the sound produced by shaking the rattle used in experiments 6b and 6c (90.2 dBa from 3.4 m away).

Experiment 6b: Human plus rattle versus Control

Of the 60 sheep tested for lateral biases, 40 were subsequently tested for their preference between a human with a noise-making device (Human) and an empty room (Control), according to the schedule shown in Table 6.2. The same male human was used for all training and free choice trials in this experiment. He was dressed in plain white overalls, and sat on a stool at approximately 1 meter above ground level.

The human treatment included the production of noise using a commercial sheep rattle sometimes used to move sheep on farms and in meat processing plants in New Zealand. The rattle consisted of a loop of metal with three sets of metal discs, which when shaken, produced a loud metallic sound (90.2 dBa from 3.4m away, Bruel and Kjaer modular precision sound level meter). When a sheep entered the treatment room, and the door was closed, the person directed his gaze at the sheep and shook the rattle for approximately 2 out of every 10 seconds. Because of the intensity of the noise produced, all handlers and human stimuli wore ear protection throughout the experiment.

177

Table 6.2 Training and testing schedule for Human plus rattle versus Control. The order of familiarization and training runs was balanced over the test population.

Day 1	**Familiarization*** 2 x group runs (15 sheep/group): R, L 2 x individual runs: R, L
Day 2 and 3	**Lateral bias testing*** 2 x individual runs: R, L 10 x free choices
Day 6	**Y Maze Preference Testing** Group 1 Day 1: Right bias group (n=15) 2 x training runs: Control, Human 10 x free choices
Day 7	Group 1 Day 2: Right bias group (n=15) 2 x training runs: Human, Control 10 x free choices
Day 8	Group 2 Day 1: Left bias group (n=15) 2 x training runs: Control, Human 10 x free choices
Day 9	Group 2 Day 2: Left bias group (n=15) 2 x training runs: Human, Control 10 x free choices
Day 10	Group 3 Day 1: No bias group (n=10) 4 x training runs: Control, Human, Control, Human 10 x free choices
Day 11	Group 3 Day 2: No bias group (n=10) 2 x training runs: Human, Control 10 x free choices

*No stimuli present in maze during familiarization or lateral bias testing

Experiment 6c: Barking dog versus Human plus rattle

Of the 60 sheep tested for lateral biases, 49 were tested for their preferences between a real barking dog (Dog) and a human with a noise-making device (Human). Because this experiment was designed to directly answer the main question of this study, larger numbers of animals, and more trials per animal were included than in experiments 6a and 6b. In addition, blood samples were taken before and after an initial exposure to the dog and human treatments on the days preceding preference testing (Table 6.3).

Several procedural changes were required in this experiment, due to the use of a barking dog rather than recorded barking, and because sheep were required to choose between two stimuli assumed to be moderately to strongly aversive. The dog used in this

experiment was a 12 month old female Huntaway (Paddy) which was trained to bark in the presence of sheep. Paddy behaved quite aggressively toward the sheep and barked almost continuously when the sheep were in the treatment room (and often at other times as well). Due to a number of sheep/dog altercations during a pilot trial, a set of metal bars was used to physically separate Paddy from the test sheep. A similar cage was in place both for the dog and human, on both sides of the maze.

On the second day of testing the 'No Bias' group (Table 6.3), Paddy lost her voice and, to prevent her injuring herself, had to be replaced by another dog. Jock was an adult male Huntaway, regularly used to move sheep on a Massey University farm, primarily by using his bark. Although this dog was expected to bark at the sheep, he was not confident in the Y maze and did not bark or behave aggressively towards the sheep in any trial. Therefore, on days 2 and 3 of testing the 'No Bias' group I had to revert to using the tape-recorded barking from experiment 6a. On the third day of testing this group, the barking was not broadcast if a sheep chose the dog; these sheep were exposed only to the replacement dog with no barking. There was some indication that non-biased sheep B27 and B46 may have changed their preference on day 2, after the dog was changed from the barking dog to the non-barking dog plus the recording.

The same male human was used for all training and free choice trials in this experiment, but was not the same person used in experiment 6b. He was dressed in plain green overalls and, in this experiment, was standing for all trials. The human treatment included the production of noise using the same sheep rattle described in the previous experiment. When a sheep entered the treatment room, and the door was closed, the person directed his gaze at the sheep and shook the rattle for approximately 2 out of every 10 seconds. Because of the intensity of the noise produced, all handlers and human stimuli wore ear protection throughout the experiment.

Table 6.3 Training and testing schedule for Barking dog versus Human plus rattle. The order of familiarization and training runs was balanced over the test population.

Day 1	**Familiarization*** 2 x group runs (15 sheep/group): R, L 2 x individual runs: R, L
Day 2	**Familiarization*** 2 x individual runs: R, L
Day 3 and 4	**Lateral bias testing*** 2 x individual runs: R, L 10 x free choices
Day 5	**Blood sampling for plasma cortisol concentration** AM: Human plus rattle (n=25) PM: Barking dog (n=25)
Day 6	**Blood sampling for plasma cortisol concentration** AM: Barking dog (n=25) PM: Human plus rattle (n=25)
Day 7	**Y Maze Preference Testing:** Group 1 Day 1: Right bias group (n=18) 2 x training runs: Dog, Human 5 x free choices
Day 8	Group 1 Day 2: Right bias group (n=18) 2 x training runs: Human, Dog 10 x free choices
Day 9	Group 1 Day 3: Right bias group (n=18) 2 x training runs: Dog, Human 10 x free choices
Day 10	Group 2 Day 1: Left bias group (n=18) 2 x training runs: Human, Dog 5 x free choices
Day 11	Group 2 Day 2: Left bias group (n=18) 2 x training runs: Dog, Human 10 x free choices
Day 12	Group 2 Day 3: Left bias group (n=18) 2 x training runs: Human, Dog 10 x free choices
Day 13	Group 3 Day 1: No bias group (n=14) 2 x training runs: Dog, Human 5 x free choices
Day 14	Group 3 Day 2: No bias group (n=14) 2 x training runs: Human, Dog[†] 10 x free choices
Day 15	Group 3 Day 3: No bias group (n=14) 2 x training runs: Dog[†], Human 10 x free choices

[†]Barking dog lost her voice and was replaced by a different dog plus recorded barking for day 2 and 3 of testing for the No Bias group

*No stimuli present in maze during familiarization or lateral bias testing

180

At the beginning of this experiment, the sheep were much less willing to make a choice in the maze than during the other experiments. This was likely because both of the options offered were aversive to some degree, and making a choice meant a close encounter with an aversive stimulus. On the first day of testing, very few sheep made a choice within the 5 minute time limit. In order to complete testing in the days available, it was necessary to reduce the maximum time allowed to enter the treatment room from 5 to 2 minutes. As described in the general procedures, sheep making no choice within this revised time limit were then forced to enter the room of their choice (e.g. the animal still got to choose which arm it entered) and the treatment was applied as usual.

As a result of the large number of 'no choice' trials in the first day, I disregarded this testing day in the statistical analysis, and treated these as additional training trials. Therefore, only 20 of the possible 25 trials completed by each sheep were included. In addition, on the third day of testing for each lateral bias group, sheep were held in the treatment room for only 10 seconds (2 sets of rattling) as opposed to the usual 30 seconds applied on the other testing days.

Blood sample collection and hormone assay

In order to assess the adrenocortical responses of the sheep to the presence of a barking dog or human with rattle in the Y maze, blood samples were collected before and after exposure to each stimulus. Blood samples were collected into heparinized tubes by repeated jugular venepuncture. Approximately 5mls of blood were collected each time, and placed on ice until the sample could be centrifuged. After centrifugation, the plasma fraction was drawn off and stored at -20°C until assayed.

The sheep (n=50) were mobbed into groups of 10 animals in the holding pens, according to the side of stimulus presentation (see below). Each sheep in the group was caught by a handler and a pre-treatment blood sample was taken. When all sheep in the group had been sampled, the group was moved into the race and individually exposed to the stimulus for 30 seconds in the treatment room. The sheep then returned to the holding pen individually, where blood samples were taken at 15, 30, 45 and 60 minutes after the end of treatment.

Samples were generally taken within 2 minutes of closely approaching the sheep. The time required, after the induction of a potential stressor, for the release and action of ACTH on the adrenal glands, and for the blood to circulate from the adrenal glands to the jugular vein where it was sampled, makes it unlikely that handling and venepuncture affected the plasma concentration of cortisol in the immediate sample (Broom and Johnson, 1993). However, it must be acknowledged that the handling and venepuncture required for each sample were likely to have evoked a rise in plasma cortisol concentration in subsequent samples (De Silva et al., 1986; Hargreaves and Hutson, 1990d). Therefore, as well as reflecting adrenocortical responses to the testing procedure and stimulus, post-exposure cortisol concentrations will also reflect responses to previous sampling procedures.

On the first day after lateral bias testing, sheep assigned to receive the human in the right side of the maze during preference testing (n=25) were exposed to the human treatment between 8:30am and 11:30am. Sheep assigned to receive the dog in the right side of the maze during preference testing (n=25) were exposed to the dog treatment between 12:30pm and 3:45pm. For each individual, the stimulus was presented on the side of the maze that it would subsequently be located in the preference test. Therefore, these exposures served as additional training trials to teach the sheep the association between side of the maze and stimulus.

On the second day, the groups were sorted to match the previous day, and pre-treatment blood samples were taken in the same order. The time of testing was kept as similar as possible between days to reduce possible confounding effects of circadian fluctuations in plasma cortisol concentration (De Silva et al., 1986; Ingram et al., 1999). Sheep were then exposed to the treatment not experienced on day one, in the left side of the maze. The order of treatment was not the same as on day one, as it was considered that the handling required to achieve this would confound the effects of the treatment itself on plasma cortisol concentration.

A commercially available cortisol radioimmunoassay kit (Cortisol GammaCoat RIA Kit CA-1549, DiaSorin Inc., Stillwater, MN), designed for measurement of cortisol in human plasma, serum or urine was used. The kit was modified for use on ovine plasma by diluting the cortisol serum calibrators (to include an extra point of 13.8 nmol/L in the

standard curve) and adding 25 μL of the plasma instead of 10 μL. These modifications were required to improve the sensitivity of the assay. The radioimmunoassay uses ^{125}I-labelled cortisol as a tracer, and the tubes are coated with a cortisol-specific antibody (rabbit). The cortisol in the sample competes with the ^{125}I-labelled cortisol for a limited number of antibody binding sites. The radioactive cortisol bound to the antibody is thus inversely proportion to the concentration of cortisol in the sample. The manufacturer's performance data gave the sensitivity of the cortisol assay as 3 nmol/L, the mean inter-assay coefficient of variation over the range of concentrations as 9.2%, and the mean intra-assay CV as 7.0% (Cortisol GammaCoat RIA Kit CA-1549 Instruction Manual, DiaSorin Inc., Stillwater, MN). However I calculated the mean inter-assay coefficient of variation over the range of concentrations to be 2.6%.

Statistical analysis

Choice behaviour

Previously, preference test data have usually been analyzed by comparing the proportion of individuals expressing a statistically significant preference for one treatment with the proportion preferring the other treatment. Such analyses have been performed using a Chi Square (χ^2) Goodness of Fit test for equal proportions or a Binomial test (P = 0.50) to test whether the proportion is significantly different from chance (Dawkins, 1982; Franklin and Hutson, 1982a; 1982b; 1982c; Pollard et al., 1994; Hosoi et al., 1995; O'Donnell et al., 2004; Izawa et al., 2005).

This method is useful, in that it allows the preferences of individual animals for different treatments to be examined. However, due to the relatively small number of subjects generally used in preference tests (usually less than 20 individuals per comparison, e.g. Dawkins, 1977; Rushen, 1986a; Grandin et al., 1994; Pollard et al., 1994; Hosoi et al., 1995; Prescott et al., 1998; Rybarczyk, 2001; O'Donnell et al., 2004; Arnold, 2005; McLeman et al., 2005), it is often difficult to achieve proportions of animals which are statistically different from chance. In addition, this method requires the imposition of a criterion defining a significant preference. While such criteria may be statistically useful, they are likely to be arbitrary from a biological point of view.

183

Another method is to compare the proportion of trials in which one treatment was selected with the proportion of trials resulting in selection of the alternative. The same tests (χ^2 or Binomial) have been used to compare the proportion of trials of each type to proportions likely to occur by chance (Leslie et al., 2004). The larger number of experimental units (trials) makes the demonstration of statistically significant differences from chance more likely. However, using individual trials may over-emphasize the contribution of a single individual to the choice of one treatment (e.g. each sheep contributes 20 data points). In addition, analysis of all trials together may obscure the significant preferences of individual subjects. For example, if half the subjects had a significant preference for treatment A and half had a significant preference for treatment B, analysis of the proportion of trials would lead to the conclusion that there was no overall preference for either treatment, which is obviously untrue on an individual basis.

For each of the three experiments, the preferences expressed by sheep in the 20 free choice trials were examined. An individual sheep was deemed to have expressed a significant 'treatment preference' if it chose the same treatment in at least 15 out of 20 trials (Binomial test 1-sided P = 0.021) (Hosoi et al., 1995; Rybarczyk, 2001; McLeman et al., 2005). A χ^2 Goodness of Fit test for equal proportions was used to test whether the proportion of individuals in each of three groups was equal: preferred treatment A, preferred treatment B, no significant preference (Siegel and Castellan, 1988; Jensen, 1999; Millman and Duncan, 2000; Kavaliers et al., 2003; Diaz and Thiel, 2004; Leslie et al., 2004). The χ^2 test was also used to compare the proportion of trials resulting in the choice of treatment A or treatment B, and the proportion of no choice trials.

Alternatively, the difference between the number of times treatment A and treatment B are chosen can be calculated for each individual (Pajor et al., 2003). The significance of this difference can then be evaluated to determine whether the test population chose one treatment more often than the other. Equation 6.1 was used to create a variable describing the difference in the number of trials resulting in the choice of treatment A and treatment B (Bisazza et al., 1997; Facchin et al., 1999; De Santi et al., 2001; Pajor et al., 2003).

$$\text{Choice Index} = \frac{(\text{Choices Treatment A} - \text{Choices Treatment B})}{(\text{Choices Treatment A} + \text{Choices Treatment B})} \times 100$$

Equation 6.1 Choice index used to describe the difference in the number of trials resulting in the choice of Treatment A and Treatment B in the Y maze preference test.

The choice index gives some indication of the relative strength of the preference of one treatment relative to the other. A positive score indicates that the individual chose Treatment A more often than Treatment B, and vice versa for a negative score. The use of this index also provides information on the magnitude of the difference between the treatments, and means that an arbitrary 'preference' criterion does not have to be applied. In addition, each animal contributes only one data unit.

The hypothesis that sheep did not prefer one treatment over the other was tested using a Sign test to compare the median value of the choice index to zero. The less powerful Sign test was used because the distributions were generally non-symmetrical (skewness always < -1.5) (Zar, 1999). However, the results of the more powerful Wilcoxon matched pairs (signed ranks) test were also examined. In most cases, the results were very similar.

Plasma cortisol concentration

Effects on plasma cortisol concentration were analyzed using mixed ANOVA with doubly repeated measures for sampling time (pre-treatment (0), 15, 30, 45, 60 minutes after exposure) and stimulus presented (barking dog, human plus rattle). Testing Group was used to approximate the time of day that sampling took place, and was included in the model as a fixed effect. In addition, 'change in concentration from pre-treatment concentration' was calculated for each post-exposure sampling time (log (x) − log (pre-treatment concentration)) and analyzed the same way as the raw concentration data (Mellor et al., 2000).

The integrated cortisol response (area under concentration x time curve) and the peak concentration reached within one hour after the start of testing were analyzed using

ANOVA with repeated measures for stimulus, and testing group as a fixed effect. All cortisol variables were log transformed to satisfy assumptions of homoscedasticity and normal distribution of the residuals. Differences between stimuli were examined using Least Squares (LS) Means post hoc tests with Bonferroni correction for multiple comparisons. Differences are significant at $P < 0.05$, two-tailed probabilities are given, and all data are presented as Least Squares (LS) means of the raw data ± pooled standard error.

6.4   Results

Choice behaviour

Experiment 6a: Dog plus recorded barking versus Control

Of 38 sheep tested, 34 (89.5%) expressed a significant preference for the control treatment, while 3 (7.9%) showed a significant preference for the dog plus recorded barking. One sheep did not meet the criterion for a significant choice between stimuli. Significantly more sheep preferred the control treatment than expected by chance (χ^2 goodness of fit test: $\chi^2_{0.05, 2} = 54.05$, $P < 0.0001$).

Similarly, significantly more individual trials resulted in the choice of the control treatment than expected by chance: control 86.1%, dog 12.2%, no choice 1.7% of trials ($\chi^2_{0.05, 2} = 962.60$, $P < 0.0001$). The median value for the choice index was positive, indicating that sheep chose the control treatment significantly more often than the dog treatment (median = 90, Sign test M = 16, $P < 0.0001$).

Experiment 6b: Human plus rattle versus Control

Of 40 sheep tested, 30 (75%) expressed a significant preference for the control treatment, while 5 (12.5%) showed a significant preference for the human plus rattle. Five sheep did not meet the criterion for a significant choice between the stimuli. Significantly more sheep preferred the control treatment than expected by chance ($\chi^2_{0.05, 2} = 31.25$ $P < 0.0001$).

Likewise, significantly more individual trials resulted in choice of the control treatment than expected by chance: control 79%, human 20.6%, no choice 0.4% of trials ($\chi^2_{0.05, 2}$ = 799.97, P < 0.0001). In those trials in which a choice was made, significantly more resulted in choice of the control treatment than by chance (Binomial test n=797, P < 0.0001). The median value for the choice index was significantly higher than zero, indicating that sheep chose the control treatment more often than the human treatment (median = 84.7, Sign test M = 13.5, P < 0.0001).

Experiment 6c: Barking dog versus Human plus rattle

Of the 49 sheep tested, 40 (81.6%) expressed a significant preference for the human plus rattle and 4 (8.1%) chose the barking dog. However, two of the sheep that chose the dog were presented with the replacement dog plus recorded barking. Five sheep (10.2%) did not meet the criterion for a significant preference between the stimuli. Significantly more sheep preferred the human treatment than expected by chance ($\chi^2_{0.05, 2}$ = 51.47, P < 0.0001).

The human treatment was chosen in significantly more trials than expected by chance: human 83.4%, dog 11.6%, no choice 5% of trials ($\chi^2_{0.05, 2}$ = 1110.47, P < 0.0001). Likewise, the median value for the choice index was positive, indicating that sheep chose the human treatment significantly more often than the dog treatment (median = 100, Sign test M = 17.5, P < 0.0001).

Effect of experiment on choice behaviour

Table 6.4 shows the choices of the 'more aversive' treatment in each experiment, defined as the treatment which was not chosen by the majority of sheep. This was dog plus recorded barking in experiment 6a, human plus rattle in experiment 6b, and barking dog in experiment 6c.

The proportion of sheep choosing the more aversive treatment did not differ between the three experiments (Table 6.4a; Fisher's exact test P = 0.691). However, a higher proportion of trials resulted in choice of the more aversive treatment when that

treatment was the human (20.6% of trials versus control) than when it was the dog (12.2% of trials versus control, 11.6% of trials versus human) ($\chi^2_{0.05, 2}$ = 29.63, P < 0.0001; Table 6.4b). In accordance with this, the choice index was significantly different according to experiment (Kruskal Wallis test $\chi^2_{0.05, 2}$ = 13.44, P = 0.001); the difference between the choices of the preferred and non-preferred treatments was smaller in experiment 6b than in experiment 6c (paired t-test on ranks t = 3.83, P = 0.001). These results suggest that the human plus rattle treatment was less aversive to the sheep than either of the dog treatments, and particularly the barking dog.

Table 6.4 Choice of the '*more aversive*' or '*less aversive*' treatment according to the pair of treatments offered in the maze (experiments 6a, b or c). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the more or less aversive treatment. *B.* Number of trials in which sheep chose the more or less aversive treatment.

A. Number of sheep

	Preferred More aversive treatment	Preferred Less aversive treatment	Total
Dog v Control	3	34	38
Human v Control	5	30	40
Dog v Human	4	40	49
Total	12	104	127

B. Number of trials

	More aversive treatment chosen	Less aversive treatment chosen	Total
Dog v Control	93	654	760
Human v Control	165	632	800
Dog v Human	114	817	980
Total	372	2103	2540

Plasma cortisol concentration: Barking dog versus Human plus rattle

Table 6.5 shows plasma cortisol concentrations before and after exposure to a human plus rattle or barking dog. Mixed ANOVA revealed no overall stimulus effect on plasma cortisol response ($F_{(1,44)} = 0.65$, $P = 0.426$). In agreement with this, there was no stimulus effect on the integrated cortisol response (Table 6.5). However, there was a significant stimulus x time effect on cortisol concentration ($F_{(4, 41)} = 7.82$, $P < 0.0001$), indicating that concentration differed according to stimulus at one or more sampling times.

Pre-treatment cortisol concentration differed according to treatment (Table 6.5). Sheep had higher cortisol concentrations before exposure to the human plus rattle than barking dog, despite the experiment being balanced for order of stimulus presentation over days. Therefore, pre-treatment concentration was included as a covariate when examining post-exposure concentrations.

After exposure to the human plus rattle treatment, plasma cortisol was significantly elevated above pre-treatment concentration at 15 and 30 minutes (paired t-test 15 mins: $t = 4.40$, $P = 0.001$; 30 mins $t = 2.94$, $P = 0.036$). Concentrations had returned to pre-treatment values 45 minutes after exposure to the human. In contrast, after exposure to the barking dog, plasma cortisol was significantly elevated above pre-treatment concentration at 15, 30 and 45 minutes (15 mins: $t = 8.26$, $P < 0.0001$, 30 mins: $t = 5.61$, $P < 0.0001$; 45 mins: $t = 2.81$, $P = 0.054$). Concentrations had returned to pre-treatment values 60 minutes after exposure to the dog.

Cortisol concentration was significantly higher 15 minutes after exposure to the barking dog than 15 minutes after exposure to the human plus rattle, and tended to be higher at 45 minutes after exposure to the barking dog (Table 6.5). There was no stimulus effect on peak concentration when pre-treatment concentration was included as a covariate. When 'change in concentration from pre-treatment concentration' was analyzed using mixed ANOVA, there was an overall stimulus effect ($F_{(1, 44)} = 7.16$, $P = 0.010$), as well as a stimulus x time effect ($F_{(3, 42)} = 9.20$, $P < 0.0001$). The change from pre-treatment concentration was significantly higher at 15, 30 and 45 minutes after exposure to the barking dog than at the same times after exposure to the human plus rattle.

Table 6.5 Plasma cortisol concentration (nmol/L) before (0) and 15, 30, 45 and 60 minutes after a 30 second exposure to a human plus rattle or barking dog (n=50). Peak concentration within one hour after exposure and the integrated response (area under concentration x time curve nmol/L*mins) are also shown. In addition, change from pre-treatment concentration is shown for each post-treatment sample. LS means and pooled standard errors are presented, as well as stimulus effect statistics.

	Human plus rattle	Barking Dog	Pooled SE	F value	P value
Concentration					
0 mins	68.0	56.6	4.9	7.96	*0.007*
15 mins	100.0	113.3	5.4	4.68	*0.033*
30 mins	86.9	89.3	6.1	0.00	*0.988*
45 mins	59.2	70.2	5.0	3.06	*0.084*
60 mins	66.4	64.6	4.6	0.09	*0.760*
Peak	113.7	121.3	5.3	1.08	*0.302*
Integrated response	4782	4899	262	0.52	*0.473*
Change from pre-treatment concentration					
15 mins	34.1	54.1	6.2	11.03	*0.002*
30 mins	21.4	29.9	7.3	3.92	*0.054*
45 mins	-6.7	11.1	6.3	8.98	*0.005*
60 mins	0.0	6.1	5.7	1.38	*0.247*
Peak	48.2	61.6	6.1	7.84	*0.008*

6.5 Discussion

Validity of Y maze preference test

The first objective of this study was to validate the use of the Y maze preference test for assessing the relative preferences of sheep presented with treatments considered to be aversive. Sheep were offered the choice between an empty room and a treatment expected to be moderately aversive, a dog plus recorded barking or a human plus rattle, and it was hypothesized that sheep would prefer the empty room to the stimulus treatment in both cases. In accordance with this, more sheep chose the control treatment than chose either stimulus treatment. Likewise, sheep chose the control treatment in significantly more trials than either the dog or human treatment. This was corroborated by the significantly positive difference between choices of control and choices of the stimulus (choice index) in both cases.

The significant population preference for the control treatment compared to a treatment presumed to be aversive appears to validate the use of the Y maze test for assessing relative preferences of sheep presented with treatments differing greatly in aversiveness. Pajor et al. (2003) achieved similar validation for the use of the Y maze test for assessing the preferences of dairy cattle between treatments differing greatly in aversiveness; heifers showed a clear preference for the least aversive treatment, choosing food and a control treatment over hit/shout treatment, and food over the control treatment.

One of the major advantages of the Y maze test, and indeed preference tests in general, is that they allow direct comparison of widely differing treatments or stimuli (Rushen, 1986a). A significant problem in the field of applied ethology relates to the necessity for subjective interpretation of the behavioural responses of animals to stressful or aversive stimulation (Rushen, 2000). The fact that behavioral responses are strongly context-specific makes it extremely difficult to directly compare the effects of different treatments on animal stress or fear (Rushen, 1990). For example, locomotor activity in sheep increases during social isolation, but decreases in the presence of a predator,

although both situations are known to be stressful for sheep (Torres-Hernandez and Hohenboken, 1979; Romeyer and Bouissou, 1992).

Preference tests offer one solution to the problem of interpreting behavioural responses. Choice behaviour in preference tests provides a common behavioural response which can be used to rank the subjects' relative aversion to different situations (Rushen, 1996). Because the behavioural response is the same in each case, widely differing treatments can be compared on a single scale (Rushen, 1990). Stimulus-specific behavioural responses do not have to be interpreted in terms of aversiveness; the animal is simply given the opportunity to choose between the treatment alternatives offered. This makes choice behaviour easier to interpret than either physiological or behavioural measures of fear or stress, and preference tests may be more sensitive to the additive effects of different treatments combined (Rushen, 1986a).

There are also ethical advantages to the use of preference tests for measuring aversion. Alternative methods often require that the animal be repeatedly subjected to an aversive treatment that cannot be avoided (Rushen, 1986c; 1990). The anticipation of unavoidable punishment, or the motivational conflict between gaining a reward and avoiding punishment, may be stressful or frustrating to the animals involved. This can result in learned helplessness or species-specific fear behaviour, such as freezing in rodents, which can interfere with the performance of the behaviours required to avoid punishment (Rushen, 1986c). By using preference tests such as the Y maze test, after a small number of initial training trials, the animal is able to choose to avoid the aversive treatment in all further trials.

In some types of aversion test, failure to learn the association between the required (or punished) behaviour and the aversive treatment results in continued or increased frequencies of punishment (Rushen, 1986c; 1996). Therefore, the learning ability and memory of subjects, as well as the appropriateness of the conditioned behaviour to the species and population under study, can affect the ability of animals to express aversion. As such, negative results may be a consequence of methodology and not an indication that the treatments were not aversive (Rushen, 1996).

The Y maze test requires only that the animal learn the association between location and treatment, and the expression of aversion is not dependent on species-specific behavioural responses, making interpretation of choice behaviour more straightforward. However, the necessity for learning this association can also be a limitation to the use of this methodology. Choice behaviour may be confounded by individual differences in learning ability and memory. An associated problem is that the amount of training given before choice testing can affect the apparent preferences expressed by individuals (Pollard et al., 1994).

In the present study, at least two forced training runs were provided at the beginning of each day of preference testing. Additional training trials may have facilitated faster learning, however the majority of individuals selected their preferred option from the first or second trial. This suggests that most animals learned the association between side and treatment very quickly. Two forced training trials before preference testing were also found to be adequate to allow detection of the preferences of dairy cows between treatments differing greatly in aversiveness (Pajor et al., 2003).

Occasionally, an animal took a number of trials to 'settle' on the preferred treatment. This may have been due to incomplete learning, but may also have been related to other factors influencing choice behaviour (see below and Chapter 7). In addition, many animals made occasional 'incorrect' choices of the less preferred treatment, even though the difference in aversiveness between the treatments was large. This behaviour was seen in both dairy cows and sheep tested in a Y maze (Rushen, 1986a; Pajor et al., 2003). In the present study, such choices were usually followed by an immediate reversion to the preferred treatment, supporting the notion that such choices were due to lapses of memory or curiosity (Rushen, 1986a).

Preferences between a noise-making human or barking dog

The second objective of this study was to use the Y maze test to assess the relative aversiveness of the two treatments compared to each other directly. The adrenocortical responses of the sheep to the initial exposure to the dog and human treatments were also compared. It was hypothesized that both the choice behaviour and physiological stress responses would indicate a relative preference for the human plus rattle treatment. In

accordance with this hypothesis, more sheep chose the human plus rattle than the barking dog. The human treatment was chosen in significantly more trials than was the dog, and the positive choice index corroborated the relative preference of sheep for the human with rattle.

The choice behaviour was supported by the adrenocortical responses; the treatment that most sheep chose to avoid in the Y maze was also the treatment which elicited the larger increase in plasma cortisol concentration. Concentrations were significantly higher 15 minutes after exposure to the dog than at the same time after exposure to the human. This is a logical sampling time to expect differences in plasma cortisol concentration in response to treatments differing in aversiveness, as it would most closely reflect the animal's experience of the treatment in the Y maze. In addition, the increase in cortisol concentration above pre-treatment concentration was significantly larger 15, 30 and 45 minutes after exposure to the dog, and was sustained longer after exposure to the dog than to the human. Therefore, it is concluded that the barking dog, presented in the Y maze test, was more aversive to the sheep than the human with a rattle.

The fact that preference tests give only a relative measure of aversion is often noted as a limitation of the methodology (Fraser and Matthews, 1997). Preference for one treatment is relevant only to the alternative offered. Accordingly, the preference for one treatment over the other does not imply that the non-preferred treatment is aversive, only that it is *less preferred* than the chosen treatment. It is entirely possible that neither treatment is aversive, or alternatively, that both treatments are aversive.

In the present study, the relativity of the treatment preferences is not regarded as problematic. My primary interest was in whether sheep find a barking dog more aversive than a human with a rattle; I was interested in the relative aversiveness of each treatment for the sheep. In addition, as noted by Van Rooijen (1983), in reality, all measures of aversiveness are relative in nature. No stimulus or treatment inherently holds an objective 'amount' of aversiveness; it may be aversive in relation to one alternative and not at all in relation to another.

Having said this, measurement of a physiological indicator of stress as well as choice behaviour unquestionably improved the understanding of the aversion of sheep to the

194

dog and human treatments. Both treatments elicited a significant increase in cortisol concentration above the pre-treatment concentration. The addition of the recorded or real barking in the case of the dog treatment, or rattling noise in the case of the human treatment is likely to have added to the aversiveness of each respective stimulus, as loud noises have been shown to elicit behavioural and physiological stress responses in sheep and other farm species (Ames and Arehart, 1972; Croney et al., 2000; Kanitz et al., 2005).

However, this result does not indicate that the presence of a barking dog or human with rattle was aversive per se. The post-exposure cortisol concentrations reflect not only the sheep's response to the stimulus in the treatment room, but also its response to the other potentially stressful elements of the Y maze testing procedure. These include close proximity to humans, the novelty of handling, repeated venepuncture and movement through the apparatus, and social isolation, all of which have been shown to elicit significant increases in plasma cortisol concentration in sheep (e.g. Pearson and Mellor, 1976; Moberg and Wood, 1982; Parrott et al., 1987; Parrott et al., 1988; Cockram et al., 1994; Mears and Brown, 1997; Degabriele and Fell, 2001).

Therefore, it is not possible, based on the results of the present study, to conclude that the presence of the human or the dog alone was aversive to sheep. In order to show this, the change in cortisol concentration of a group subjected only to repeated blood sampling and movement through the facility, without the presence of a stimulus in the treatment area, would have to have been measured. The lack of such a control group in the experimental design limits my ability to judge the aversiveness of the barking dog or human with rattle, independent of the aversiveness of the testing procedure itself. However, it is possible to conclude that the addition of the barking dog in the treatment area enhanced the aversiveness of the testing procedure to a greater degree than did the addition of the human.

The results also indicate that exposure to either a barking dog or a human with rattle *during Y maze testing,* with all its potentially stressful components, appears to be aversive. The fact that the majority of sheep also chose to avoid both the dog and the human treatment, when compared to social isolation alone in the same testing environment, suggests that both treatments were aversive to some degree. However, the

195

magnitude and duration of the plasma cortisol responses in the present study indicate that the Y maze testing procedure including the treatments was only moderately aversive to the sheep.

Although it is not possible to directly compare concentrations from different studies because of differences in assay sensitivity, the peak concentrations and peak increases above pre-treatment concentration obtained in this study fall in the middle of the general range of cortisol responses of young and adult sheep subjected to a variety of physical and psychological stressors (e.g. Fulkerson and Jamieson, 1982; Harlow et al., 1987; Fell and Shutt, 1989; Hargreaves and Hutson, 1990b; 1990c; 1990d; 1990a; Coppinger et al., 1991; Apple et al., 1993; Niezgoda et al., 1993; Apple et al., 1994; Chapman et al., 1994; Cockram et al., 1994; Kilgour and Szantar-Coddington, 1997; Cook, 2004). Without conducting an ACTH challenge test to determine maximum plasma cortisol concentrations in the sheep used in this study, it is not possible to conclusively judge the magnitude of the responses to the treatments imposed here. However, the concentrations did not even approach maximum concentrations reported in other studies on sheep (e.g. Fulkerson and Jamieson, 1982; Kilgour and Szantar-Coddington, 1997).

The peak concentration was only about twice the pre-treatment concentration after exposure to the dog, and only about 1.5 times the pre-treatment concentration after exposure to the human. In Chapter 5 of this thesis, I reported that a 10-minute exposure to a non-barking dog in a much larger area elicited peak plasma cortisol concentrations around 3 times higher than pre-treatment concentrations. Even these concentrations were only moderate compared to peak increases reported for adult sheep exposed to barking dogs (e.g. Harlow et al., 1987; Canny et al., 1990; Kilgour and Szantar-Coddington, 1997; Cook, 2004).

In the present study, the moderate increases in cortisol concentration after exposure to the stimuli may have been partly due to the relatively high pre-treatment concentrations obtained in this study. These high values were probably due to the handling required to group the sheep according to stimulus-side presentation group. However, the actual peak concentrations were still only moderate in magnitude, so that a "ceiling effect", where maximum concentrations cannot rise higher, may be ruled out (Mellor et al., 2000). In addition to the moderate magnitude of these responses, sheep appeared to

habituate quickly to repeated handling and blood sampling, as indicated by the return of cortisol concentrations to pre-treatment concentrations within one hour of exposure to the treatment. The moderate magnitude and duration of the cortisol responses in this study indicate that the sheep were not excessively stressed by either treatment (Mellor et al., 2000).

The agreement of the physiological and choice results lends further support to the use of the Y maze test for assessing the relative preferences of sheep for stimuli similar in aversiveness. Other studies have also shown that preferences expressed in the Y maze test agreed with other behavioural and physiological indicators of aversion. For example, both choice behaviour and adrenocortical responses showed that sheep found capture and inversion more aversive than capture alone (Rushen, 1986a; Hargreaves and Hutson, 1990b). Likewise, similar results were obtained using a Y maze test and an 'aversion race' for determining the aversion of sheep to electro-immobilization or mechanical restraint (Grandin et al., 1986; Rushen, 1986b) and of dairy cows to milking shed noise (Arnold, 2005).

The results of this Y maze test and the adrenocortical responses corroborate results from past studies. The presence of a human has been shown to elicit behavioural and physiological responses indicative of fear or stress in sheep (Fulkerson and Jamieson, 1982; Baldock and Sibly, 1990; Romeyer and Bouissou, 1992; Bouissou and Vandenheede, 1995; Goddard et al., 2000; Hansen et al., 2001; Beausoleil et al., 2005). Not surprisingly, Kendrick et al. (1995) found that of those sheep which showed discriminative ability in a Y maze test, most individuals chose a photograph of a sheep's face which granted access to a sheep, rather than a photograph of a human's face which granted access to a human. However, sheep preferred close proximity with a human in the presence of conspecifics to restraint in a cage or social isolation (Rushen, 1986a), once again highlighting the fact that aversion is only relative.

The presence of a dog has also been shown to elicit behavioural and physiological stress responses in sheep (Torres-Hernandez and Hohenboken, 1979; Harlow et al., 1987; Canny et al., 1990; Kilgour and Szantar-Coddington, 1997; Hansen et al., 2001; Cook, 2004). Rather than explicitly testing the effects of dog presence on stress responses, most of these studies have designated this treatment as a 'severe stressor' in order to

compare the effects of other stressors on behaviour and physiology (e.g. Harlow et al., 1987; Canny et al., 1990; Cook, 2004; Roussel et al., 2004). The reason for this choice is often given as the high biological relevance of a potential predator to sheep (Cook, 2004). The results of the present study call into question the validity of using a dog as a *severe* stressor, however, the duration of the treatment in this study was short, and the magnitude of the peak cortisol concentration was small relative to the peak concentrations reported in other studies.

The present study is one of only a few to provide direct scientific evidence that dog presence is more aversive to sheep than human presence. Harlow et al. (1987) found that a brief exposure (10 seconds) to a shouting human caused a significant increase in both peak heart rate and plasma cortisol concentration in adult ewes. A longer exposure to a more aggressive human (shouting and banging on sheep stalls) delayed recovery of both parameters, although the magnitude of the response was no higher than the milder treatment. However, a 5-minute exposure to a barking dog elicited adrenocortical and cardiovascular responses much greater than those resulting from either human treatment (Harlow et al., 1987). Likewise, the approach of a man and dog elicited higher cardiovascular and behavioural responses in sheep than did approach of a man alone (MacArthur et al., 1979; Baldock and Sibly, 1990; Martinetto and Cugnasse, 2001).

Effect of difference in aversiveness on choice behaviour

The agreement between the choice behaviour and adrenocortical responses in experiment 6c lends validity to the use of the Y maze test for assessing preferences between stimuli similar in aversiveness. However, choice behaviour in the Y maze was expected to be affected by the relative difference in aversiveness between the two stimuli offered. This expectation was based on reports that individual differences in preferences were found in both sheep and cattle when the treatments offered were similar in aversiveness (Rushen, 1986a; Pajor et al., 2003). Rushen also postulated that the relative distribution of choices between two alternatives should reflect the degree of difference in aversiveness between them.

The degree of difference in the first two experiments was large, whereas the treatments offered in experiment 6c were considered to be relatively similar in aversiveness.

198

Therefore, I expected the relative distribution of choices to be more equal in experiment 6c than in the first two experiments. However, there was no evidence of this. In fact, there was evidence that sheep were less likely to choose the more aversive stimulus in experiment 6c (barking dog) than in experiment 6b (human plus rattle); that is that the distribution of choices was less equal in experiment 6c. This result suggests that the difference in aversiveness between the treatments offered in experiment 6c was larger than anticipated, with the barking dog being significantly more aversive than the human with rattle to most sheep.

In the present study, a small proportion of sheep appeared to prefer the more aversive treatment in each experiment. Three sheep chose the dog plus recorded barking treatment, while 5 sheep appeared to prefer the human plus rattle, when the alternative was an empty room. Likewise, two sheep expressed a significant preference for the real barking dog as opposed to the human with rattle, while two sheep chose the replacement dog plus recorded barking.

A similar phenomenon has been observed in previous Y maze tests. For example, four out of 13 dairy heifers continued to choose the side of the maze in which an electric shock was applied (Arnold, 2005). Likewise, while most cows or deer preferred to walk through a crush without being restrained, a number of individuals chose restraint in almost every trial (Grandin et al., 1994; Pollard et al., 1994). Using an aversion learning technique, Pajor et al. (2000) found that cows ranked handling treatments from least to most aversive as follows: Control (human presence), Tail Twist, Hit, Shout, Cattle Prod. However, when tested in a Y maze, 2 of 8 cows chose Shout over Hit, 2 of 8 chose Cattle Prod over Shout, and 2 out of 8 chose Tail Twist over Control (Pajor et al., 2003).

There are several possible explanations for this behaviour. Firstly, it is possible that a minority of individuals in any population actually prefer the more aversive stimulus. In this case, some sheep may prefer the presence of a dog or human to social isolation alone. Likewise, a proportion of individuals may prefer confinement with an aggressive barking dog to confinement with a noise-making human. My lack of knowledge about the past experience of each individual sheep precludes an understanding of the potential factors leading to such a preference. However, the relatively constant proportion of

individuals expressing the minority preference suggests that some factor other than the presence of the stimulus may have influenced choice behaviour in the Y maze.

In the present study, the most likely explanation for this phenomenon is the effect of different behavioural response patterns on choice behaviour in the Y maze (Rodriguez et al., 1992; Arnold, 2005). Briefly, response patterns which may affect choice behaviour include Spontaneous Alternation Behaviour (SAB), Perseveration Behaviour (PB) and Lateralized Behaviour (Rodriguez et al., 1992). SAB refers to a higher than random propensity to alternate between one side of the maze and the other in consecutive trials. PB refers to the tendency to persist in choosing the same option in consecutive trials, and suggests the formation of a routine or stereotyped behavioural response. However, an animal may show a high degree of perseveration without showing a consistent preference for one option over the other in successive testing sessions e.g. consistently chooses right in all sessions on day one, but left in all sessions on day two (Rodriguez et al., 1992).

In contrast, lateralized behaviour refers, in this case, to a consistent preference for one particular side of the maze (Rodriguez et al., 1992). Lateralized behaviour is not simply a consequence of perseverative behaviour. As noted above, an animal may show perseveration without showing a consistent preference for one side. Lateral biases may relate to some feature of the testing facility or experimental design. For example, if one arm of the maze facilitates easier movement or is closer to the exit than the other, animals may express a preference for this side, independent of the treatment applied there (e.g. Hopster et al., 1998). Alternatively, lateral biases may reflect inherent behavioural asymmetry in the individual, perhaps relating to functional asymmetry at higher levels of the central nervous system (Vallortigara and Rogers, 2005). The potential for lateralized behaviour to affect choice behaviour and therefore the expression of treatment preferences has been explicitly tested and is discussed in Chapter 7.

In general, the choice of one treatment over the other in a preference test provides no information about the strength of the preference; it could be a minor partiality, or one with significant biological relevance to the animal (Fraser and Matthews, 1997). In the current study, sheep that ultimately chose the control treatment (or human plus rattle in

6c) made this choice in an overwhelming majority of trials, and very few individuals failed to express a significant preference one way or the other. This suggests that the preferences expressed were biologically significant to the sheep. However, because preference tests tend to minimize extraneous variation (e.g. time or location effects), they may detect statistically significant differences even when the magnitude of the preference is small (Fraser and Matthews, 1997).

The significant difference in adrenocortical responses to the dog and the human supports the idea that the preference for the human plus rattle in the Y maze was biologically relevant. However, in order to formally assess the strength of the preferences, the choice or avoidance of each treatment would have to be related to some cost (Fraser and Matthews, 1997). This cost could be experimentally varied to assess the willingness of sheep to 'pay' to avoid each treatment (e.g. Dawkins, 1983; 1990). Future studies could use cost-benefit analyses to determine the strength of sheep's preferences between the dog and human treatments presented in this study.

Implications

Limiting the use of dogs prior to slaughter has been recommended in the operational guidelines for sheep welfare of several countries (Canadian Food Inspection Agency, 1990; Australian Quarantine and Inspection Service, 1995; New Zealand National Animal Welfare Advisory Committee, 1996). In addition, avoiding the use of dogs to reduce stress in the period between mustering on the farm and slaughter has been identified as one of the critical points for controlling meat quality in the Australian meat supply chain (Young et al., 2005). However, the present study is one of the first to directly assess the potential for dog presence to increase pre-slaughter stress in sheep.

The results suggest that the presence of either a barking dog or a human with a rattle was aversive to the sheep. However, it is important to note that there are significant differences between the Y maze environment in which these responses were measured and the pre-slaughter environment in meat processing premises. For example, the Y maze procedure involved temporary social isolation of the test sheep, while visual and physical contact with other sheep is generally maintained during pre-slaughter handling. Social isolation is known to be stressful to sheep (Parrott et al., 1987; Parrott et al.,

1988; Cockram et al., 1994; Degabriele and Fell, 2001), and may have significantly contributed to the aversiveness of each treatment in the Y maze. The lack of an appropriate control group in the experimental design limits my ability to judge, by reference to plasma cortisol concentrations, the aversiveness of the barking dog or human with rattle, independently of the testing procedure itself.

Moreover, the exposure of test sheep to the dog and human in the Y maze was relatively short, and may not represent the typical pre-slaughter experience of sheep in New Zealand. Additionally, in meat processing premises, barking dogs are invariably accompanied by their human handlers. The additive effect of a dog and human may lead to higher stress levels in sheep than those elicited by the presence of a dog or human alone. Because of the differences between the experimental and practical situations, it would be beneficial to conduct further studies on the stress responses of sheep to the presence of barking dogs or humans with rattles in the actual pre-slaughter environment.

Although the present study suggests that both dogs and humans may be aversive to sheep, in practice, sheep must be moved from lairage to slaughter by some method, and it is unrealistic to suggest that the use of both dogs and humans be avoided before slaughter. Despite the fact that sheep avoided both stimuli in the present study, the plasma cortisol responses to both were only moderate compared to those elicited by other procedures occurring during sheep management (e.g. Fell and Shutt, 1989; Hargreaves and Hutson, 1990b; 1990c; 1990d; 1990a; Coppinger et al., 1991; Niezgoda et al., 1993; Apple et al., 1994; Chapman et al., 1994; Dinnis et al., 1997; Kilgour and Szantar-Coddington, 1997; Cook, 2004).

Accepted management practices such as shearing, castration, and tail removal elicit higher physiological stress responses than were found in the present study (Fulkerson and Jamieson, 1982; Hargreaves and Hutson, 1990b; 1990c; 1990d; 1990a; Dinnis et al., 1997; Molony, 1997; Mellor, 2002). Therefore, if further testing in the pre-slaughter environment demonstrates similarly moderate stress responses to those found here, the use of dogs or humans with rattles in meat processing plants would create little animal welfare concern. However, whether such stress levels would adversely affect meat quality is an issue which remains to be tested directly in the pre-slaughter environment.

Both the choice behaviour measured in the Y maze and the adrenocortical responses suggest that the barking dog was more aversive than the human plus rattle. Therefore, replacement of barking dogs with humans using noise-making devices in meat processing premises may reduce pre-slaughter stress in sheep. However, as noted above, without examining stress responses within the context of the real pre-slaughter environment, I can do little more than point to the potential for barking dogs to elicit more stress than humans with rattles.

It is also important to note that the behaviour of the human with the rattle was relatively passive in the present study. In practice, people attempting to move sheep are likely to behave differently, and may elicit more stress than was indicated here. Yarding and drafting of sheep by humans who shouted and waved their arms was shown to elicit significant increases in plasma cortisol concentration compared to sheep which remained undisturbed in a pen (Fulkerson and Jamieson, 1982; Hargreaves and Hutson, 1990d). In contrast, because it was not possible to control its behaviour, the relatively aggressive nature of the particular dog used in experiment 6c may have enhanced the aversion sheep expressed towards it. However, even in experiment 6a, in which the dog was relatively passive, sheep showed a significant preference for the alternative treatment.

The present observations suggest that additional research in this area would be beneficial. Future studies should compare changes in plasma cortisol concentration, and other indicators of stress, after handling with or without dogs in a real pre-slaughter situation. Such responses should more appositely indicate the potential for dog presence to increase stress in sheep over and above the level elicited by human handling. Direct comparison, within the same experiment, of stress responses to pre-slaughter handling (with or without dogs) with responses elicited by other accepted management practices such as mustering, shearing or castration, will provide further information on the potential for such pre-slaughter handling to negatively affect animal welfare.

There are clearly benefits to minimizing unnecessary animal stress at all stages of production. If the results of the present study are confirmed by further experiments conducted in the real pre-slaughter environment, it may be advantageous to continue to minimize the use of dogs for moving sheep prior to slaughter.

6.6   Conclusions

The results of the present study appear to validate the use of the Y maze test for assessing the relative preferences of sheep presented with treatments differing greatly in aversiveness. In addition, the results tend to support avoiding or limiting the use of dogs for moving sheep prior to slaughter. Both the choice behaviour in the Y maze and the adrenocortical responses indicate that sheep found confinement with a barking dog more aversive than confinement with a noise-making human.

Although there was some evidence that sheep found both the barking dog and human with rattle aversive, the adrenocortical responses to both stimuli presented separately were only moderate in magnitude and duration compared to other accepted sheep management practices. Therefore, if the results of this study are confirmed by experimental testing in the real pre-slaughter environment, handling either with dogs or by people with rattles should cause little concern on the basis of animal welfare. However, minimizing animal stress in all stages of production is advantageous, and this preliminary study provides some support for the replacement of barking dogs with people using noise-making devices before slaughter.

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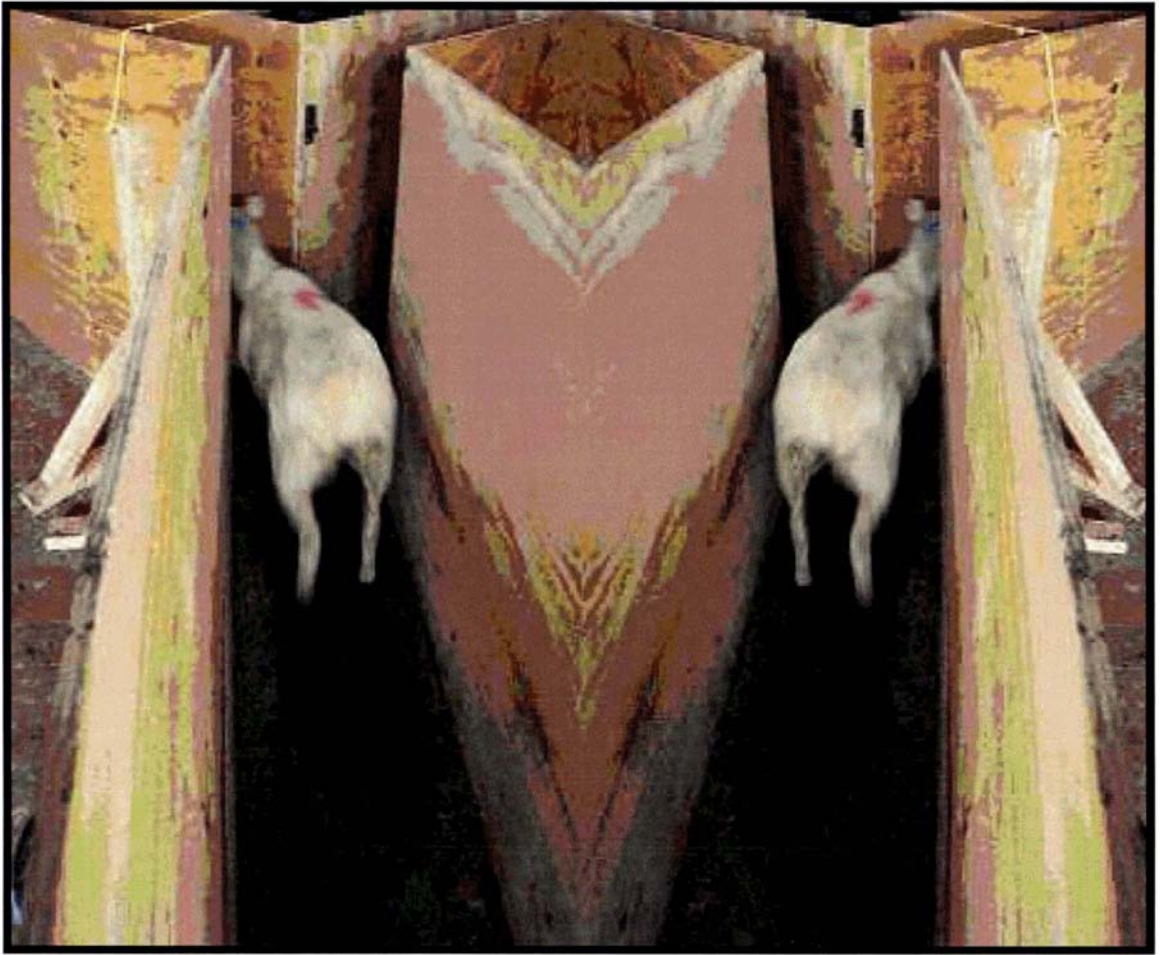
CHAPTER 7

The effect of lateral biases on the choice
behaviour of sheep in a Y maze preference test

7.1 Abstract

Lateralized behaviour is a common phenomenon across a wide range of species. Significant laterality can occur at the level of the individual without the existence of a directional bias at the population level. Previous studies using two-choice tests, such as Y or T maze tests, have found that individual lateral biases, or side preferences, are common in domestic species. In addition, there is indirect evidence that lateral biases can influence choice behaviour in the Y maze test, which may distort the expression of preferences for the treatments presented. Individual sheep were tested for the expression of lateral biases in an empty Y maze apparatus. Sheep were considered to have a significant bias if they chose one side of the maze 8 or more times in 10 trials. Treatments were then allocated according to bias: on the preferred side or non-preferred side of biased sheep, or randomly for non-biased sheep. The choice behaviour of biased and non-biased sheep was compared when offered the choice between, a. dog plus recorded barking and control (empty room), b. human plus rattle and control, c. barking dog and human plus rattle. The results confirm that individual lateral biases may influence choice behaviour in the Y maze test. Biased sheep chose the more aversive stimulus more often than did non-biased sheep when both groups were offered the same pair of treatments (experiments 7a and b). In addition, the side of presentation affected the choice behaviour of biased sheep; the more aversive stimulus was chosen more often when presented on the preferred than non-preferred side. Finally, the degree to which lateral biases affect choice behaviour appears to be related to the relative aversiveness of the treatments presented; sheep were more willing to switch away from their preferred side if the treatment presented there was a dog than if it was a human. These results suggest that individual lateral biases expressed in two-choice tests are biologically relevant to the individual, have the potential to affect choice behaviour, particularly when the treatments are similar in aversiveness, and may therefore distort the expression of animal preferences. It is concluded that individual lateral biases should be characterized before the commencement of Y maze preference testing, and test treatments should be allocated in such a way as to standardize the effects of lateral biases on choice behaviour.

7.2 Introduction

There is a surfeit of evidence for the existence of lateralized behaviour in a wide range of species, including humans, primates and other mammals, birds, fish, reptiles, amphibians and even invertebrates (e.g. Rodriguez et al., 1992; Andrews and Rosenblum, 1994; Bisazza et al., 1997; Bisazza et al., 2000; De Santi et al., 2001; Seligmann, 2002; Byrne et al., 2004; Csermely, 2004; Malashichev and Wassersug, 2004; Heuts and Brunt, 2005; Izawa et al., 2005). For recent reviews see Rogers (2002) and Vallortigara and Rogers (2005).

Laterality is evident in naturally expressed behaviours, such as predator inspection and avoidance, prey capture, food manipulation and intraspecific interactions (e.g. Sovrano et al., 1999; De Santi et al., 2001; Franklin and Lima, 2001; Csermely, 2004; Izawa et al., 2005). It is also manifested in a number of experimentally induced behaviours, such as the direction of amphetamine-induced circling, negotiation of barriers, and in preferences toward one side of the testing apparatus in two-choice tests (e.g. Zimmerberg et al., 1978; Rodriguez et al., 1992; Bisazza et al., 1997; Facchin et al., 1999; Bisazza et al., 2000; Wynne and Leguet, 2004).

Laterality can be measured either at the level of the individual or across the test population. Individual lateralization refers to the propensity of the individual to express lateralized behaviour (e.g. person A uses his right hand significantly more often than his left hand), while population lateralization refers to a bias in the distribution of individuals with significant lateralized behaviour within the population (e.g. significantly more people are right-handed than left-handed). It is possible for individuals within the population to exhibit highly lateralized behaviour while the distribution of biases within the population is balanced (e.g. half the individuals are right-handed and half are left-handed). For the purposes of this study, I am primarily interested in the effects of individual lateral biases on the expression of choice behaviour in a two-choice behavioural test, the Y or T maze preference test, and I will henceforth refer only to lateralized behaviour at the individual level, unless otherwise stated.

Y and T maze preference tests offer subject animals the choice between the two maze arms, each of which is associated with a particular treatment. By selecting either the right or left arm of the maze, individuals can theoretically express their preferences for one treatment relative to the other. However, factors other than the reinforcing presence of the treatments may influence choice behaviour in the Y maze, and most tests fail to account for variables such as different response patterns or the existence of lateralized behaviour (Arnold, 2005).

Briefly, response patterns in two-choice tests include spontaneous alternation behaviour (SAB), perseverative behaviour and lateralized behaviour (Rodriguez et al., 1992). SAB refers to a higher than random propensity to alternate between one side of the maze and the other in consecutive trials. SAB has been commonly reported in rodents and some other mammals, and may reflect motivation to explore novel environments (see Dember and Richman, 1989). However, to date, there is no evidence of SAB in domestic ungulates (Arnold, 2005).

Perseverative behaviour (PB) refers to a lower than random propensity to alternate, or the tendency to persist in choosing the same option in consecutive trials. It is important to note that an animal may show a high degree of perseveration without showing a consistent preference for one option over the other in successive testing sessions e.g. it consistently chooses right in all sessions on day one, but left in all sessions on day two (Rodriguez et al., 1992).

Lateralized behaviour refers, in this case, to a consistent preference for one particular option (Rodriguez et al., 1992). Laterality is not simply a consequence of PB; as noted above, an animal may show perseveration without showing a consistent preference for one option. Laterality can be defined as the probability of choosing a particular side in a single two-choice trial, while perseveration refers to the likelihood of repeatedly choosing the same side in a series of trials in one testing session, when there is no other reinforcement mediating the choices (Arnold, 2005). In practice, it may be difficult to differentiate between perseveration and lateral biases during Y maze testing, however, both factors have the potential to mask the effect of the test treatment on choice behaviour, and can potentially confound the expression of treatment preferences (Arnold, 2005).

Individual preferences for one arm of a Y or T maze over the other, independent of the side/treatment association, have been noted in a variety of domestic species, including sheep (Hansen et al., 1978; Grandin et al., 1986; Hosoi et al., 1995; Kendrick et al., 1995; Perez-Barberia et al., 2005), goats (Hosoi et al., 1995), cattle (Grandin et al., 1994; Phillips and Morris, 2001; Pajor et al., 2003; Arnold, 2005) and horses (Murphy et al., 2005). In addition, consistent individual side preferences in other two-choice situations, such as milking parlours, are commonly reported (Hopster et al., 1998; Da Costa and Broom, 2001; Phillips et al., 2003).

Studies testing sheep in two-choice situations with no treatments imposed report that the majority of individuals in the test population showed significant preferences for one side or the other. For example, 70% of sheep were found to be biased, when a significant bias was defined as 3 out of 4 trials to the same side (Hansen et al., 1978). Likewise, Hosoi et al. (1995) reported that 63% of sheep and 100% of goats tested showed side preferences when a significant preference was defined as at least 69% of 23 trials to the same side. Similar proportions have been reported in cattle (Hopster et al., 1998; Da Costa and Broom, 2001; Arnold, 2005).

In a Y maze test which allowed treatments to be balanced across sides for each individual, 30-96% of sheep expressed a preference for one side of the maze, regardless of the treatment presented on that side (Kendrick et al., 1995). The proportion of animals expressing position preferences was influenced by breed and by the pairs of treatments presented, indicating the potential for lateral biases to differentially affect choice behaviour.

Individual side preferences appear to be consistent over time and with varying social and environmental factors (Glick et al., 1977; Hosoi et al., 1995; Hopster et al., 1998; Arnold, 2005). In addition, side preferences appear to be strongly persistent; in studies on both rodents and cows, despite being trained to choose the option opposite to that originally preferred, most animals reverted back to their initial preferences after the training reinforcement was discontinued (Glick et al., 1977; Arnold, 2005). Therefore, individual lateral biases likely represent stable characteristics of the individual animal, and there is evidence that side preferences relate to structural and neurochemical asymmetries in the brain (e.g. Zimmerberg et al., 1978; Peirce et al., 2000; Peirce and

Kendrick, 2002; Rogers, 2002; Rizhova and Kokorina, 2005; Vallortigara and Rogers, 2005).

However, it is also possible that lateralized behaviour in a two-choice system is induced by some feature of the experimental apparatus or testing procedure. For example, one side of the maze may appeal to the individual more than the other. However, evidence suggests that sheep do not use external cues in the maze, nor olfactory or visual cues from previous subjects to direct their choices in maze tests (Liddell, 1925; Franklin and Hutson, 1982; Hosoi et al., 1995). In addition, most studies on domestic species report equal proportions of animals expressing significant right and left biases e.g. no population bias (Hansen et al., 1978; Hopster et al., 1998; Da Costa and Broom, 2001; Murphy et al., 2005). This suggests that the expression of lateral biases are not related to one side of the apparatus being inherently more or less aversive than the other.

It is also possible that what appears to be a biologically relevant preference for one side is simply the expression of perseverative behaviour after an initial random choice of sides. Stress and fear have been shown to increase the expression of perseverative behaviour in rodents (Rodriguez et al., 1992). Kendrick et al. (1995) noted that directional biases appear particularly strong and difficult to overcome in sheep, and suggest that this may be due to the expression of stereotyped behaviour when sheep are temporarily isolated from conspecifics. As social isolation is known to be particularly stressful for sheep, fear or stress related to the Y maze testing procedure may increase the expression of perseverative behaviour. Confinement in the testing apparatus and close contact with humans may also increase the stress experienced by the animals and may reinforce particular response patterns during lateral bias testing (Arnold, 2005). However, regardless of the origin of individual lateral biases, their existence necessitates further investigation into their potential to influence the expression of preferences in the Y maze test.

Although the potential for individual lateral biases or side preferences to influence choice behaviour, and therefore expression of preferences, in Y and T maze tests has been acknowledged (e.g. Grandin et al., 1986; Hosoi et al., 1995; Prescott et al., 1998; Phillips and Morris, 2001; Pajor et al., 2003), very few studies to date have attempted to quantify or control for such effects (however, see Arnold, 2005). The aim of the present

221

study was to assess the effect of lateral biases on the choice behaviour of sheep in a Y maze preference test. This was done by characterizing each individual's lateral bias (or lack thereof) in the empty maze, and comparing the choice behaviour of biased and non-biased sheep after the addition of the stimulus treatments in the maze.

It was hypothesized that biased sheep would be more likely than non-biased sheep to choose an aversive treatment when it was offered against a control (empty room). When two aversive treatments were offered, it was hypothesized that biased sheep would be more likely than non-biased sheep to choose the more aversive treatment (defined as the treatment not preferred by the majority of sheep). It was also hypothesized that if lateral biases were affecting choice behaviour, biased sheep would be more likely to choose an aversive treatment if it was presented in their preferred side of the maze than if it was presented in their non-preferred side.

7.3 Methods and Materials

The animals, experimental setup and Y maze testing procedures and schedules for each experiment were described in Chapter 6. Experiments 7a, b and c are the same as experiments 6a, b and c. In each experiment, after familiarization with the testing facility, and before preference testing, each sheep was tested for any lateral bias in the empty Y maze (no stimuli present). The use of all animals and procedures was approved by the Massey University Animal Ethics Committee (protocol 03/113).

Lateral bias testing

On the day of lateral bias testing, each sheep received two training runs, being forced first to one side and then the other using the direction-forcing gate. The training runs ensured that each sheep had experienced both sides of the maze before testing for lateral biases (Pollard et al., 1994). The order of training runs (R, L) was balanced across the 60 sheep in each experiment. Each sheep was then allowed to make 10 free choices in the empty maze. After entering the chosen treatment room, the sheep was held for 10 seconds in the empty room, before being released.

Originally, a lateral bias was defined as 7 or more choices to one side of the maze. However, using a one-tailed binomial test, it was determined that a sheep must choose the same side at least 8 times to be statistically different from chance in 10 trials (P = 0.055) (Hosoi et al., 1995; Rybarczyk, 2001; McLeman et al., 2005). Therefore, for statistical analysis, a lateral bias was recognized if the sheep went to the same side 8 out of 10 times. Sheep that were originally classed as having a lateral bias (7/10) were placed in the No Bias group for statistical analysis. The difference in the number of animals in each bias group from Chapter 6 (Tables 6.1, 6.2, 6.3) to Chapter 7 (Figures 7.1, 7.2, and 7.3) reflects this change in definition of lateral bias.

Treatments were allocated to each side of the maze according to lateral biases expressed in the empty maze (see Figures 7.1, 7.2, and 7.3). In experiments 7a and 7b, one of the main goals was to determine whether the addition of a treatment (dog or human) on the animal's preferred side of the maze would cause it to shift away from that side, if the alternative was an empty room. Therefore, for the Right and Left bias groups, the majority of animals received the treatment on their preferred side of the maze. For the No bias group, half the sheep received the treatment in the right, and half in the left. The primary aim of experiment 7c, was to determine which of the two treatments was least preferred, without making any assumption about which was more aversive. Therefore, in each group, half the animals received the dog on the right side and half received the dog on the left side.

Statistical analysis

A Fisher's exact test or Yates-corrected χ^2 test (depending on the number in each cell of the table) was used to test whether the existence of a lateral bias affected the proportion of sheep preferring each treatment or expressing no preference (Bias vs No bias). The same tests were used to compare the number of trials resulting in the choice of each treatment, according to the same groupings. Within the Bias groups, I tested whether choice behaviour differed according to the direction of the bias (Right bias vs Left bias).

223
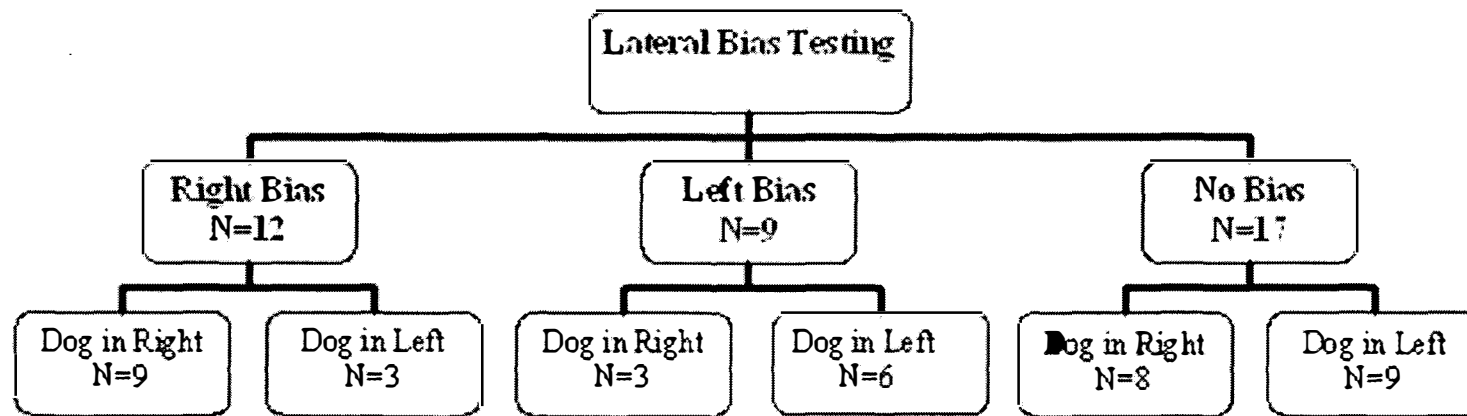
Figure 7.1 Experiment 7a: presentation of dog plus recorded barking (versus control) in the Y maze, according to lateral biases expressed in the empty maze. N = number of sheep in each side/stimulus group.
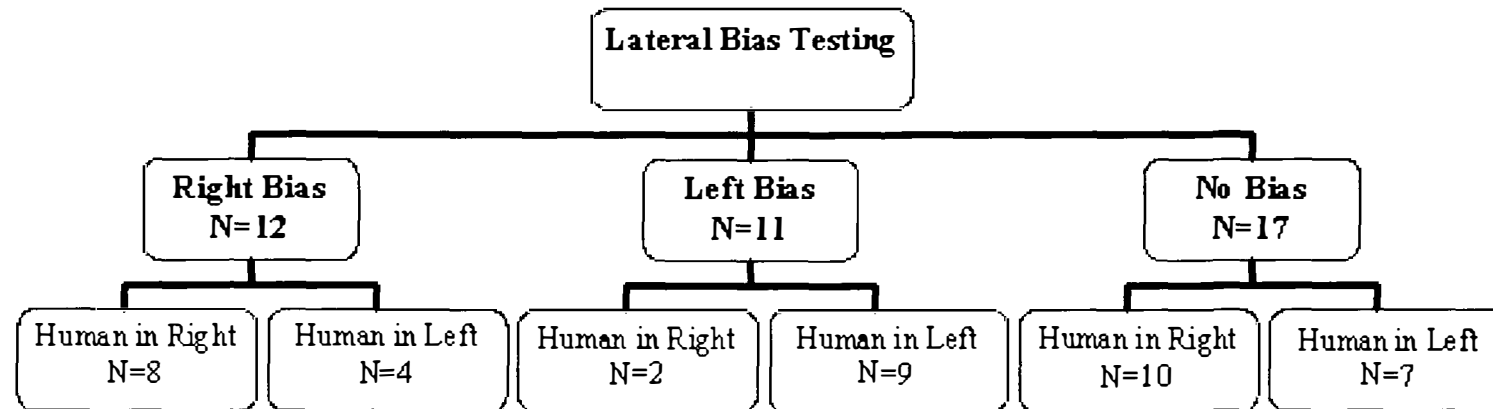
Figure 7.2 Experiment 7b: presentation of human plus rattle (versus control) in the Y maze, according to lateral biases expressed in the empty maze. N = number of sheep in each side/stimulus group.
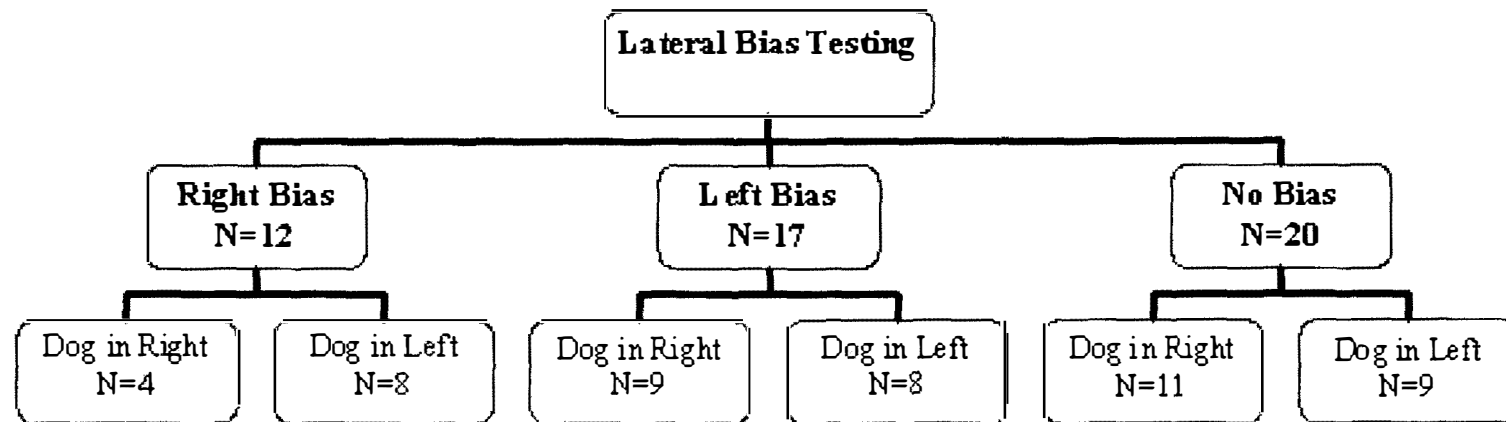
Figure 7.3 Experiment 7c: presentation of barking dog (versus human plus rattle) in the Y maze, according to lateral biases expressed in the empty maze. N = number of sheep in each side/stimulus group.

The formula shown in Equation 7.1 was used to create a variable describing the difference between the number of times treatment A and treatment B were chosen (Bisazza et al., 1997; Facchin et al., 1999; De Santi et al., 2001; Pajor et al., 2003). The value of this variable was calculated for each individual sheep. The effect of lateral biases on 'choice index' was determined using a Mann-Whitney test (Zar, 1999).

$$\text{Choice Index} = \frac{(\text{Choices Treatment A - Choices Treatment B})}{(\text{Choices Treatment A + Choices Treatment B})} \times 100$$

Equation 7.1 Choice index used to describe the difference in the number of trials resulting in the choice of Treatment A and Treatment B in the Y maze preference test.

Within the biased group, a Fisher's exact test or Yates-corrected χ^2 test was used to determine whether the side of presentation (preferred or non-preferred) affected the number of sheep choosing the 'more aversive' treatment (defined as the treatment not chosen by the majority of sheep). The same tests were used to compare the number of trials resulting in the choice of the more aversive treatment, presented either on the preferred or non-preferred side.

I was also interested in whether the relative aversiveness of each treatment (treatment pair in each experiment) affected the proportion of sheep which switched away from their preferred side of the maze to avoid the 'more aversive' treatment presented there. To ascertain whether the addition of the stimulus elicited a significant switch from the preferred side, the proportion of trials to the right side of the empty maze was used as the expected value in the Binomial test, and I examined whether the proportion of right choices was significantly different from this expected value after the addition of the stimulus (corrected for multiple comparisons). A χ^2 test was used to compare the proportion of sheep switching away from their preferred side in each experiment. In addition, the proportion of trials in which biased sheep chose their preferred side when the more aversive treatment was presented there was compared between the three experiments.

227

7.4 Results

Effect of lateral biases on choice behaviour

Experiment 7a: Dog plus recorded barking versus Control

Two sheep with right biases and one sheep with a left bias chose the dog, while none of the non-biased sheep chose the dog. Direction of lateral bias (right or left) did not affect the number of sheep choosing the dog or control (Fisher's exact P = 1.0), nor the number of trials in which sheep chose the dog ($\chi^2_{0.05, 1}$ = 3.69, P = 0.055). Therefore, all sheep with a lateral bias (Biased) were compared with those not having a bias.

Table 7.1 Choice of the dog plus recorded barking, control or no choice, according to the presence or absence of a lateral bias in the empty maze. The '*Biased*' group includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the dog or control treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the dog or control, or made no choice.

A. Number of sheep

	Preferred Dog	Preferred Control	No Preference	Total
Biased	3	18	0	21
Non-Biased	0	16	1	17
Total	3	34	1	38

B. Number of trials

	Dog chosen	Control chosen	No Choice	Total
Biased	67	345	8	420
Non-Biased	26	309	5	340
Total	93	654	13	760

The number of sheep choosing the dog was not affected by whether or not the sheep had a lateral bias (Table 7.1a, Fisher's exact test P = 0.157). In contrast, the existence of a lateral bias affected the number of trials in which sheep chose the dog or control (Table 7.1b, $\chi^2_{0.05, 2}$ = 12.47, P = 0.002). Considering only trials in which a choice was made (i.e. excluding 'no choice' trials), sheep with biases chose the dog more often (16.3% of choices) than sheep with no bias (7.8% of choices) ($\chi^2_{0.05, 1}$ = 11.48, P = 0.001). However, the choice index was not significantly different between sheep with lateral biases and those with no bias (Mann Whitney test S = 324.50, P = 0.836).

Experiment 7b: Human plus rattle versus Control

Two sheep with right biases and two sheep with left biases chose the human, while one sheep with no bias chose the human. Direction of lateral bias (left or right) did not affect the number of sheep choosing the human (Fisher's exact P = 1.0), nor the number of trials in which sheep chose the human ($\chi^2_{0.05, 2}$ = 0.29, P = 0.866). Therefore, all sheep with a lateral bias (Biased) were compared with those without a bias.

The number of sheep choosing the human was not affected by whether or not the sheep had a lateral bias (Table 7.2a, Fisher's exact test P = 0.506). However, biased sheep chose the human in significantly more trials than did non-biased sheep (Biased 24.6%, Non-biased 15.3% of trials, Table 7.2b, $\chi^2_{0.05, 2}$ = 12.80, P = 0.002). The choice index was not significantly different between sheep with lateral biases and those with no bias (Mann Whitney test S = 367, P = 0.615). It is interesting to note that the single non-biased sheep which chose the human went left 7 out of 10 times during bias testing, and was subsequently presented with the human in the left side of the maze.

229

Table 7.2 Choice of the human plus rattle, control or no choice, according to the presence or absence of a lateral bias in the empty maze. The '*Biased*' group includes all sheep with a lateral bias, regardless of the direction (e.g. right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the human or control treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the human or control, or made no choice.

A. Number of sheep

	Preferred Human	Preferred Control	No Preference	Total
Biased	4	17	2	23
Non-Biased	1	13	3	17
Total	5	30	5	40

B. Number of trials

	Human chosen	Control chosen	No Choice	Total
Biased	113	344	3	460
Non-Biased	52	288	0	340
Total	165	632	3	800

Experiment 7c: Barking dog versus Human plus rattle

One right-biased sheep, one left-biased sheep and two non-biased sheep chose the barking dog when compared to the human plus rattle. The direction of lateral bias (right or left) affected neither the number of sheep choosing the dog, nor the number of trials in which sheep chose the dog (Fisher's exact test P = 1.0; $\chi^2_{0.05, 2}$ = 1.15, P = 0.563). Therefore, all sheep with a lateral bias were compared with those without a bias. The number of sheep choosing the human or dog was not affected by whether or not the sheep had a lateral bias (Table 7.3a, Fisher's exact test P = 0.558). Nor was the choice index significantly different between sheep with lateral biases and those with no bias (Mann Whitney test S = 510, P = 0.798).

The existence of a lateral bias affected the number of trials in which sheep chose the barking dog (Table 7.3b, $\chi^2_{0.05, 2} = 18.46$, P < 0.0001). In contrast to the first two experiments, non-biased sheep chose the dog more often (16.3% of trials) than biased sheep (8.4% of trials). However, this difference may be due to the fact that after the training trials, non-biased sheep were offered the choice between a timid dog plus recorded barking and a human plus rattle, while biased sheep were offered the choice between an aggressive barking dog and human plus rattle. Excluding the two non-biased sheep that appeared to alter their behaviour after the dog was replaced, there were no differences between biased and non-biased sheep ($\chi^2_{0.05, 2} = 1.65$, P = 0.438).

Table 7.3 Choice of the dog plus recorded barking, human plus rattle or no choice, according to the presence or absence of a lateral bias in the empty maze. The '*Biased*' group includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the dog or human treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the dog or human, or made no choice.

A. Number of sheep

	Preferred Dog	Preferred Human	No Preference	Total
Biased	2	25	2	29
Non-Biased	2	15	3	20
Total	4	40	5	49

B. Number of trials

	Dog chosen	Human chosen	No Choice	Total
Biased	49	508	23	580
Non-Biased	65	309	26	400
Total	114	817	49	980

All experiments combined

Table 7.4 shows the choices of the 'more aversive' treatment in all three experiments combined. The more aversive treatment was defined as the treatment which was not chosen by the majority of sheep in each experiment. This was dog plus recorded barking in experiment 7a, human plus rattle in experiment 7b, and barking dog in experiment 7c.

Table 7.4 All experiments combined: choice of the 'more aversive' treatment or 'less aversive' treatment, according to the presence or absence of a lateral bias in the empty maze. The '*Biased*' group includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the more or less aversive treatment. *B.* Number of trials in which sheep chose the more or less aversive treatment.

A. Number of sheep

	Preferred More aversive treatment	Preferred Less aversive treatment	Total
Biased	9	60	69
Non-Biased	3	44	47
Total	12	104	116

B. Number of trials

	More aversive treatment chosen	Less aversive treatment chosen	Total
Biased	229	1197	1426
Non-Biased	143	906	1049
Total	372	2103	2475

When the results of all three experiments were considered together, the existence of a lateral bias had no significant effect on the number of sheep choosing the more aversive treatment (Table 7.4a, Fisher's exact test $P = 0.201$), nor on the number of trials in which sheep chose the more aversive treatment (Table 7.4b, $\chi^2_{0.05, 1} = 2.60$, $P = 0.107$). However, in experiment 7c, the comparison between biased and non-biased sheep was confounded by the fact that the groups were offered different treatments (see above).

Excluding the results of this experiment, biased sheep were found to choose the more aversive stimulus in significantly more trials (20.5%) than did non-biased sheep (11.5%) ($\chi^2_{0.05, 1}$ = 22.24, P < 0.0001).

Effect of presentation side on choice behaviour of biased sheep only

Experiment 7a: Dog plus recorded barking versus Control

The number of biased sheep choosing the dog was not affected by whether it was presented in the preferred or non-preferred side of the maze (Table 7.5a, Fisher's exact test P = 0.657). Nor was the number of trials resulting in choice of the dog affected by whether the dog was presented in the preferred or non-preferred side (Table 7.5b, $\chi^2_{0.05,1}$ = 0.10 , P = 0.752).

Right-biased sheep chose the dog more often if it was presented in the preferred side (25% of choices) than in the non-preferred side (1.6% of choices) ($\chi^2_{0.05, 1}$ = 13.61, P = 0.001). In contrast, left-biased sheep chose the dog more often if it was presented in the non-preferred side (26.7% of choices) than in the preferred side (4.2% of choices) ($\chi^2_{0.05, 1}$ = 18.68, P < 0.0001). However, it is important to note that the differences in the proportion of trials resulting in choice of the dog were predominated by the repeated choices of three individual sheep: two right-biased sheep and one left-biased sheep.

The addition of the dog plus recorded barking in the preferred side of the maze significantly altered the behaviour of 13 out of 15 biased sheep (86.7%); these sheep switched away from their preferred side to avoid choosing the dog. The remaining two sheep (22.2%) maintained their original choice behaviour, even when the dog was presented in their preferred side. When the dog was presented in the non-preferred side, 5 out of 6 sheep (83.3%) continued to choose their preferred side. One sheep switched away from its preferred side to choose the dog.

233

Table 7.5 Biased sheep only: choice of the dog plus recorded barking, control or no choice, according to whether the dog was presented on the preferred or non-preferred side of the maze. This analysis includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the dog or control treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the dog or control, or made no choice.

A. Number of sheep

	Preferred Dog	Preferred Control	No Preference	Total
Dog in Preferred side	2	13	0	15
Dog in Non-preferred side	1	5	0	6
Total	3	18	0	21

B. Number of trials

	Dog chosen	Control chosen	No Choice	Total
Dog in Preferred side	50	248	2	300
Dog in Non-preferred side	17	97	6	120
Total	67	345	8	420

Experiment 7b: Human plus rattle versus Control

Whether the human was presented on the preferred or non-preferred side of the maze did not affect the number of biased sheep choosing this treatment (Table 7.6a, Fisher's exact P = 0.425). However, significantly more trials resulted in choice of the human when the stimulus was presented in the preferred side (31.2% of trials) than in the non-preferred side (5.8% of trials) (Table 7.6b, $\chi^2_{0.05, 2}$ = 30.74, P < 0.0001). Both right- and left-biased sheep chose the human in significantly more trials when the human was presented in their preferred side.

Table 7.6 Biased sheep only: choice of the human plus rattle, control or no choice, according to whether the human was presented on the preferred or non-preferred side of the maze. This analysis includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the human or control treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the human or control, or made no choice.

A. Number of sheep

	Preferred Human	Preferred Control	No Preference	Total
Human in Preferred side	4	11	2	17
Human in Non-preferred side	0	6	0	6
Total	4	17	2	23

B. Number of trials

	Human chosen	Control chosen	No Choice	Total
Human in Preferred side	106	232	2	340
Human in Non-preferred side	7	112	1	120
Total	113	344	3	460

The addition of the human plus rattle in the preferred side of the maze significantly altered the choice behaviour of 13 out of 17 biased sheep (76.5%); these sheep switched away from their preferred side to avoid the human treatment. The remaining four sheep (23.5%) continued to choose their preferred side, even though the human was located there. All 6 sheep presented with the control in their preferred side maintained their original choice behaviour to choose the control treatment; no biased sheep switched from its preferred to non-preferred side to choose the human treatment.

Experiment 7c: Barking dog versus Human plus rattle

The number of biased sheep choosing the dog was not affected by whether the dog was presented on the preferred or non-preferred side of the maze (Table 7.7a, Fisher's exact test $P = 0.304$). In contrast, biased sheep chose the barking dog in significantly more trials if the dog was presented on their preferred side (20% of trials) than on their non-preferred side (less than 1% of trials) (Table 7.7b, $\chi^2_{0.05, 2} = 71.17$, $P < 0.0001$). Both right- and left-biased sheep chose the dog significantly more often if it was presented in their preferred side.

Table 7.7 Biased sheep only: choice of the barking dog, human plus rattle, control or no choice, according to whether the dog was presented on the preferred or non-preferred side of the maze. This analysis includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for the dog or human treatment or with no significant preference between treatments. *B.* Number of trials in which sheep chose the dog or human, or made no choice.

A. Number of sheep

	Preferred Dog	Preferred Human	No Preference	Total
Dog in Preferred side	2	9	1	12
Dog in Non-preferred side	0	16	1	17
Total	2	25	2	29

B. Number of trials

	Dog chosen	Human chosen	No Choice	Total
Dog in Preferred side	48	182	10	240
Dog in Non-preferred side	1	326	13	340
Total	49	508	23	580

Seven biased sheep failed to make a choice in at least one trial. Therefore, only those trials which resulted in a choice were included in the binomial analysis. Nine of 12 sheep (75%) switched away from their preferred side when the barking dog was presented there. The other 3 continued to choose their preferred side, despite the presence of the dog. When the human plus rattle was presented on the preferred side (dog on non-preferred side), all 17 sheep continued to choose their preferred side; no biased sheep switched away from its preferred side to choose the dog.

All experiments combined

Considering the choice behaviour of biased sheep from all three experiments, there was moderate evidence for a higher proportion of sheep choosing the more aversive treatment when it was presented on their preferred side than when it was presented on their non-preferred side (Table 7.8a, Fisher's exact test P = 0.053). Likewise, sheep chose the more aversive treatment in significantly more trials when it was presented on their preferred side of the maze (Table 7.8b, $\chi^2_{0.05, 1}$ = 91.96, P < 0.0001).

Effect of treatment pair on proportion of biased sheep switching away from preferred side

The treatment pair presented (experiment) had no effect on the proportion of biased sheep switching away from their preferred side when the more aversive treatment was presented there (Table 7.9a, Fisher's exact test P = 0.880). However, sheep were more likely to stay with their preferred side when the human plus rattle was presented there than when the dog was (31.4% of trials in experiment 7b, 16.8% in experiment 7a, 20.9% in experiment 7c; Table 7.9b, $\chi^2_{0.05, 2}$ = 19.96, P < 0.0001). In other words, sheep were less likely to switch away from their preferred side when the human plus rattle was presented there than when either of the dog treatments was presented there.

Table 7.8 Biased sheep only: choice of the *'more aversive'* treatment or *'less aversive'* treatment, according to whether the more aversive treatment was presented on the preferred or non-preferred side of the maze. This analysis includes all sheep with a lateral bias, regardless of the direction (e.g. all right and left biased sheep). *A.* Number of sheep expressing a significant preference (at least 15/20 trials) for more or less aversive treatment. *B.* Number of trials in which sheep chose the more or less aversive treatment.

A. Number of sheep

	Preferred More aversive treatment	Preferred Less aversive treatment	Total
More aversive treatment in Preferred side	8	33	41
More aversive treatment in Non-preferred side	1	27	28
Total	9	60	69

B. Number of trials

	More aversive treatment chosen	Less aversive treatment chosen	Total
More aversive treatment in Preferred side	204	662	866
More aversive treatment in Non-preferred side	25	535	560
Total	229	1197	1426

Table 7.9 Choice behaviour of only those biased sheep for which the *'more aversive'* treatment was presented in their preferred side of the maze; staying with or switching away from the originally preferred side (determined in the empty maze), according to the pair of treatments offered (experiment). This analysis includes both right and left biased sheep. *A.* Number of sheep staying with their originally preferred side of the maze or switching away from it to the non-preferred side, after the addition of the treatments in the maze *B.* Number of trials in which sheep chose their originally preferred side or the originally non-preferred side, after the addition of the treatments in the maze.

A. Number of sheep

	Stayed with originally preferred side	Switched away from originally preferred side	Total
Dog in preferred side (Experiment 7a)	2	13	15
Human in preferred side (Experiment 7b)	4	11	15
Dog in preferred side (Experiment 7c)	2	9	11
Total	8	33	41

B. Number of trials

	Originally preferred side chosen	Originally non-preferred side chosen	Total
Dog in preferred side (Experiment 7a)	50	248	298
Human in preferred side (Experiment 7b)	106	232	338
Dog in preferred side (Experiment 7c)	48	182	230
Total	204	662	866

7.5 Discussion

Effect of lateral biases on choice behaviour

The aim of this study was to assess the effect of lateral biases on the choice behaviour of sheep in a Y maze preference test. This was done by characterizing lateral biases for individual sheep and comparing the choice behaviour of biased and non-biased sheep after the addition of the treatments. It was hypothesized that biased sheep would be more likely than non-biased sheep to choose the more aversive treatment.

The existence of a lateral bias had no effect on the number of sheep choosing the dog or human treatment when compared to the control. Likewise, the number of sheep choosing the barking dog as opposed to the human did not differ between biased and non-biased sheep. Nor did the existence of a lateral bias affect the median value of the choice index variable (difference in number of times each stimulus was chosen) in any experiment. However, this may be a statistical consequence of the relatively small number of individuals in each group (17-29), and the fact that very few individuals expressed a statistically significant preference for the more aversive treatment in each case.

In contrast, when I compared the number of trials in which sheep chose each treatment, biased sheep chose the stimulus more often than did non-biased sheep in the first two experiments. Likewise, when the results of these two experiments were combined, biased sheep chose the stimulus over the control in more trials than non-biased sheep. It is important to acknowledge the fact that this method may over-estimate the differences between groups, as each animal contributes 20 choices to the analysis, and these choices are likely to be correlated. Nevertheless, there was some evidence of a difference in choice behaviour between biased and non-biased sheep.

In the third experiment, non-biased sheep chose the barking dog more often than did biased sheep. However, this comparison was confounded by the fact that non-biased sheep were offered a timid non-barking dog plus recorded barking, rather than the aggressive barking dog offered to biased sheep. The fact that the relative difference in

aversiveness between the treatments was not comparable for the two groups makes it difficult to draw any meaningful conclusions about the effect of lateral biases from this particular experiment.

In accordance with the idea that lateral biases affect choice behaviour, when sheep and goats were trained to expect the same food reward in both sides of a T maze, the percentage of times a win/shift strategy was employed was strongly correlated with the strength of the individual's lateral bias (Hosoi et al., 1995). Animals that expressed strong lateral biases based on their first choices (tested in 23 sessions of 2 consecutive trials) shifted less frequently on the second choice than did animals with weaker biases.

Effect of presentation side on choice behaviour of biased sheep

The first two experiments provide some evidence that the existence of a lateral bias increased the probability that an individual sheep would choose the arm associated with the more aversive stimulus, at least when the alternative was an empty room. It is postulated that this effect was primarily due to the fact that for some biased individuals, their preference for one side of the maze over-rode their motivation to avoid the aversive treatment.

It is interesting to note that the only non-biased sheep to choose the more aversive treatment in the first two experiments was originally classified as left biased (7/10 choices to left), and was subsequently presented with the human on the left side. Although the present definition of bias was based on a statistical procedure, it is possible that this ratio represented a biologically significant preference for the left side of the maze. Therefore, it is possible that this particular sheep was left-biased and consequently 'chose' the human on the left due to its strong motivation to maintain that original side preference.

Presentation of an aversive treatment on the individual's preferred side of the maze would result in a conflict of motivation between maintaining the original preference and avoiding the treatment. If the lateral bias is of sufficient biological relevance to the individual, or the treatment is only mildly or moderately aversive, then the animal's motivation to maintain the original preference may over-ride its motivation to avoid the

241

aversive treatment. This could result in an apparent preference for the more aversive stimulus when, in fact, no such preference exists.

In contrast, presentation of the aversive treatment in the non-preferred side would stimulate no such motivational conflict, as the individual could both maintain its original preference and avoid the aversive treatment by making the same choice. In this case, we would expect biased individuals to continue to choose their preferred side, resulting in an apparent preference for the treatment presumed to be less aversive. Based on this proposition, it was hypothesized that biased individuals would be more likely to choose the more aversive treatment when it was presented on their preferred side of the maze than when it was presented on their non-preferred side.

No differences were found, in any of the experiments, in the number of sheep choosing the more aversive stimulus according to side of presentation. In experiment 7a, there was also no difference in the number of times biased sheep chose the dog when it was in the preferred or non-preferred side. This was primarily due to the fact that all three sheep that made a significant choice of the dog did so when it was presented in the right side. For two of these sheep (right-biased), the dog was in their preferred side, while for the one left-biased sheep, the dog was chosen in its non-preferred side. Therefore, the number of choices of the dog in the preferred and non-preferred side was not different.

In contrast, biased sheep chose the more aversive stimulus in more trials if it was presented on their preferred side of the maze, in both experiments 7b and c. This was true of both right- and left-biased sheep. Similarly, when the results of all three experiments were combined, a higher number of trials resulted in choices of the more aversive treatment when it was presented in the preferred side. In addition, significantly more sheep overall chose the more aversive stimulus when it was on their preferred side than did when it was on their non-preferred side.

While the majority of biased sheep switched away from their preferred side to avoid the aversive treatment, approximately one quarter of biased sheep in each experiment maintained their original preferences, despite the presence of the more aversive treatment in their preferred side. However, as predicted, no biased sheep switched away from its preferred side to choose the more aversive treatment in experiment 7b and c.

These results support the hypothesis that, for some biased individuals, the motivation to maintain the original preference outweighed the motivation to avoid the more aversive stimulus. It appears that the side of presentation can affect the likelihood that sheep will apparently 'prefer' a treatment, once again supporting the idea that lateral biases can affect choice behaviour in sheep in the Y maze test.

The fact that one biased sheep in experiment 7a switched away from its preferred side to choose the dog plus recorded barking is perplexing. This phenomenon was not seen in either of the other experiments, although a similar number of individuals chose the more aversive treatment in each case. During lateral bias testing, this sheep expressed a strong preference for the left side of the maze (9 out of 10). However, it is possible that this simply reflected perseverative behaviour, rather than a lateral preference (Rodriguez et al., 1992). As lateral biases were characterized during a single session, it is not possible to differentiate between the two response patterns in this study. Therefore, the significant choice of the right side of the maze in the following two (testing) sessions could simply reflect a switch in the direction of perseveration. However, Arnold (2005) tested lateral preferences in an empty maze over two sessions on consecutive days and found that most cows exhibited consistent side preferences.

Several other factors suggest that this individual actually preferred the dog treatment to temporary social isolation. On both testing days, at least one choice of the control treatment in the preferred side was made somewhere in the middle of the session. This choice was followed by an immediate reversion to the dog treatment in the right room, suggesting that choice of the control was a 'mistake' (Rushen, 1986). It is possible that the discrepancy between the dog's behaviour (lying quietly or even sleeping) and the playback of the barking may have attracted the curiosity of this sheep. Kendrick et al. (1995) found that mismatching appropriate sight and sound stimuli reduced the ability of sheep to discriminate between sheep and human stimuli in a Y maze. Whatever, the reason, there is no evidence to refute the possibility that this particular sheep simply preferred the dog treatment.

243

Effect of presentation side on choice behaviour of other species

Studies of other species support the proposition that presentation side can influence behaviour in two-choice tests. Milking biased cows on their non-preferred side of the parlour resulted in slower entry into the parlour and milking stall, more pauses in feeding behaviour during milking, and increased heart rate in the first minute of milking when compared to milking in the preferred side (Hopster et al., 1998). The higher initial heart rates on the non-preferred side were attributed to the fact that the cows often had to be encouraged by a handler to enter this side. However, there were still behavioural indications that the cows were less contented when milked on their non-preferred rather than preferred side. In contrast, Da Costa and Broom (2001) found no evidence that cows with highly consistent side preferences were more stressed when milked on their non-preferred side. Measurements of stress included cow reactivity during cup-fitting (leg-lifting), time to fit cups and milking duration. However, there was some indication that the cows had higher milk yields when milked on their non-preferred side.

Biased rats also showed differences in T maze behaviour, depending on whether they could escape electric shock on their preferred or non-preferred side (Zimmerberg et al., 1978). Each rat was repeatedly tested in an electrified maze until a criterion of 5 out of 6 consecutive choices of the safe arm (shock turned off upon entry) was reached. On the first day of testing, there was a tendency for rats whose safe arm was on the preferred side to reach the criterion in fewer trials than rats whose safe arm was on the non-preferred side. On day two, this difference was significant; rats learned and retained knowledge of the location of the safe arm better if it was on their preferred side.

These studies indicate that lateral biases expressed in two-choice tests are biologically relevant to the individual animal, and may affect behavioural and physiological variables other than the choice of arms. This is supported by evidence of persistent lateral asymmetries expressed by individual animals over time and with varying social and environmental conditions (Hopster et al., 1998). In studies on both rodents and cows, despite training or reinforcement to choose the option opposite to that originally preferred, most animals reverted back to their initial preferences after the treatment was discontinued (Glick et al., 1977; Arnold, 2005).

Other studies provide evidence of linkages between lateralized behaviour and autonomic and endocrine function. For example, removal of the adrenal glands increased the probability of right lateral biases expressed in a T maze by female rats (Rizhova and Kulagin, 1994). There is even some evidence that chronic presentation of food from the left side improves reproductive performance and lactation in cows, due to asymmetrical brain control of the autonomic nervous system (Rizhova and Kokorina, 2005). These and other studies, along with the results of the present study, support the hypothesis that the lateral position of emotionally meaningful stimuli (e.g. food, predators, electric shocks) can influence somatic function and behaviour in animals (Rizhova and Kokorina, 2005).

Effect of relative treatment aversiveness on influence of lateral bias on choice behaviour

In the present study, the relative aversiveness of the treatments appeared to affect the degree to which lateral bias affected choice behaviour. Biased sheep were more likely to choose the non-preferred side of the maze when a dog was located in their preferred side than when a human plus rattle was located there. This suggests that the choice behaviour of sheep with lateral biases is more likely to be influenced by their bias when the treatment is only mildly aversive relative to the alternative than when it is strongly aversive. In other words, when the preference for one stimulus over the other is only mild, lateral biases are more likely to affect individual choice behaviour.

Several other studies provide evidence that the degree of influence that lateral biases have on choice behaviour is modulated by the relative aversiveness of the treatment, or the strength of the preference. When sheep were offered choices between photographic images of a sheep, human, dog or symbol, (each one indicating access to the stimulus pictured, except the dog, which was replaced with an unfamiliar human), 30-96% of individuals exhibited a significant preference for one side of the maze (Kendrick et al., 1995). The proportion of individuals with significant biases varied according to breed and the stimulus pair presented. However, when the same sheep were offered the choice between a dark or illuminated arm in the same maze, with treatments balanced across sides for each individual, all sheep consistently avoided the dark arm.

245

Likewise, no individual sheep expressed a significant preference for electro-immobilization (I) compared to mechanical restraint (S), although there was a tendency for sheep to prefer the I side of the maze before the treatments were applied (Grandin et al., 1986). These results support the idea that biased animals are willing to switch away from their preferred side if the treatment presented there is strongly aversive, such as darkness or electro-immobilization, or if the alternative is strongly attractive, while they persevere with their preferred choice if the treatment is only mildly aversive or neutral compared to the alternative. Therefore, lateral bias becomes an additional weighting factor on an animal's choice between the two treatment alternatives.

Implications for preference testing

The Y maze test is a commonly used methodology for assessing the preferences of animals, and has been used to evaluate the choices of domestic species for a variety of treatments, including different feeds, housing and environmental features, handling treatments, familiar or unfamiliar conspecifics and mates (e.g. Dawkins, 1982; Grandin et al., 1986; Rushen, 1986; Grandin et al., 1994; Hosoi et al., 1995; Prescott et al., 1998; Jensen, 1999; Millman and Duncan, 2000; Phillips and Morris, 2001; Pajor et al., 2003; Leslie et al., 2004). It has been acknowledged that individual lateral biases are commonly expressed by a variety of species when tested in a two-choice paradigm (Kendrick et al., 1995). Indeed a number of authors have directly commented on the potential for individual lateral biases to affect the expression of preferences in Y or T maze tests (Grandin et al., 1986; Grandin et al., 1994; Hosoi et al., 1995; Pajor et al., 2003).

However, to my knowledge, this is the first study to explicitly test the effects of individual lateral biases on choice behaviour in the Y maze test. This despite evidence, outlined above, that lateralized behaviour is a widespread phenomenon among vertebrates and invertebrates, that lateral biases or side preferences are expressed by a high proportion of individuals in two-choice tests, that such biases appear to be a stable characteristic of the individual animal, and that individual biases appear strongly persistent over time and varying contexts.

This study provides direct evidence that the existence of lateral biases in sheep can influence choice behaviour in a Y maze test, and subsequently affect the expression of treatment preferences. While other Y maze studies have not explicitly characterized individual lateral biases or side preferences, at least one study shows indirect evidence that side preferences may have influenced choice behaviour. In a two-choice study, cows were trained to expect milking on one side of a parlour and no milking on the other side, and then re-trained after 9 days of testing to expect the treatments on the opposite sides (Prescott et al., 1998). The choice behaviour of some cows appeared to be primarily influenced by treatment location (e.g. they continued to choose milking after the switch), while others appeared primarily motivated by preference for a side (e.g. milked before the switch, but not after it).

It was concluded that these latter individuals had low motivation to be milked. However, all cows chose to be fed rather than milked, both before and after the treatments were switched, suggesting that the refusal to switch during the milking trial was due to a persistent lateral bias, rather than to an inability to re-learn the side/treatment association. By this rationale, individuals which were milked during the first half, but not the second half of the trial may simply have had strong preferences for one side of the maze which over-rode their motivation to be milked. However, without characterizing individual lateral biases, it is not possible to resolve the motivations underlying the expression of this pattern of behaviour.

These results highlight the importance of characterizing individual lateral biases and allocating treatments accordingly. Using the most common method of randomly allocating treatments to sides of the maze, without any knowledge of the individual lateral biases, a choice of the stimulus presumed to be less aversive could simply reflect maintenance of the individual's original side preference. Therefore, failure to allocate treatments according to individual lateral biases can cast significant doubt on the results of preference tests. This is particularly true of those tests in which the treatments are similar in aversiveness. Importantly, these are the kinds of treatment comparisons of greatest interest to researchers, and indeed the similarity of the treatments in terms of aversiveness is often the reason for employing a preference test in the first instance. In such cases, individual lateral biases could obscure mild preferences between treatments, which could nonetheless be significant in terms of productivity or animal welfare.

247

How to deal with lateral biases in preference tests

The fact that individual lateral biases can influence choice behaviour in two-choice preferences tests makes it imperative that such biases be characterized and their effects controlled or standardized in the experimental design. Balancing treatment-side associations across the test population by random allocation does nothing to control for the effects of lateral biases on the choice behaviour of individuals, which ultimately determine the expression of preference at the population level. For example, without any knowledge of individual lateral biases, the test treatment could be allocated to a biased individual's non-preferred side of the maze. In this case, a choice of the side opposite the test treatment could simply reflect maintenance of the individual's original side preference, rather than reflecting a true preference for that option relative to the test treatment.

Several two-choice studies have been designed in a way that allows identification of lateral biases during testing. For example, the apparatus used by Jensen (1999) allowed calves to choose each treatment on both their right and left sides e.g. treatment A on the left in trial 1 and on the right in trial 2. This was achieved by having two entrances to the maze (cross shaped maze), so that individuals could alternately enter from the top or bottom. A similar design was used by Hosoi et al. (1995), while Kendrick et al. (1995) simply allowed each animal visual access to both treatments at the decision-making point.

However, while such approaches allow balancing of treatments across the maze for individuals, a significant bias for one side could obscure any preference the animal might have, particularly if the preference is only mild (e.g. half of choices for treatment A and half of choices for treatment B). This is, in fact, what Kendrick et al. (1995) reported when sheep were given the choice between treatments considered to be only mildly aversive.

Only one other study to date has explicitly incorporated information on individual lateral biases into the Y maze preference test methodology (Arnold, 2005). Arnold exploited the fact that the effect of lateral biases on choice behaviour appears to be related to the relative aversiveness of the treatments offered. She suggested that

challenging the animal's initial preference with a test treatment presumed to be aversive could provide a conservative measure of the relative aversiveness of that treatment, while controlling for variation in individual lateral biases.

For individuals with consistent lateral biases, Arnold (2005) always applied the test treatment on the animal's preferred side of the maze. For animals showing no significant side preference, the treatment was applied in the side chosen in the first free choice after the animals were familiarized with the testing apparatus. Using this treatment allocation regime, choice of the treatment presumed to be less aversive (or control) reflected a switch away from the animal's preferred side, and indicated a significant aversion to the test treatment. In addition, the proportion of biased animals switching away from their preferred side could be used as an indication of the relative aversiveness of the treatment; the more aversive the treatment, the more willing biased animals would be to switch away from their preferred side. This methodology could be modified for testing the relative attractiveness of treatments, by always offering the attractive test treatment (e.g. type of food) in the non-preferred side. In this case, a choice of the test treatment would reflect a switch away from the preferred side and indicate a significant attraction to that treatment.

One limitation of this approach is the method employed for characterizing lateral biases. Arnold (2005) defined the preferred side of the maze as the side chosen in the first free choice after familiarization, and assigned the test stimulus to this side for all individuals. However, the assumption that an initial choice was consistent with the direction of a lateral bias was subsequently found to be correct in only 64% of cases (Arnold, 2005). Arnold measured lateral biases in dairy cows in an empty Y maze, and defined a significant bias as 70% of choices to the same arm over two sessions of 5 trials (10 trials over 2 consecutive days). For each animal choosing a side in the first free choice, there was a 30% chance that the animal had no lateral bias, a 45% chance that it had a significant lateral bias consistent with the initial side chosen, and a 25% chance that the lateral bias was not consistent with the initial side chosen. Therefore, the allocation of the test treatment to the first side chosen did not ensure that the animal's original preference was challenged by the test treatment (Arnold, 2005). This leads me to suggest that some of the individual cows that 'chose' the less aversive treatment may simply have been maintaining their original preference for the other side.

249

Arnold (2005) concluded that it may be more appropriate to directly determine initial lateral preferences before administering the test treatment in the Y maze preference test. More comprehensive lateral bias testing, such as that undertaken in the present study, will ensure that the relationship between bias and treatment allocation is standardized for each animal. One additional improvement to the methodology used in the present study would be to test lateral biases over a number of testing session, rather than all in one session, to enable differentiation between perseverative behaviour and true lateralized behaviour.

7.6 Conclusions

This study provides evidence that lateral biases can influence choice behaviour in two-choice paradigms, such as the Y maze preference test. Biased sheep chose the more aversive stimulus more often than non-biased sheep when both groups were offered the same pair of treatments. In addition, the side of presentation affected the choice behaviour of biased sheep; the more aversive stimulus was chosen more often when presented on the preferred than non-preferred side of the maze. Finally, the degree to which lateral biases affect choice behaviour appears related to the relative aversiveness of the treatments presented; sheep were more willing to switch away from their preferred side if the treatment presented there was a dog than if it was a human.

These results suggest that individual lateral biases expressed in two-choice tests are biologically relevant to the individual, and have the potential to affect choice behaviour, especially when the treatments are similar in aversiveness. Individual lateral biases may distort the expression of animal preferences, particularly preferences which are mild, but which may nonetheless be significant in terms of productivity or animal welfare. Whether such lateral biases are inherent (e.g. the result of cerebral asymmetry), or environmentally induced is largely irrelevant, as their effect on choice behaviour, tested in the apparatus in which the bias was expressed, will be the same. It is concluded, therefore, that individual lateral biases should be characterized before the commencement of Y maze preference testing, and test treatments should be allocated in such a way as to standardize the effects of lateral biases on choice behaviour.

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CHAPTER 8

General Discussion

The way in which domestic animals experience components of routine management procedures is important in terms of animal welfare, productivity, product quality and increasingly, to facilitate access to international product markets. There are clearly benefits to minimizing unnecessary animal stress at all stages of production. Humans and dogs are an integral part of the social environment of domestic sheep, and it is difficult to imagine how large flocks of sheep could be managed otherwise. However, as a prey species, sheep are likely to perceive both humans and dogs as biologically relevant, and both may potentially elicit fear in sheep.

The processes of domestication and selective breeding have altered not only morphological and behavioural traits, but are also likely to have altered the emotional significance of elements of the captive environment (Price, 1984; Zohary, 1998). Man, as the pivotal figure in the domestication process, is almost certainly perceived differently by domestic sheep than he was by their wild antecedents. However, whether domestic sheep continue to perceive humans as predators or whether humans have been adopted into the social structure as dominant conspecifics, as has been suggested, is unknown. Likewise, whether domestication has also altered the relationship between sheep and man's ubiquitous associate, the domestic dog, has not been previously investigated.

Because the presence of humans and dogs is integral to sheep management, and may cause stress in domestic sheep, it is important to improve our fundamental understanding of the relationships between these species. If necessary, it may then be possible to reduce the stress experienced by domestic sheep, either by minimizing contact between the species at certain stages of production (e.g. before slaughter), or by selectively breeding for animals which exhibit reduced stress responses to the presence of humans and dogs.

A note on the use of terminology in the study of sheep experience and emotion

In Chapters 4 and 5, I discussed the problems associated with using behavioural and physiological variables to infer knowledge of subjective internal phenomena such as emotional experiences and motivational states. Throughout this thesis, terms such as 'aversion', 'stress' and 'fear' have been used to describe the responses of sheep to the

presence of humans and dogs. The use of a variety of terms to describe similar or related concepts reflects the complexity of this issue and the uncertainty, both in my own mind, and in the ethological literature about our current ability to use observable parameters to infer subjective experiences in non-human animals.

To clarify the present discussion, I interpret the term 'aversion' as relating most closely to the behavioural responses of animals exposed to situations assumed to be associated with a negative emotional experience. In contrast, 'stress' is used to describe physiological responses to such events. The term 'fear' refers to the emotional experience itself and with our present state of knowledge and technology, fear can only be implied, not directly measured.

While the terms 'aversion' and 'stress' implicitly refer to the associated emotional experience, the relationships between fear and measured changes in behaviour and physiological variables are poorly understood and are unlikely to be direct. For example, behaviours interpreted as relating to aversion may be directly indicative of the level of fear experienced, or alternatively, represent a method of avoiding or reducing the elicitation of fear. To illustrate: by staying further away from the dog than from the box in the arena, sheep may be able to avoid experiencing a higher level of fear when the dog is present. Alternatively, stronger avoidance may directly reflect a higher level of fear experienced in the presence of a dog.

Although there are limitations to using measurable responses to infer emotional activation, the concurrent measurement of behavioural aversion and physiological stress responses in this research improved my ability to interpret sheep's responses in terms of their subjective experience. Continuing with the previous example, I found that sheep in the arena not only stayed further away from the dog than from the box or human, but also mounted significantly larger adrenocortical responses to the dog's presence. Taken together, these results provide stronger evidence that behavioural aversion reflects a higher level of emotional activation, at least in this experimental situation.

Likewise, the significantly larger adrenocortical responses to the dog than to the human presented in the Y maze facility support the conclusion that voluntary avoidance of the dog in the Y maze was related to higher levels of emotional activation or fear in its

258

presence. Based on the results of the research presented in this thesis, it is reasonable to conclude that the presence of a dog elicited not only more behavioural aversion and higher physiological stress responses, but also more fear in domestic sheep than did a human.

8.1 Major findings

This thesis provides the first direct comparison of the behavioural and physiological responses of domestic sheep to the presence of humans and dogs. These studies show that the presence of both humans and dogs elicited behavioural aversion and physiological responses indicative of stress in sheep. However, sheep exhibited more behavioural aversion, higher adrenocortical responses and greater avoidance in response to the presence of a dog than to a human. The results of each study have been discussed in detail in Chapters 2 to 7. In the following sections, the consistency of the observations made using different experimental tests is highlighted and any discrepancies between results achieved using different methodologies are discussed.

Behavioural responses to humans and dogs

The presence of a human or a dog in an arena test elicited behavioural responses indicative of aversion in domestic sheep (Chapters 2, 3, 4 and 5). These results are supported by the choice behaviour expressed in the Y maze test (Chapter 6); when given the opportunity, the majority of sheep avoided both a human (plus rattle) and a dog (plus recorded barking), when the alternative was temporary isolation. The behavioural responses observed in these two tests suggest that sheep found the presence of a human or a dog aversive.

In the arena test, the presence of the dog elicited more behavioural aversion than did the human. Similarly, in the Y maze test, the majority of sheep chose confinement with a noise-making human rather than confinement with a barking dog. These results suggest that sheep experienced more fear in the presence of a dog than with a human. This pattern was observed in two different breeds of sheep, from different management systems in two different countries (Chapters 2 and 5), making the conclusion that sheep are relatively more fearful of dogs than of humans more robust.

Physiological responses to humans and dogs

In accordance with the behavioural responses, the presence of a human or a dog in the arena test elicited a significant rise in plasma cortisol concentration, indicating increased activation of the HPA axis (Chapter 5). However, while the presence of a dog elicited a significantly greater adrenocortical response than a novel object (box) in the arena, the magnitude of the response to the human was no greater than was elicited by the box. This suggests that while the presence of a stationary human in the arena did not significantly add to the stress caused by the arena testing procedure itself, the presence of a dog in the arena did.

In the Y maze test, sheep mounted significant plasma cortisol responses to the presence of a human with a rattle, and to a barking dog (Chapter 6), supporting the notion that sheep experienced stress in the presence of both stimuli. However, interpretation of the adrenocortical responses in the Y maze test is limited by the absence of a control group, the inclusion of which would have allowed differentiation of the effects of the testing procedure from the effects of the stimulus itself on plasma cortisol concentration (see below). Although I am unable to judge whether the presence of a human increased the stress experienced due to the Y maze testing procedure, it is likely that the addition of the noise-making device intensified the fear-eliciting properties of the human stimulus.

Based on these results, it is possible that the presence of a stationary human alone is insufficient to elicit a significant rise in plasma cortisol concentration in domestic sheep. In contrast, sheep did show significantly more behavioural aversion to the human than to a novel object in the arena (Chapters 2 and 5), and most sheep chose to avoid a human when given the opportunity to do so (Chapter 6). In addition, a number of other studies have reported that human presence elicited both behavioural and physiological responses indicative of fear in domestic sheep (e.g. Pearson and Mellor, 1976; Harlow et al., 1987; Baldock and Sibly, 1990; Romeyer and Bouissou, 1992; Bouissou and Vandenheede, 1995; Goddard et al., 2000; Hansen et al., 2001; Erhard, 2003; Vierin and Bouissou, 2003).

This discrepancy can be attributed to the significant methodological differences between the previous studies and the arena test described in this thesis (Chapters 2, 3, 4 and 5).

For example, some of the previous studies measured sheep responses to a moving or approaching human (Baldock and Sibly, 1990; Hansen et al., 2001), or to a human behaving in a threatening manner (Harlow et al., 1987), while others involved close contact or handling by humans (Pearson and Mellor, 1976). In my studies, the human presented in the arena test was stationary and, except in one experiment (Chapter 3), did not even make eye contact with the test sheep. Therefore, differences in the behaviour of the human are likely to have altered the fear-eliciting potential of this stimulus (see below).

In the experiments described in this thesis, differences in the magnitude of the adrenocortical responses to the human and dog between the arena test and Y maze test may have related to the age, breed, management and previous experience of the test sheep used in each experiment. In addition, differences may relate to the experimental methodology. For example, in the arena, test sheep were able to withdraw to a distance of 7 metres from the stimulus and had visual and limited physical access to other sheep during the test (Chapter 5). In contrast, sheep could withdraw only about 3 m from the stimulus in the Y maze and they were socially isolated during Y maze testing (Chapter 6).

Despite the differences between the two experimental methodologies, in both cases, the adrenocortical responses suggest that the presence of a dog was stressful to individual sheep. In addition, there is some suggestion that close confinement with a human using a noise-making device was stressful. However, the magnitudes of the adrenocortical responses to the human or dog were only moderate in both studies.

In the UWA arena test, the average peak change in plasma cortisol concentration above pre-treatment concentration (calculated over all three flocks) was 40.1 nmol/L after exposure to the box, 40.3 nmol/L after exposure to the human, and 46.4 nmol/L after exposure to the dog in the arena (Chapter 5). These changes represent about a 3-fold increase in plasma cortisol concentrations above pre-treatment values. In the Y maze test, the average peak change in plasma cortisol over pre-treatment concentration was 48.2 nmol/L after exposure to the human shaking the rattle and 61.6 nmol/L after exposure to the barking dog (Chapter 6). The peak values were approximately twice as large as the pre-treatment concentrations.

Previous studies have reported larger increases in plasma cortisol concentration in sheep exposed to a range of currently accepted husbandry practices. For example, surgical mulesing elicited peak changes in plasma cortisol concentration of 107-137 nmol/L (3-fold increase) above pre-treatment concentrations (Fell and Shutt, 1989; Chapman et al., 1994), while various methods of castration or tail docking elicited peak changes of 40-95 nmol/L above pre-treatment concentrations in lambs (3-11 fold increase) (Lester et al., 1996; Dinnis et al., 1997). Prolonged physical restraint in social isolation (6 hours) elicited peak changes of more than 150 nmol/L above pre-treatment plasma cortisol concentrations (2.5-4 fold increase) (Coppinger et al., 1991; Apple et al., 1993).

Common sheep handling procedures such as dipping, crutching, shearing and drafting (without dogs) elicited peak plasma cortisol concentrations that were approximately 3 times higher than concentrations measured in non-handled control animals (Hargreaves and Hutson, 1990c). Likewise, shearing was reported to elicit peak changes of 130–165 nmol/L above pre-treatment values, representing a 4-fold increase in plasma cortisol concentration (Hargreaves and Hutson, 1990b; 1990a).

As well as the relatively moderate magnitude of the increases in cortisol concentration, in both studies described in this thesis, plasma concentrations had returned to pre-treatment values within one hour of the start of testing. In contrast, other studies report that plasma cortisol concentrations in lambs remained significantly elevated for 3 to 4 hours after the implementation of currently accepted surgical husbandry treatments such as castration and tail docking (Lester et al., 1996; Dinnis et al., 1997).

The relatively moderate magnitude and duration of the adrenocortical responses measured in the arena and Y maze studies suggest that the presence of the human or dog was not excessively stressful for the sheep. However, it is important to note that the stimuli used in my experiments may not accurately represent humans and dogs as they behave during real husbandry practices. In reality, humans seeking to move sheep from one place to another are likely to approach sheep and use more threatening behaviour than did the human stimuli presented in these studies. As noted above, the behaviour of the stimulus may influence the neurophysiological, behavioural, and physiological responses of the sheep to which it is presented (e.g. MacArthur et al., 1979; Harlow et

al., 1987; Kendrick and Baldwin, 1989). Therefore, in practice, humans may elicit more stress than was measured in these studies.

Likewise, the dogs used in the arena tests were generally passive and motionless (in fact, often asleep). Therefore, the presence of actively working dogs is likely to elicit more stress in sheep than was measured in these studies. In accordance with this idea, the dog used in the final Y maze test described in Chapter 6 was alert and relatively aggressive for the most of the time, and plasma cortisol concentrations elicited by confinement with this dog were higher than those measured in the arena tests discussed in Chapter 5 (however, note discussion of other differences in experimental methodology above).

For the reasons outlined above, the adrenocortical responses of sheep to the presence of humans and dogs need to be confirmed in practical situations. If the observations reported in the present experimental studies are confirmed in sheep exposed to stimuli behaving in a manner typical to the practical farming environment, then the presence of a human or dog during sheep management is unlikely to cause undue concern on the basis of animal welfare.

In both the arena test and Y maze test, the presence of a dog elicited higher adrenocortical responses than did the presence of a human (Chapters 5 and 6). This supports the behavioural evidence that the presence of a dog is more aversive or fear-eliciting to domestic sheep than the presence of a human. The fact that this pattern was seen not only in behavioural responses tested using two very different experimental methodologies, but also in the physiological responses measured in the two different situations, lends credibility to the conclusion that dogs elicit more fear in domestic sheep than do humans.

Selection for fearfulness in sheep?

The measurement of both behavioural and physiological responses enhanced the understanding of the differences between the More Active (MA) and Less Active (LA) flocks at the University of Western Australia (UWA). Both the behavioural and

263

adrenocortical responses suggest that, contrary to what was previously believed, MA sheep have not been selected for higher levels of fearfulness (Chapters 4 and 5).

While MA sheep were consistently more behaviourally active than LA sheep in the arena test with a range of stimuli, there was behavioural and some limited physiological evidence that they experienced less fear in the presence of a human. However, this apparent lower level of fear in MA sheep was specific to the presence of a stationary human in the arena test; there were no inter-flock differences in fear-related behaviour or adrenocortical responses to other stimuli (Chapter 5).

In addition, inter-flock differences in adrenocortical response to the human were evident only in the sample taken immediately after the arena test. There were no differences between the flocks in overall (integrated) adrenocortical responses, which likely reflected the experience of repeated handling by humans during the general testing procedure, as well as exposure to the stationary human in the arena. Therefore, inter-flock differences in adrenocortical response to a human appeared relevant only within the context of the arena test, and it is unlikely that selection of the UWA flocks has been based on some consistent underlying predisposition to react fearfully i.e. fearfulness.

The results discussed in Chapters 4 and 5 illustrate the potential for genetic manipulation of the behavioural and possibly physiological responses of domestic sheep to specific stimuli e.g. human presence. However, whether the selection process at UWA has also altered the perceptions and emotional experiences of the flocks is, as yet, uncertain.

Explaining the discrepancies between studies reporting context-specific or domain-general fear responses

Behavioural and physiological studies have shown evidence supporting both the existence of domain-general and context-specific fear responses in a wide range of species. Such inconsistency in results may relate to differences in testing procedures, and the nature of the population being tested (Boissy, 1995). As noted in Chapter 5, consistency in behavioural or physiological responses may relate to common features in the different testing environments, rather than to the existence of a domain-general

264

temperament trait. However, there are also many examples of consistent individual responses in contexts varying greatly in nature (e.g. Lyons et al., 1988; Jones et al., 1991; Hessing et al., 1993; Launay, 1993; Jones et al., 1994a; Jones et al., 1994b; Jones and Saterlee, 1996; Jones et al., 1997; Capitanio, 1999; Malmkvist and Hansen, 2002).

Differences in the consistency of fear-related responses may also relate to the relevance of the testing environment. For example, ecologically relevant tests, including elements such as foraging and predators, may reveal differences in the indicators of temperament traits such as fearfulness or boldness that are not elucidated in artificial testing situations (e.g. Wilson et al., 1993). This suggests that stability in the observable manifestations of temperament (e.g. behavioural and physiological responses) does not necessarily reflect the existence of innate tendencies (domain-general traits); rather that environmental conditions may facilitate the expression of differences between phenotypically plastic individuals (Wilson et al., 1993).

The degree to which the study subjects have been selected for particular traits may also alter the consistency of behavioural and physiological responses. The study outlined in Chapter 5 shows how selection has altered the consistency of individual behavioural and adrenocortical responses within the selected flocks. By this rationale, there may be differences in the specificity or generality of fear responses between wild and domestic populations. Natural selection may favour context-specific fear responses in wild populations, while relaxation of natural selective pressures may allow for the generalized reduction in emotional reactivity/fearfulness that has characterized the domestication of many species (Price, 2002).

Reale et al. (2000) note that natural selection appears to have favoured co-selection for certain combinations of context-specific traits. Boldness (willingness to enter a salt trap) and docility (response to human handling) were evaluated in a wild population of Bighorn sheep (*Ovis canadensis*). While the study population contained both individuals that were bold and non-docile/aggressive, and individuals that were shy and docile, there were very few examples of shy and aggressive sheep. In wild populations, co-selection of boldness and aggression towards humans or shyness and docility may confer a selective advantage. In support of this, Reale and Festa-Bianchet (2003) reported evidence of predator-induced selection favouring bold and non-docile ewes in

265

years of high cougar predation. However, there was no evidence of predator-induced selection of these traits in years of low predation, supporting the idea that domestic animals, which experience little or no predation, are released from selective pressure acting on context-specific temperament traits.

As a continuation of this line of thinking, strong artificial selection for divergent behavioural or physiological responses to challenge may alter the consistency of the expression of individual temperament traits. The results presented in Chapter 5 provide some support for this idea, as both MA and LA sheep tended to show more consistent peak and integrated adrenocortical responses to the different stimuli than did unselected sheep. Likewise, while several studies on pigs found no consistency in individual responses using unselected populations, they reported that individual responses to different challenges tended to be more consistent when the top and bottom performing sections of the populations were compared (e.g. Lawrence et al., 1991; Hessing et al., 1993; Ruis et al., 2000).

Lines of poultry and mink divergently selected for high or low behavioural or physiological responses to specific events also showed individual consistency in fear-related responses to other challenging situations (e.g. Jones et al., 1991; Launay, 1993; Jones et al., 1994a; Jones et al., 1994b; Jones and Saterlee, 1996; Jones et al., 1997; Malmkvist and Hansen, 2002). Likewise, Kagan et al. (1988) found that children at the extremes of the bold/shy continuum were more phenotypically stable than those exhibiting intermediate responses. The authors postulated that phenotypically inflexible individuals occupy the extremes of temperament trait distributions, while more phenotypically plastic individuals occupy the middle range (Kagan et al., 1988). Therefore, rigorous selection for a particular trait over many generations may artificially increase the apparent consistency of individual fear responses.

Neurophysiological basis of flock differences

I would like to briefly speculate on the neurophysiological differences upon which the UWA flocks may have been indirectly selected. Research has revealed widespread differentiation in the state and reactivity of the central nervous system associated with variation in the behavioural manifestations of temperament. Divergent genetic selection

based on behavioural responses to putative fear-eliciting stimulation in a wide range of species has revealed associated differences in a host of neuroendocrine, hormonal and physiological systems, including the serotonergic, noradrenergic and dopaminergic brain systems, and sympathetic and hypothalamic-pituitary-adrenal systems (e.g. Naumenko et al., 1989; Popova et al., 1991; Koolhaas et al., 1997; Ramos and Mormede, 1998; Koolhaas et al., 1999; Trut et al., 2000; Popova, 2004; Trut et al., 2004).

Arena behaviour is reported to change with immunological and hormonal status, parasite burden, prenatal under-nutrition, and exposure to potential toxins (Fell et al., 1991; Behrendt, 1998; Hohenhaus et al., 1998; Erhard et al., 2004; Erhard and Rhind, 2004), indicating the complexity of the underlying relationships between brain activity, immunology, neuroendocrinology, physiology and behaviour. Differential secretion of endogenous opioids is also purported to play a role in altering arena behaviour in sheep (Gates et al., 1992; Behrendt, 1998). Endogenous opioids seem to act by reducing the subjective intensity of noxious stimulation (Boissy, 1998), which could subsequently alter physiological and behavioural responses to stress.

There is also evidence to support the central and multiple roles of corticotropin releasing factor (CRF) in controlling behaviour and physiology in response to acute stress (Johnson et al., 1994). In sheep, the presence of a barking dog induced CRF secretion, not only in the hypothalamus, where it would subsequently regulate pituitary-adrenal activity, but also in other brain regions believed to be involved in an individual's subjective experience of stress, e.g. the amygdala (Johnson et al., 1994; Cook, 2004).

In pigs, intracerebroventricular injection of CRF increased plasma ACTH as well as the expression of locomotor and vocal behaviour (Johnson et al., 1994). In rodents, CRF infusion into the ventricles of the brain was associated with an increase in the expression of active behaviours such as locomotion, grooming and digging, as well as indicators of autonomic nervous system activation (Diamant and Dewied, 1991; Wiersma et al., 1995). Different levels of active behaviour have also been associated with differential activation of the sympathetic nervous system, with higher sympathetic-adrenal-medullary (SAM) activation being related to increased behavioural activity

(Syme and Elphick, 1982; Fokkema et al., 1988; Koolhaas and Oortmerssen, 1989; Hessing et al., 1994; Jones and Saterlee, 1996).

8.2 Methodological developments and considerations

Arena test

The results discussed in Chapters 2 and 3 verify that the arena test is appropriate for detecting differences in the relative aversion of sheep to various stimuli (Erhard, 2003). Using univariate analysis of arena behaviour, I was able to detect differences in the behavioural responses of sheep to stimuli which were very different in aversiveness. However, neither univariate analysis of arena behaviour over the entire duration of the test, nor the use of an additive index of fearful behaviour (fearfulness score) was sensitive enough to detect differences in behavioural responses to stimuli very similar in aversiveness (Chapter 3). Only by comparing behavioural responses in the initial part of the test were subtle differences in behavioural response to similar stimuli revealed.

The additive fearfulness score was devised in an attempt to provide an integrated behavioural measure of fear in sheep (Romeyer and Bouissou, 1992). However, I discontinued the use of this index after the first set of arena tests due to concerns about the inclusion of behaviours which had ambiguous relationships with fear in sheep. In particular, the inclusion of locomotor activity and vocalization in the index was considered problematic, and these behaviours were excluded in the calculation of the index in Chapters 2 and 3. While social isolation increases locomotor activity in sheep, the presence of a human or dog has been found to reduce the relative expression of locomotion (Torres-Hernandez and Hohenboken, 1979; Romeyer and Bouissou, 1992; Erhard, 2003). The fact that locomotor frequency changes in opposite directions in two situations that are both assumed to represent negative experiences for sheep, suggests that the frequency of this behaviour alone cannot be considered a reliable indicator of the experience of a negative affective state, such as fear (Paul et al., 2005).

While univariate analysis of arena behaviour failed to detect differences between stimuli similar in aversiveness, multivariate analysis allowed differentiation of responses to both very different and very similar stimuli (Chapters 2 and 3). Canonical discriminant

analysis identified integrated patterns of sheep behaviour which differed according to the relative aversiveness of the stimulus presented. This illustrates one of the advantages of using multivariate analysis of behaviour to complement univariate techniques, particularly when differences in behaviour are expected to be subtle.

In the studies discussed in Chapters 4 and 5, I used a different multivariate technique to analyze behavioural responses in the arena test. Rather than attempting to differentiate groups based on stimulus aversiveness, I used factor analysis to identify patterns of behaviour which may have been related to the same underlying motivational or emotional state. I then examined inter-flock differences in these patterns in an effort to understand the basis of 'temperament' selection in the UWA flocks. Multivariate factor analysis allowed for more meaningful interpretation of the expression of behaviour in terms of the underlying motivation than could be ascertained using univariate analyses of a multitude of individual behaviours.

The concurrent measurement of a physiological indicator of stress also aided in the interpretation of the behavioural responses of the three UWA flocks (Chapters 4 and 5). Whereas increased locomotor and vocal activity in the arena were previously interpreted as indicative of higher levels of fear in MA sheep, the concurrent measurement of adrenocortical responses suggests otherwise. While the behavioural and physiological variables were not significantly correlated, the patterns of response were at least coherent; both the behavioural and adrenocortical results supported the idea that MA sheep experienced lower levels of fear in the presence of the human. This highlights the necessity for measuring both behavioural and physiological variables when attempting to make inferences about intangible phenomena such as temperament traits, and motivational and emotional states.

In future studies using the UWA flocks, it will be important to ensure that all flocks have had similar previous experience with the testing environment and procedures. In addition, it would be prudent to run all three flocks together prior to testing, to allow direct comparison of the behavioural and physiological responses of the selected flocks and the Reference flock to the presence of different stimuli in the arena test.

The sensitivity of the arena test may, in future, be improved by allowing each individual animal to act as its own control. In doing so, individual variation in behavioural responses to the testing procedure may be reduced, allowing better resolution of the effects of the stimulus on behaviour. This could be achieved by presenting each animal with the control treatment for several minutes, then comparing the change in frequency of behaviour after the addition of the stimulus between groups.

In addition, the use of analytical software to analyze video-recorded behaviour rather than using an observer to record behaviour may improve the sensitivity of the test. The use of such software would aid quantification of behaviours which are difficult to measure in real time. In addition, it would allow independent scoring by more than one observer, improving the reliability of behaviour measurements.

Finally, the use of video analysis would allow the quality as well as the quantity of expressed behaviour to be compared between groups. Future studies should consider the sequential expression of behavioural responses, rather than just the total frequency expressed during the test. Sequential analysis identifies segments of successive behavioural items, allowing for the analysis and interpretation of behaviour within the context in which it is expressed (Calatayud et al., 2004). 'Context' in this case refers to the situation as it is subjectively experienced by the individual, rather than just the physical environment and this behavioural context may vary within a single testing period.

The concept of 'behavioural context' is useful when using behavioural indicators to infer temperament traits, and motivational and emotional states, as individual behaviours may relate to different subjective states when expressed within different sequences of behaviour (Calatayud et al., 2004). As an example, high bleating expressed by an individual sheep in the arena test may have one meaning when it occurs in a sequence such as 'Approach-Glance at group sheep-High bleat', and mean something different when occurring in 'Depart-Sniff side-High bleat-Trot'. Using univariate analysis, or even traditional factor analysis, we are limited to examining the frequency of high bleating, without any information about the context in which it occurred. In addition, the fact that a behavioural item may occur in more than one context may obscure differences between experimental groups or correlations between different

behaviours. Therefore, the structural organization of behavioural items may reveal more about the subjective state of the individual than analyses based solely on the frequency of expression of individual behavioural items.

Y maze test

One of the major advantages of using the Y maze test to measure relative aversion is that choice behaviour provides a common behavioural response which can be used to rank the subjects' relative aversion to different situations (Rushen, 1996). Stimulus-specific behavioural responses do not have to be interpreted in terms of aversiveness; the animal is simply given the opportunity to choose between the treatment alternatives offered. This makes choice behaviour easier to interpret than either physiological or behavioural measures of fear or stress (Rushen, 1986). The fact that sheep chose to avoid stimuli that also elicited fear-related behavioural responses in the arena lends credibility to my interpretation of behaviour expressed in the arena test.

The major methodological developments in the use of the Y maze preference test in this thesis were the measurement of a physiological stress indicator in the same testing environment (Chapter 6), and the explicit testing of the effects of lateral biases on choice behaviour (Chapter 7). Concurrent measurement of adrenocortical responses confirmed that the sheep chose to avoid the stimulus which caused the larger rise in plasma cortisol concentration i.e. the more aversive stimulus.

The inclusion of a control group would have improved my ability to interpret the physiological results of the experiment described in Chapter 6. The inclusion of a group which was handled, blood sampled and moved through the Y maze, but not exposed to any stimulus, would have provided an indication of the amount of stress elicited by the testing procedure itself, independently of the stimulus presented. The absence of such a group means that I can interpret the adrenocortical responses to the stimuli only within the total context of the Y maze testing procedure.

In addition, it may have been advantageous to familiarize the animals with the testing environment and procedures to a greater extent before measuring the behavioural and adrenocortical responses of the test sheep to the stimuli. This would reduce the

271

confounding effects of novelty on their responses, and provide a better idea of the stress induced by the presence of the stimuli themselves.

Chapter 7 describes the very first study to explicitly test the effects of individual lateral biases on choice behaviour expressed in a Y maze. This study shows that individual lateral biases are commonly expressed by sheep in the Y maze apparatus, and that they have the potential to confound the expression of stimulus preferences. Individual biases appear more likely to influence choice behaviour when the stimuli are similar in aversiveness or when preferences are mild, which is precisely the situation in which preference testing is most valuable in applied ethology. The results of this study indicate the importance of characterizing individual lateral biases, regardless of their origin, and allocating test treatments in such a way as to standardize the effects of such biases on choice behaviour.

One improvement to the characterization of individual lateral biases would be to measure biases in the empty maze over a number of testing sessions. For example, one session would consist of ten individual runs through the empty maze and sessions could be repeated on consecutive days, or even once a week for a number of weeks before stimulus preferences were tested. In addition, individual biases should be tested in a different two-choice apparatus. The use of such protocols would allow us to determine whether individual biases expressed by sheep in a Y maze represent consistent lateralized behaviour, or whether they simply reflect perseverative behaviour in a single testing facility.

Potential effects of experimental methodology on results

When using experimental methods such as the arena and Y maze tests, it is important to consider the influence on the results of experimental features other than the presence of the stimulus. For example, in the arena tests discussed in this thesis, the novelty of the testing environment and procedure, as well as the novelty of the stimulus itself may have had a considerable influence on the behavioural and physiological responses of the sheep. Repeated testing of the sheep's responses to the same stimulus may have resulted in a reduction in the differences between the stimuli. In support of this, Erhard (2003) found that initially a human facing towards the sheep elicited stronger behavioural

aversion than a human facing away; however, after repeated testing, the differences in behavioural response to the two stimuli disappeared.

Likewise, the differences between the behavioural and adrenocortical responses of the UWA flocks in the arena may have related, in part, to the novelty of the testing environment and procedure. In fact, there was some evidence that as the weeks of testing progressed, the inter-flock differences in locomotor and vocal activity declined.

It is also important to consider the nature of sheep, relative to other domestic species. For example, rather than reflecting an active choice of one stimulus over another in the Y maze, it is possible that consistent choices for one side of the maze simply reflected species-typical persistent behaviour. However, I did observe some behavioural indications that sheep were making considered choices in the maze. For example, the number of vacillations (looking from one side to the other) performed at the crux of the maze was highest in the first few trials.

The behavioural and physiological responses of sheep to various stimuli must be interpreted bearing in mind the limitations imposed by the experimental methodology and the nature of the species under study. However, in terms of the relative aversiveness of humans and dogs to domestic sheep, it may be most appropriate to examine responses to relatively novel or unfamiliar stimuli. Many sheep in New Zealand are handled relatively rarely, and in large flocks, close encounters with humans and dogs may occur infrequently.

8.3   Implications for animal welfare and management

If we accept that changes in behavioural aversion and physiological parameters can be used to infer emotional activation in sheep, the results of this research have practical implications for the use of humans and dogs for handling sheep. Sheep expressed behavioural aversion and significant rises in plasma cortisol concentration in response to both human and dog presence, suggesting that handling with either stimulus may elicit fear in domestic sheep. However, as noted above, the magnitude and duration of the adrenocortical responses were only moderate compared to responses reported in other studies exposing sheep to currently accepted sheep husbandry practices.

Therefore, if these observations are confirmed in the practical environment, handing sheep using either a human or dog is unlikely to cause concern on the basis of animal welfare.

However, it is always advisable to minimize animal stress wherever possible. As noted in Chapter 6, limiting the use of dogs on farms and prior to slaughter has been recommended to reduce sheep stress for reasons relating to animal welfare, productivity and product quality. This idea is supported by the present research, with dog presence eliciting more fear in sheep than human presence, as indicated by behavioural aversion and adrenocortical responses. Therefore, avoiding the use of dogs may be recommended to reduce the detrimental effects of excessive fear or stress in domestic sheep, particularly in certain stages of sheep husbandry, e.g. perinatal period, prior to slaughter. Further research is required to determine whether handling with dogs or humans prior to slaughter has the potential to negatively affect meat quality.

As discussed above, we are currently limited in our ability to use behavioural and physiological indicators to examine the emotional experiences of domestic sheep. This also imposes a limitation on determining the practical implications of handling sheep using humans and dogs, particularly in terms of animal well-being, which relates most closely to the animal's perceptions and subjective experiences. However, even if behavioural aversion does not directly reflect levels of emotional activation or fear in sheep, there are still management implications to the stronger avoidance of a dog than of a human.

Sheep stayed further away from the dog in the arena and chose to avoid the dog in the Y maze, suggesting that they are more likely to move away from a dog on the farm or in the meat processing plant. This has implications both for handling efficiency and for the occurrence of injuries in sheep. On one hand, removing dogs from such facilities may reduce the efficiency with which sheep can be handled, as the sheep show less behavioural aversion to humans. On the other hand, there may be a reduction in the prevalence of injuries resulting from strong avoidance of a dog.

Likewise, even if the physiological stress responses measured in these studies do not relate directly to the level of fear experienced by the sheep, the potential for increased

adrenocortical activity to negatively impact on animal productivity, health and product quality is well known. Therefore, handling sheep with humans rather than dogs may reduce the impact of physiological stress responses on economically important factors.

Implications of selection for lower fear of humans

Genetic selection for sheep experiencing lower levels of fear or stress in the presence of humans could be used to improve the well-being of the sheep and potentially, productivity and handling efficiency (Jones, 1997; Faure and Mills, 1998). However, there are also management implications related to lowering fear of humans in domestic livestock.

Firstly, animals with reduced fear of humans can be potentially dangerous for their handlers. Over-tame animals, particularly male herbivores, may direct behaviours towards humans that they would be reluctant to exhibit if they were more fearful (Grandin and Deesing, 1998). In addition, although tameness would be advantageous for handling in confinement, over-tameness may be undesirable both in confined and non-confined situations if humans are the primary stimulus for moving/controlling animals using their natural aversion.

Secondly, if selection for lower fear of humans has been based on alteration of a domain-general temperament trait (e.g. fearfulness), sheep showing lower fear of humans might also be expected to exhibit decreased responses to dogs. As dogs are currently the most effective means of moving sheep maintained in extensive pastural managements systems, this could create serious problems for sheep farmers.

However, the results of the study presented in Chapter 5 suggest that lower fear responses of MA sheep in the arena test were specific to the presence of a human. The behavioural and adrenocortical responses of MA sheep were similar to those of the LA and unselected flocks in the presence of the dog. This points to the possibility of selecting domestic sheep for lower fear of humans while maintaining higher levels of fear in the presence of a predator. If possible, such a selection regime would facilitate efficient movement of the sheep using dogs, as well as minimizing the fear or stress experienced due to necessary routine handling by humans.

Alternatively, if we consider that the inter-flock differences in adrenocortical responses to the human were relatively minor (Chapter 4 and 5), we might conclude that the flocks showed differences in behavioural aversion to a human and different activity levels, but did not experience significantly different levels of fear or stress due to the human's presence. In this case, it may be possible to select animals that humans can move more easily (MA sheep), but that do not experience any more fear or stress than sheep that are more difficult to move (LA sheep).

There is some empirical evidence that context-specific traits can be genetically separated in other domestic species (Mills and Faure, 1991). In addition, the existence of the Lasater Beefmaster cattle breed points to the possibility of co-selecting domestic animals for a number of context-specific temperament traits. For almost 70 years, Lasater Beefmaster cows have been strictly selected for strong maternal behaviour, including strong anti-predator behaviour, but also for high docility (e.g. cows seek to interact with humans) (Lasater, 1972; Grandin and Deesing, 1998). Selection is implemented as follows: cows living in North American range environments, which include predators such as coyotes, are required to raise a calf to weaning every year. The calves are then tested at weaning for their willingness to approach and feed from a human in a pen. Calves and cows which do not satisfy these criteria are culled (Dale Lasater, personal communication in Grandin and Deesing, 1998).

The combination of highly docility and strong anti-predator behaviour would be especially well suited to areas in which natural predation still constitutes an economic cost for sheep producers. In areas such as Northern Europe and North America sheep producers experience significant lamb and ewe mortality to carnivorous predators such as wolves, coyotes, cougars, wolverines and bears (Hansen et al., 2001). Domestic ewes with reduced anti-predator skills would be expected to lose more lambs to predators than those with strong defensive behaviour. This is supported by experimental evidence that unimproved breeds show stronger anti-predator behaviours and have lower lamb losses to predation than do more 'improved' breeds (Hansen et al., 2001).

The physiological responses of Lasater Beefmaster calves to human or predator presence have not been systematically investigated. However, there is scientific support

276

for the notion that an animal that approaches quickly and interacts frequently with a human experiences lower adrenocortical activation than one that is reluctant to approach and interact (Lyons et al., 1988; Goddard et al., 2000; Van Reenen et al., 2005), suggesting that higher docility, as measured in the Lasater Beefmaster, indicates lower fear of humans.

Although the Lasater Beefmaster is selected partially on the basis of strong anti-predator behaviour, we do not know whether this necessarily implies higher levels of fear in the presence of a predator. It is possible that less fearful cows (i.e. more docile) protect their calves more vigorously. In fact, Reale and Festa-Bianchet (2003) found that bolder (less fearful) Bighorn ewes were more likely to survive in years of high predation pressure. In addition, piglets that were more socially aggressive also appeared to be less fearful of a novel object (Hessing et al., 1994). If less fearful individuals exhibit more successful anti-predator behaviour, then the Lasater Beefmaster could simply be exhibiting lower levels of fear, both in the presence of humans and predators in range conditions. However, Hansen et al. (2001) reported that more 'domesticated' sheep breeds (implying lower fear of humans, although this was not experimentally validated), also exhibited lower levels of anti-predator behaviour. This is an area that requires further investigation.

Implications of individual lateral biases

Lateral biases expressed by individual sheep in the Y maze facility may reflect inherent lateralized tendencies, or may simply relate to features of the experimental facility. As discussed in Chapter 7, both the present research and previous studies provide evidence that lateral biases are biologically relevant to the individual, and that a significant proportion of domestic ungulates (60 -70% of test population) exhibit such biases (Hansen et al., 1978; Hosoi et al., 1995; Hopster et al., 1998; Arnold, 2005).

Lateralized individuals have been shown to differ from non-lateralized animals in aspects such as problem solving, learning ability and memory, behavioural responses to novelty, and physiological stress responsiveness (e.g. Zimmerberg et al., 1978; Rizhova and Kulagin, 1994; Hopster et al., 1998; Larose et al., 2006). Therefore, the existence of individual biases in sheep populations may have potential implications for facility

design (e.g. direction of races) and management (e.g. side of food presentation: Rizhova and Kokorina, 2005) and for breeding animals for certain behavioural or physiological traits. However, further research is required to ascertain whether individual biases expressed in a Y maze represent consistent behavioural or functional asymmetries which have the potential to influence other biological responses in sheep.

8.4   Future studies

As noted above, the experiences of domestic sheep and their responses to the presence of humans and dogs as measured in an experimental context may be significantly different from those occurring during routine husbandry procedures on a farm and in meat processing premises. Because of the differences between the experimental and practical situations, it would be beneficial to conduct further studies on the stress responses of sheep to the presence of humans and dogs in the actual environment in which such interactions routinely take place.

It may be difficult to measure behavioural responses in actual farm and pre-slaughter environments without altering the normal methods used to handle sheep in these situations. However, remote sampling methods applied to freely-behaving animals could be used to measure physiological stress indicators such as plasma cortisol concentration, body temperature and heart rate (Cook et al., 2000) in response to human presence and handling as it occurs on the farm and in the pre-slaughter environment. The effect on physiological stress responses of adding dogs during handling should then be evaluated. Physiological variables, such as plasma cortisol concentration, could also be measured in samples taken immediately after slaughter, as well as measuring the ultimate pH of muscle tissue to determine the relative effects of pre-slaughter handling with and without the presence of dogs.

With regard to the UWA flocks, the behavioural and physiological responses of the flocks to a wider range of challenging situations of different qualities would help elucidate the results of the selection process, and whether the selected trait is context-specific or domain general. For example, a startle test would specifically test the responses of the flocks to novelty and suddenness, whereas a human approach test may reveal more about the relative aversion of the flocks to human presence. Testing

individuals in a thoroughly familiar environment would reduce the effects of novelty on stress responses, whereas testing groups of animals from the same flock together would determine the extent to which social isolation differentially affects the behaviour and adrenocortical responses of the flocks. The flocks could also be compared in terms of the ease of taming, i.e. reduction in the behavioural and physiological indicators of fear or stress due to the presence of, and contact with humans (Pearson and Mellor, 1976).

To date, the adrenocortical and other physiological responses of the UWA flocks to social isolation have not been reported. Such variables should be measured, both in the Box test (one of the selection environments) and in a testing environment not involved in the selection process. The results would aid in interpreting the relationship between the 'selected behaviours' and fear in UWA sheep. In the long term, selective breeding experiments could be used to determine whether selection has been based on two separate traits (sociality and fear of a human), or one consistent predisposition to react fearfully (fearfulness).

Additional neurophysiological and hormonal variables should be measured to help elucidate the physiological basis of the behavioural differences between the flocks. There is evidence to support the central and multiple roles of corticotropin releasing factor (CRF) in controlling behavioural and physiological responses to acute stress (Johnson et al., 1994). Therefore, if possible, the CRF responses of the flocks should be compared, particularly in brain areas involved in perception of and emotional response to fear-eliciting stimulation e.g. the amygdala. In addition, an ACTH challenge test may reveal differences in adrenal responsiveness, although Kilgour and Szantar-Coddington (1997) found no differences in adrenocortical response to an ACTH challenge between flocks selected for behavioural differences similar to those seen in the UWA flocks. Finally, the relative activation of the sympathetic nervous system in response to acute challenge should be compared between flocks e.g. heart rate, catecholamine responses.

Methods to improve the characterization of individual lateral biases have been discussed above. In order to determine the strength of relative preferences expressed in the Y maze, demand function or cost-benefit tests could be used. For example, for studying the aversiveness of human or dog presence, we could measure the cost individual sheep are willing to pay in order to avoid each stimulus. This could be achieved by

279

experimentally manipulating the cost of exiting a treatment room containing each stimulus. Alternatively, we could measure the willingness of individuals to forfeit other valuable resources such as companions or food in order to avoid each stimulus.

8.5 Conclusions

The results of the studies presented in this thesis lead to the following conclusions:

- The presence of a dog elicited behavioural aversion and physiological stress responses in domestic sheep tested in a variety of experimental contexts.

- Sheep also exhibited behavioural aversion to the presence of a human in an arena test and a Y maze test. In addition, there is some limited evidence that confinement with a human (plus rattle) in the Y maze also elicited significant physiological stress responses in sheep.

- The presence of a dog elicited more behavioural aversion and avoidance and higher rises in plasma cortisol concentration than the presence of a human. These results suggest that sheep experienced more emotional activation or fear in the presence of a dog than with a human.

- The physiological stress responses elicited by the presence of a human or a dog in the experimental situations described were only moderate in magnitude and duration compared to those reportedly elicited by currently accepted sheep husbandry procedures. However, the behaviour of humans and dogs in practical situations may be expected to elicit higher physiological stress responses than were measured in these experiments. Therefore, these observations need to be confirmed in practical sheep management situations.

- If the physiological observations are confirmed in practical situations, the presence of humans and dogs during routine handling of sheep should cause little concern on the basis of animal welfare.

- However, it is advisable to minimize animal stress wherever possible, and limiting the presence of dogs in certain situations (e.g. before slaughter) may reduce stress in domestic sheep.

- Future studies should measure sheep stress responses to the presence of humans and dogs in practical situations.

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283

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Appendix 1

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Appendix 2

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Appendix 3

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Appendix 4

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