Chapter 1 Literature Review

1.1 Introduction

Temperament is defined here as an animal's inherent fearfulness of humans and strange, novel or threatening situations. It has long been known that temperament has an influential effect on many different animals, including cattle, sheep, poultry and rodents (Hall 1938; Hughes *et al.* 1977; Satterlee and Jones 1995; Torres-Hernandez and Hohenboken 1979; Tulloh 1961). Research has further shown important correlations between temperament and other production traits, including immune function (Fell *et al.* 1999), reproduction, (e.g. sexual behaviour, maternal behaviour and neonatal survival (Gelez *et al.* 2003; Gelez *et al.* 2003; Kilgour and Szantar-Coddington 1997; Putu 1988), adaptation (Ruis *et al.* 2001), growth (Zulkifli *et al.* 2002) and meat quality (Burrow and Dillon 1997; Fordyce *et al.* 1988). Moreover, temperament can influence the welfare of the animal and its conspecifics and the welfare of the stockperson handling the animals. Associations between temperament and other animal production traits are discussed in more detail in section 1.2.4.

It is generally recognised that selection for temperament in farm animals offers benefits with regard to animal welfare, productivity and ease of handling and management of animals, particularly cattle (Burrow 1997). However, the underpinning neuro-regulatory mechanisms responsible for variations in temperament between animals are not well known. Specific behavioural and physiological responses are of importance when considering the adaptability of an animal to changing situations, improvement of performance, welfare and the handling of the animals. The selection for these responses to such challenges has until recently, not been studied extensively. In recent years there has been more focus on these responses across a range of species, including cattle (Boissy and Bouissou 1995; Hagen and Broom 2004), poultry (Faure and Mills 1998); (Muir and Craig 1998), mink (Malmkvist and Hansen 2002), pigs (Désautés *et al.* 2002; Malmkvist) and sheep (Boissy 1995; Boissy *et al.* 2005; Viérin and Bouissou 2003; Blache and Ferguson 2006; Fisher *et al.* 2006).

Activation of the stress system to threatening or potentially threatening situations is considered a normal adaptive response and as such, helps to bring the animal back to homeostasis and improve its chances of survival. Additionally, the the stress response system is also activated

by other activities, not considered to be stressful *per se*, such as sexual activity and eating (Chrousos 1998). However, prolonged or excessive exposure to the same stressful situation/s can then develop into pathological states, which can impact on the welfare of the animal (Ninan 1999; Sandford *et al.* 2000). Standard farming procedures, such as restraint for veterinary procedures, human handling and transport, amongst others, can induce a stress response, resulting in fear and anxiety (Arthington *et al.* 2003; Boissy and Bouissou 1988; Coleman *et al.* 2005; Saco *et al.* 2003). Fearfulness is a trait which is inherent in the evaluation of animal temperament and the expression of fearfulness can also have a significant effect on the animal's welfare (Colditz and Hennessy 2001) as well as being of considerable economic and ethical importance (Boissy 1995; Cockram *et al.* 1994; Moberg 2000). It is therefore clear that fear responses and their regulation are central to the mechanisms that underpin animal temperament. Further understanding of these mechanisms may facilitate the identification of particular responses that may help to improve the capacity and ability of animal breeders to select for animals that have the optimal temperament for their production environments.

There has been considerable research focus on the neurophysiological regulation of fearfulness and anxiety in animals, but not necessarily as it relates to animal temperament. The large majority of the research has centered on the neurotransmitters, such as the monoamines (e.g. serotonin (5-HT) and norepinephrine (NE)), γ-amino-*n*-butyric acid (GABA), corticotropin releasing hormone (CRH), cholecystokinin, neuropeptide Y and opioid peptides (Sandford *et al.* 2000). A common approach to this type of research has been to utilize pharmacological treatments that target specific neuro-regulatory systems and their specific neurotransmitters.

The review that follows will focus on the links between particular neurotransmitter systems (GABA and 5-HT), fear and fearfulness and what affect these might have on livestock temperament. It will also examine why the temperament of livestock is important and how it is currently measured. The two neurophysiological receptor pathways will be examined in some detail to determine their major functions in the body and their relationship to fear and fearfulness and therefore, associated animal temperament.

1.2 Fear, fearfulness and temperament

1.2.1 Fear and fearfulness

Fear is a universal emotion in the animal kingdom and it could be argued that it is primarily a defensive response to predators, threats and adverse situations (Grandin 1988) as well as helping to facilitate a learned response (Maren 2005). Boissy (1995) defined fearfulness as a basic psychological characteristic of an individual that predisposes it to perceive and react in a similar manner to a wide range of potentially frightening events. The expression of fearfulness is the result of interactions related to prior experience, the environment and the animal's own genetic background. These factors are discussed further in section 1.2.5. Fearfulness can influence a variety of different parameters, including social rank in cattle (Solano *et al.* 2004), maternal behaviour in sheep (Murphy 1999; Putu 1988), reproductive success in sheep and goats (Réale *et al.* 2000), meat quality in cattle (Grandin 1980) and immune parameters in cattle (Colditz and Hennessy 2001). Excessive fearfulness can lead to chronic stress and psychopathologies (see review by (Korte 2001)and decreased productivity (Boissy *et al.* 2005), whilst a reduction in the level of fearfulness may therefore result in improved welfare of farmed animals (Malmkvist and Hansen 2002) . Responses attributed to fear are also involved in the adaptation of animals to their environment, as well as production situations for livestock (Bouissou and Vandenheede 1995).

1.2.3 Temperament

Temperament can be defined here as an animal's inherent fearfulness of humans and strange, novel or threatening situations, and generally refers to the animal's behavioural fear response. Responses can range from exploration and grazing/eating, regarded as calm or good temperament, to animals that express behavioural manifestations of fear and anxiety, such as non-responsiveness, freezing and aggression, regarded as bad or nervous temperament (Burrow 1997).

1.2.3.1 Temperament tests

Many different methods and tests have been used and evaluated to study temperament and fear responses in animals. Whilst there are a large number of tests available to assess fearfulness and temperament in livestock, it is not always clear what each test measures (see review by (Burrow 1997). Furthermore, Archer (1979) commented that some methods/tests are not relevant to the animal species and therefore do not take into account the biological relevance

of the subsequent behaviour, therefore the test may not illustrate relevance to the behavioural repertoire of the species.

Some of the tests currently used for assessment of temperament for production animals have been adapted from laboratory methods used in rodent fear and anxiety models. The results obtained from the different tests are often context specific, as well as being species specific. For example, some animals will freeze in fearful situations (e.g. poultry), whilst others will try to escape. Dependent on the type of test used, the behaviours identified may be misinterpreted. For example, differentiating between an animal's fear response of increased locomotion and that of investigatory behaviour is difficult. Furthermore, some tests utilize physical restraint of the animal, which may not reflect the same fear-inducing situations if the animal was un-restrained and was able to freely interact.

In the case of livestock, the majority of tests attempt to quantify approach and escape/avoidance behaviours. The general features of these types of tests include exposing the animals to novel or threatening contexts and subjectively or objectively measuring the desire of the animal to escape/avoid or approach and explore the situation /object/event. Examples of these tests include the open-field test, (e.g. cattle, (Kilgour 1975) and sheep, (Murphy *et al.* 1994), the arena test (e.g. sheep, (Fell and Shutt 1989) and horses, (Le Scolan *et al.* 1997)), restraint or crush tests (e.g. cattle, (Fordyce *et al.* 1982; Grandin 1993), novelty tests (e.g. horses, (Le Scolan *et al.* 1997) and lambs (Désiré *et al.* 2004)), predator challenge models (e.g. sheep, (Beausoleil *et al.* 2005) and rodents (Korte and De Boer 2003; Adamec and Shallow 1993; Hansen *et al.* 2001), maternal temperament tests and dominance tests (see review by Burrow 1997). Burrow (1997) also stated that the different types of tests could be generally categorized into several groups; 1) restrained tests, 2) non-restrained tests, 3) movement tests, 4) dairy temperament scoring, 5) dominance testing and 6) maternal temperament scoring.

Temperament, as defined by the behaviours in the various different test types has been shown to be moderately to highly heritable in cattle, indicating that there is significant genetic potential to modify and improve temperament (Burrow 1997). This has also been examined in wild and domestic animals. Trut (1999) and Malmkvist and Hansen (2002) have shown a genetic basis for temperament in foxes and mink, respectively. Temperament is also

postulated to be a relatively stable individual characteristic, which results from a combination of genetic and epigenetic factors, as stated earlier.

If a given behaviour is measured from a group of animals over a period of time, there is normally considerable difference in the expression of that behaviour. Some of this individuality would be random and unpredictable, whilst some would be more consistent in their responses over time (Erhard and Schouten 2001). Selection for specific traits, during domestication and for current usage in farming practices is based on this concept. The consistency of individual responses is the essential aspect and reinforces the view that study at the level of the individual is important. An understanding of the mechanisms underpinning variations in behaviour between individuals and the consequences of such in terms of adaptability is important. Research into how and why animals respond differently in similar environments, supports the view that animals have different ways to adapt to changing situations. There are different theories regarding the variety of ways this may occur. One theory (discussed below) suggests that animals have two basic types or styles of coping mechanisms, which they use in the face of adverse situations. The *active* coping style is where animals will try to actively remove the stressor or move away from it, whilst the second is a more *passive* style where the animal may freeze and/or withdraw from the situation (Benus *et al.* 1987; Bohus *et al.* 1987), which is postulated to indicate intense fear and anxiety (Gallup 1979; Jones and Satterlee 1996). Each profile appears to be correlated with different physiological, behavioural and neuroendocrine responses (Schouten and Wiepkem 1991), for example (Sapolsky 1990; Van Holst 1986), animals considered to have an active coping style show more aggression, higher general activity and a predominant sympathetic nervous reaction (fight or flight). In contrast, animals considered to have a passive coping style respond more with immobility and avoidance behaviours and a predominant parasympathetic/hypothalamic activation (Ruis *et al.* 2000). Hessing *et al.* (1994) illustrated that the coping profiles of pigs that were aggressive (based on social confrontation tests) and resistant (based on number of escape attempts from the back-test) were found, in general, to react with an increased sympathetic stress response, whilst the non-aggressive, non-resistant pigs responded in a para-sympathic manner. However, other researchers, as shown below, have not found evidence to support the hypothesis put forward by Hessing (1993). Forkman *et al.* (1995) suggested an alternative view to the coping style theory, suggesting that there were three possible personality traits (aggressiveness, sociability and exploration) to explain the

variation in the response of pigs. Van Reenan *et al.* (2005) suggested an alternative interpretation to those above, that the variable responsiveness of the animal to a challenge may reflect actual differences in fear.

1.2.4 Associations between temperament and production traits

Voisinet *et al.* (1997) stated that the economic implications of animal temperament have been unrecognized for a long period of time. However, there is now an abundance of research that indicates animal temperament has significance due to its association with production traits. Kovalciková and Kovalcik (1982) have shown that cows with good temperament have better milk production efficiency and welfare. Similarly, Gupta and Mishra (1979) found that dairy cows with a good temperament had higher production characteristics, such as milk yield, shorter milking times and decreased milk letdown times. Moreover, Lyons (1989) illustrated that goats classified as reactive (nervous) had higher rates of problems with milk ejection, suggesting their increased susceptibility to stressors in the environment could affect productivity. In sheep, Murphy (1999) showed that ewes selected for calm temperament were better mothers, resulting in lower lamb mortality compared to ewes with a more nervous temperament. Bighorn sheep, classified as having a bold temperament were found by Reale *et al.* (2000) to have faster and higher reproductive success than those classified as shy, suggesting the female's temperament characteristics affects their reproductive success and sexual behaviour (Gelez *et al.* 2003). Research by Knott *et al.* (2004) showed that sheep with a decreased efficiency to utilise feed, showed an increased HPA response to a ACTH challenge, than those with better feed efficiency. Voisinet *et al.* (1997) showed significant correlations between temperament, feedlot average daily gain and beef quality in cattle. Collectively, these research findings highlight that selection for temperament can potentially yield increases in animal production.

1.2.5 Factors affecting the expression of temperament

The expression of animal temperament and indeed any stress response is a function of genetics, experience and environmental factors or epigenetic factors. Examples of the influence of these genetic and epigenetic factors include, yard-weaning of calves (confinement of calves in a yard for a period of time after weaning from their dam, allowing exposure of the calves to novelty and social interaction with conspecifics). This procedure has been shown to decrease the stress response of the calves during subsequent handling procedures, thereby

being a positive influence (Lefcourt and Elsasser 1995). Stressors, such as the use of electric shocks have also been shown to negatively affect later response to other environmental stressors (Kennett *et al.* 1986; Weiss *et al.* 1981).

Behavioural responses to stress are also seen to vary markedly within breeds and strains. For example, considerable variation has been observed in rats from the same strain (File and Vellucci 1979; Bohus *et al.* 1987; Koolhaas *et al.* 1986). Such differences are also correlated with physiological response, including heart rate, level of hormone release, and general overall hypothalamic-pituitary-adrenal (HPA) axis activation. Additionally, observations of cloned pigs has found temperament characteristics differ between and within litters and in responses towards litter mates and humans (Archer *et al.* 2003). Additional genetic studies have also been conducted to determine what effect the sire has on the temperament phenotype of his daughters. Dickson *et al.* (1970) investigated temperament in dairy cows and found that the sire's genetics played a significant role in influencing the behaviour of daughters in the milking shed, indicating sires with better temperament scores produced daughters with similar scores. Further genetic influence can come from effects of maternal stress on fetal development, which can lead to alteration of the regulation of the HPA axis affecting anxiety later in life (Weinstock 2004).

Environment can also influence temperament, however, in some respects this can be confounded by experience. Raising young animals in situations devoid of stimulation or novelty can affect the development of the nervous system, and lead to an animal being highly reactive and nervous in challenging situations, or lead to expression of apathetic behaviour in later life (Grandin and Deesing 1998). The introduction of an enriched environment to young animals has been shown to have a positive effect on decreasing fearfulness, resulting in animals that are more willing to explore novel situations or challenges (Vandenheede and Bouissou 1998). For example, pigs that were given access to toys were found to be calmer and less excitable than a control group, which did not have toys to interact with (Grandin *et al.* 1987). Additionally, enriching the environment can have positive effects in older animals, as well as younger animals, as it enhances neuron proliferation in the brain, which in turn leads to improved memory and learning (Segovia *et al.* 2006) . Enrichment has been used widely for laboratory and zoo animals to reduce reactivity to novel situations and improve learning (Mellen and MacPhee 2001; van Praag *et al.* 2000). The influence that genetics, experience

and environment have on temperament can be clearly observed; however the interaction between these factors on the expression of the trait is clearly a complex process.

Genetic and environmental factors (Grandin 1988) also play varying roles in how animals respond to a specific challenge. The expression of a stress response is highly variable and what may be stressful for one individual may not be so for another. This has been well illustrated when animals are subjected to repeated stress exposure (such as loud noises or isolation). It can lead to either habituation, sensitization or have no effect at all on particular behavioural measures (Briones-Aranda *et al.* 2005; McCormick *et al.* 1998; Pitman *et al.* 1988). Research has also shown that an animal's reaction to an adverse stressor can be very specific and dependent on the type of stressor and its duration (Djordjevic *et al.* 2003; Charmandari *et al.* 2005).

1.3 Neurological regulation of fear and temperament

How an animal reacts to a stressful situation will vary depending on the nature of the challenge. The response to a stressor consists of a series of behavioural, physiological and neuroendocrinological changes that are regulated by the brain, including a variety of changes at many different neurotransmitter and neuropeptide systems (Vaccarino and Kastin 2001). Failure to adapt to stressors or stressful situations can lead to altered brain function and this in turn can lead to pathologies (van Praag 2004; Kessler *et al.* 1996).

Several neurotransmitters and their pathways have been investigated in the context of the stress response and the regulation of fearfulness, and particularly the GABA and serotonin pathways have received considerable research attention. There is a large body of evidence of the role that these neurotransmitters (GABA and 5-HT) play in the context of the stress responses, fear and anxiety and this is reviewed in the following sections. However, the evidence from this area is primarily related to the effect of pharmacological treatments administered to animals during different states, challenge models and situations and are not always directly related to animal temperament, therefore the role of these systems in the control or establishment of temperament is not clear cut. However, if they affect the expression of fearfulness, then it is fairly likely that they are relevant to temperament.

1.3.1 γ-aminobutyric acid (GABA)

GABA is a major inhibitory neurotransmitter, widely distributed in the vertebrate central nervous system (CNS), occupying approximately one-third of all neurons (Miczek *et al.* 2002), whilst the remainder use glutamate as the primary neurotransmitter. In addition to the CNS, GABA receptors are also found in other tissues including endocrine glands, smooth muscle, and in the female reproductive system (Krogsgaard-Larsen *et al.* 1994). Alterations in GABAergic activity and GABA receptor function affect a number of normal mammalian behaviours, such as eating, sleeping and sexual behaviour, as well as influencing learning and memory (Shiah and Yatham 1998). Furthermore, dysfunction within the GABAergic system is associated with several neuropathological disorders, such as depression (Krogsgaard-Larsen *et al.* 1994; Petty 1995; Shiah and Yatham 1998).

1.3.1.1 GABA receptors

GABA acts through two different classes of receptors, defined as either ionotropic or metabotropic. The receptors within each and their primary anatomical location are shown in Table 1.1. The $GABA_A$ and $GABA_C$ are ionotropic, ligand-gated ion receptors (Chebib and Johnston 2000), which when activated result in increased chloride (Cl^-) ion conductance and mediate the majority of fast inhibitory neurotransmission (Korpi *et al.* 2002). Metabotropic receptors, such as the $GABA_B$ receptor, rely on secondary messenger systems *via* activation of G-proteins (Frolund *et al.* 2002). They are located pre- and post-synaptically and are responsible for slow inhibitory synaptic transmission (Smart and Thomas 2004).

Table 1.1: GABA receptor subtype and location in mammals

Ionotropic ligand gated ion receptors		**Metabotropic secondary messenger system receptors**
$GABA_A$ receptor	*$GABA_C$ receptor*	*$GABA_B$ receptor*
Cortex, substantia nigra, cerebellum, hippocampus, striatum and spinal cord	Retina and areas of the visual pathway	Cortex, cerebellum, lateral septum, nucleus accumbens, periaquaductal gray, mammillary bodies, hippocampus, amygdala, locus coeruleus and raphé nuclei

$GABA_A$ receptor

When GABA binds to the $GABA_A$ receptor, the Cl^- ion flux through the channel increases causing hyper-polarization and inhibition of neurotransmission (Bormann 2000). The $GABA_A$ receptor consists of transmembrane pentamers of at least 19 different subunit compositions (ά 1-6, β1-4, γ1-4, δ, ε, π and θ), which have been cloned and localised on different genes (Korpi *et al.* 2002; Mehta and Ticku 1999). Different combinations of the subunits form receptor subtypes which vary in their expression in different brain cells and regions to confer functional diversity (Korpi *et al.* 2002). Further functional diversity arises as some subunit mRNAs undergo alternative splicing, producing two alternative subunit proteins (S-short and L-long) (Smart and Thomas 2004). The postsynaptic $GABA_A$ receptor contains a large number of binding sites which enables a large diversity of compounds to act on $GABA_A$ receptors (Chebib and Johnston 2000), which can positively or negatively modulate the inhibitory action of GABA. Numerous compounds allosterically modulate $GABA_A$ receptors by binding to the different sites (e.g. benzodiazepine (BZP), barbiturate, neurosteroid, picrotoxin, t-butylbicyclophosphorothionate (TBPS), flourosemide, alcohol and loreclezole) (Chebib and Johnston 2000; Wang *et al.* 1999). Research into the association between GABA and stress and anxiety has mainly focused on the $GABA_A$ receptor as several GABA antagonists were recognized to have anxiogenic and pro-convulsant properties (Frolund *et al.* 2002).

$GABA_B$ receptor

$GABA_B$ receptors are heterodimeric G-protein coupled receptors, with $GABA_{B1}$ and $GABA_{B2}$ subunits (Jackson and Kuehl 2002; Bowery *et al.* 2002). These receptors are located both pre- and post-synaptically and underpin slow inhibitory synaptic transmission (Barnard *et al.* 1998). $GABA_B$ receptors are also widely distributed in the mammalian brain (Liang *et al.* 2000; Margeta-Mitrovic *et al.* 1999), as well as in peripheral autonomic terminals (Bowery *et al.* 1981) and are able to mediate responses in other organs (Bowery *et al.* 2002). Activation of the $GABA_B$ receptor causes hyperpolarisation via changes by either, increasing potassium (K^+) or decreasing Cl^- conductance and a reduction of neuronal firing through G-proteins and secondary messenger systems (Martin and Dunn 2003).

$GABA_C$ receptor

$GABA_C$ receptors are also ligand-gated receptors and are homomeric, composed only of ρ1 subunits (Chebib and Johnston 2000). These receptors are different in their pharmacological

profile from $GABA_A$ receptors in that they are more sensitive to GABA than $GABA_A$ receptors, have slower activation times, do not readily desensitize with maintained GABA application (Chebib and Johnston 2000) and are insensitive to many $GABA_A$ receptor ligands, particularly BZPs and barbiturates (Enz and Cutting 1998). The results of *in situ* tissue hybridization studies show that $GABA_C$ receptors are located primarily in the retina and other parts of the visual pathway indicating a predominant influence in vision (Enz and Cutting 1998;Johnston 1996). The limited nature of selective $GABA_C$ ligands makes it the least characterized of the GABA receptors (Frolund *et al.* 2002).

1.3.1.2 Ligands

The large number of $GABA_A$ binding sites has facilitated identification and development of ligands specific to the other GABA receptor subtypes. The multiple recognition sites found typically on the $GABA_A$ receptors for various pharmacological compounds are classified as agonists, antagonists and inverse agonists (Wang *et al.* 1999).

One prominent example is that of Benzodiazepines (BZPs), first discovered in the 1970's (Costa *et al.* 1975). Most BZPs work to enhance the action of GABA by increasing the frequency of the channel opening and increasing the affinity of the receptor for GABA (Barnard *et al.* 1998). This causes an anxiolytic action, inducing muscle relaxation and lowering plasma corticosterone and cortisol concentrations (Sanger *et al.* 1994; Bohus *et al.* 1990) and can also produce secondary addictive side-effects (McNaughton *et al.* 1996; File and Pellow 1987). Benzodiazepine action has been studied in many different animal anxiety models, mostly using conflict tests in rodents (File and Pellow 1987). Several other ligands have binding sites on the $GABA_A$ receptor. Barbiturates act like BZPs, without affecting channel opening frequency, and at high concentrations, open the $GABA_A$ receptor channels directly (Martin and Dunn 2003). Selected steroids also have a high affinity-binding site on $GABA_A$ receptors causing increases in channel opening times and opening frequency, and like barbiturates, can directly activate the receptor at high concentrations (Mehta and Ticku 1999). Other sites include some general anaesthetics (Martin and Dunn 2003) and a picrotoxin site (Wang *et al.* 1999).

1.3.1.3 GABA and fearfulness

GABAergic neurotransmission has been consistently shown to play a key role in the modulation of stress mediated changes in behaviour and physiology (Biggio *et al.* 1990; Drugan *et al.* 1994). Furthermore, a decrease in GABA neurotransmission has been related to several neurological disorders, such as anxiety and depression (Clément 1996). Relationships between GABAergic dysfunction and fearfulness and altered stress responses have been well documented (Martijena *et al.* 1997). For example, using a mouse knockout model, Mombereau *et al.* (2004) demonstrated that mice deficient for the $GABA_{\beta(1)}$ subunit were more anxious than wild type mice in several behavioural challenge models, particularly the light dark box test. Additionally, GABA receptor agonists have been shown to attenuate stress-induced behaviours, while antagonists cause the opposite effect (Biggio *et al.* 1990; Drugan *et al.* 1985). The amygdala and frontal cortex contain high densities of $GABA_A$ receptors (Swanson and Petrovich 1998) and these brain regions are involved centrally in mediating fear and changes in GABAergic transmission caused by stress. Low concentrations of GABA in cerebral spinal fluid (CSF) and plasma have been found in patients diagnosed with depression compared with healthy controls (Gold *et al.* 1980). Additionally, GABA has been implicated in a number of animal models of depression (Cheetham *et al.* 1988). For example, anxiogenic behaviours can be reversed with the chronic administration of antidepressant treatments (those that target/mimic GABA) (Petty and Sherman 1981). Overall, this large body of evidence shows an association between GABA dysfunction and the etiology of anxiety and excessive fearfulness.

The GABAergic system has also been well researched in the context of neurological disorders. In 1980, Emrich *et al.* (1980) found valproate (a GABA agonist) to be effective in treating bipolar affective disorder, leading to the hypothesis that GABA played a role in the development of mood disorders. Subsequently, several different BZPs were found to enhance GABA transmission and were effective in relieving social anxiety disorders, while decreases in GABA neurotransmission were associated with some key neurological disorders (anxiety, epilepsy, pain, depression and Huntington's disease) (Clément 1996). Since this, $GABA_A$ receptor dysfunction has been implicated in other CNS pathologies (e.g. sleep disorder, Alzheimer's disease, schizophrenia and muscle spasm) (Frolund *et al.* 2002). There is clinical evidence of reduced GABA function and stress pathologies in patients suffering with depression. Depressed patients had significantly lower GABA levels in cerebrospinal fluid

(CSF) and plasma compared with the control group (Petty 1995). In addition, glutamate decarboxylase (GAD), a synthesizing enzyme for GABA, was found to be reduced in patients with depression, further supporting a GABA deficit hypothesis in relation to depression (Kaiya *et al.* 1982).

Exposure to particular stressors and administration of anxiogenic pharmacological treatments have been shown to induce changes in Cl^- conductance and in BZP ligand receptor occupancy indicating changes in the functional properties of $GABA_A$ receptors (Clément 1996). Montpied *et al.* (1993) showed decreases in the GABA receptor $\alpha 1$ mRNA subunits in the hippocampus of rats after swim stress. Using another animal model of depression, Petty and Sherman (1981) demonstrated that injection of a $GABA_A$ receptor antagonist into the hippocampus blocked the GABA receptors to produce learned helplessness in naïve non-stressed rats. Similarly, the behavioural effects were reversed when GABA agonists were administered (Frolund *et al.* 2002). Crestini *et al.* (1999) developed a mouse heterozygous for the $GABA_A$ receptor $\gamma 2$ subunit, that resulted in reduced BZP binding and the number of $\gamma 2$ subunits. The resultant animals exhibited symptoms similar to anxiety disorder patients.

There is very little information on the use of GABA receptor agonists and antagonists and their effect on the expression of fearfulness in livestock as the majority of research has been conducted in humans and rodents. This literature clearly indicates the GABAergic system, particularly $GABA_A$ receptor and GABA/BZP complex plays an important role in anxiety and fearfulness. Therefore, it is plausible that altered functionality of the GABAergic system may account for differences in the expression of temperament in livestock. The need to better understand the neurobiological mechanisms underlying anxiety and fearfulness may lead to the development of models and/or methods that generate a better quantification of animal temperament. For example, could the variation in $GABA_A$ receptor population or the genes that regulate this, underlie vulnerability to anxiety, resulting in large individual variation in the way that animals cope with different stressors, expressed as differences in temperament? The significant evidence implicates the GABA receptor, particularly $GABA_A$ as a underlying target for the expression of fearfulness and a partial deficit in GABAergic transmission in humans and animals may result in vulnerability to stress and anxiety (Crestini *et al.* 1999). However, it is unlikely that the GABA pathway is the only one involved in fear, anxiety and stress and it may play a major role in conjunction with other neurotransmitter pathways.

1.3.2 Serotonin (5-HT)

The original hypothesis regarding the involvement of serotonin in anxiety, fearfulness and stress was developed from studies indicating that the inhibition of 5-HT release produced anxiolytic effects (Tye *et al.* 1979). Since then 5-HT dysfunction which includes increased/decreased synthesis and/or release of 5-HT, has been shown in human and animal clinical studies, to account for panic disorders, generalized anxiety disorders and unconditioned fear responses (Graeff 2002).

The serotonin system consists of the 5-HT neurotransmitter and clusters of neurons, whose cell bodies are found primarily in the raphe nuclei of the brainstem and innervate an extensive area throughout the CNS, particularly the cerebral cortex, limbic system, basal ganglia and regions of the brainstem. The 5-HT system is made up of several pathways, with two particularly prominent systems: 1) the medial raphe nuclei (MRN) and the 2) dorsal raphe nuclei (DRN) (Aghajanian 2004; Hanley and Van De Kar 2003; Sandford *et al.* 2000). Other 5-HT systems are found in the peripheral nervous system (PNS), the gut, the cardiovascular system and in blood platelets (Pauwels 2003). In addition to other functions in the body, 5-HT acts as a neurotransmitter in the brain, aiding in the transmission of nerve impulses between synapses (Sternbach 1991) and mediating a variety of normal physiological functions (e.g. sleep, cognition, sensory perception, sexual behaviour, hormone secretion and appetite) by interacting with multiple receptors.

The diversity of receptors and transduction pathways of the 5-HT system leads to a wide array of actions. Dysfunction in these pathways has been linked to the etiology of disorders associated with aggression, sleep, appetite, pain, migraine and emesis (Sternbach 1991) as well as playing a major role in psychiatric disorders, such as depression, social phobias, schizophrenia and obsessive-compulsive disorders and panic disorders (Aghajanian 2004; Knight *et al.* 2004). Most importantly in the context of this review, the serotonin system has a major influence on the expression of fear and anxiety (Sternbach 1991).

1.3.2.1 5-HT receptors

The classification and nomenclature of 5-HT receptors has undergone a large review in the past 10 years and is currently organized according to the International Union of Pharmacology Subcommittee for the Classification and Nomenclature of Serotonin Receptors (IUPHAR)

(Barnard *et al.* 1998). Currently, 13 distinct human subtypes of 5-HT receptors are recognized based on structural and functional characteristics as shown in Table 1.2. A fourteenth subtype (5-HT_{5B}) has also been identified, but only in rodents (Hoyer and Martin 1997; Verheggen *et al.* 2004). Of the seven 5-HT receptor families, all are 7-transmembrane peptides coupled to G-proteins, except for the 5-HT_3 receptor, which is a ligand-gated ion channel for Na^+ and K^+ (Hanley and Van De Kar 2003). Serotonin receptors can also be classified by their localization within the body, depending on whether they are located on the target neuron or on the 5-HT neuron, also called auto-receptors (of which there are two classes, somatodendritic (5-HT_{1A}) and pre-synaptic ($5\text{-HT}_{1B/1D}$)) (Hanley and Van De Kar 2003).

Table 1.2: Distribution and function or known effect of the 5-HT receptor families

Receptor	Distribution	Function or known effect
5-HT_1 receptor family (5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5ht_{1E}/ 5-ht_{1F})		
5-HT_{1A}	CNS, brain stem and GI tract	Suggested role in regulation of ACTH
5-HT_{1B}	CNS - Basal ganglia, striatum and frontal cortex	Controls release of acetylcholine, glutamate, dopamine, noradrenaline and GABA
5-HT_{1D}	Low expression in the brain, substantia nigra and basal ganglia. Vascular smooth muscle	Suggested to be a therapeutic target for migraine/headaches
5-HT_{1E}	CNS	Unknown
5-HT_{1F}	CNS	Unknown
5-HT_2 receptor family (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C})		
5-HT_{2A}	Wide distribution in CNS/PNS. Prefrontal cortex, claustrum and basal ganglia. Vascular smooth muscle, platelets, GI tract, lungs	Causes head shaking in rats and mediates effects of hallucinogens in humans. Stimulates ACTH, corticosterone, oxytocin, renin and prolactin secretion
5-HT_{2B}	Amygdala, septum, hypothalamus and cerebellum. Stomach, vascular smooth muscle	Stimulation causes anxiolysis, hyperphagia and reduced grooming in rats
5-HT_{2C}	Cerebral cortex, hippocampus, amygdala, hypothalamus, striatum and substantia nigra	Inhibitory action on dopaminergic and adrenergic transmission and has role in neuroendocrine function
5-HT_3 receptor		
5-HT_3	High density – area postrema, nucleus tractus solitarius, substantia gelatinosa and nuclei of lower brainstem. Low density - higher brain areas (cortex, hippocampus, amygdala), gastrointestinal tract, peripheral neurons	Species variation. Dopamine release on activation. Effects similar to anti-psychotics/anxiolytics, therefore involvement in schizophrenia and anxiety pathologies is suggested
5-HT_4 receptor		

5-HT$_4$	Striatum, basal ganglia and nucleus accumbens, found on GABAergic, cholinergic interneuones and on GABAergic projections to the substantia nigra, gastrointestinal tract, heart, bladder, adrenal gland	Gut motility, to help mediate secretory responses in intestinal mucosa
5-HT$_5$ receptor family (5-HT$_{5A}$ and 5-HT$_{5B}$)		
5-HT$_{5A}$	CNS	Unknown
5-HT$_{5B}$	CNS	Unknown
5-ht$_6$ receptor		
5-ht$_6$	Striatum, amygdala, nucleus accumbens, hippocampus, cortex and olfactory tubercle	Suggested involvement in psychiatric disorders
5-HT$_7$ receptor		
5-HT$_7$	Hypothalamus, thalamus, brainstem and hippocampus. Cardiovascular, gastrointestinal tract and non-vascular smooth muscle	Acute stress shown to regulate mRNA expression of this receptor

5-HT$_1$ receptor family (5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$, 5ht$_{1E}$/ 5-ht$_{1F}$)

Currently three 5-HT$_1$ receptor subtypes are recognized, 5-HT$_{1A}$, 5-HT$_{1B}$, 5-HT$_{1D}$ with the additional two being endogenous receptors, with no currently known physiological role (5-ht$_{1E}$ and 5-ht$_{1F}$) (Pauwels 2003). Activation of the 5-HT$_{1A}$ receptors by 5-HT results in reduction of the firing rate of the serotonergic neurons to suppress 5-HT synthesis, turnover and release into targeted areas (Toth 2003). The receptors are widely distributed in the CNS, particularly the hippocampus, septum and amygdala (Toth 2003; Aghajanian 2004) and this distribution is particularly important as these are areas predominantly associated with the control of mood. The majority of the 5-HT literature focuses on this particular receptor, 5-HT$_{1A}$, due to its involvement in anxiety disorders (Overstreet *et al.* 2003; File *et al.* 1996; Gross *et al.* 2003; Groenink *et al.* 2003). For example, 5-HT$_{1A}$ receptor knockout mice demonstrate increased anxiety in several behavioural paradigms designed to assess anxiety (Heisler *et al.* 1998). Additionally, 5-HT$_{1A}$ receptor agonists (e.g. Buspirone) are used regularly in the treatment of anxiety and depressive disorders (Tunnicliff 1991), which are found to be highly effective (Stahl 1997). Furthermore, studies with rodents have also shown 5-HT$_{1A}$ agonists to induce anxiolytic-like states and antidepressant-like effects (De Vry 1995).

The effects of specific drugs acting on the 5-HT$_{1B}$ receptor have also been assessed as this receptor has also been implicated in the modulation of anxiety, particularly 5-HT$_{1B}$ autoreceptors (Benjamin *et al.* 1990). It has been demonstrated that *m*-chlorophenylpiperazine (*m*-CPP), a 5-HT$_{1B}$ agonist, can produce anxiogenic effects in humans (Charney 2002; Pellow

et al. 1983). Further evidence of involvement of this receptor in anxiety was put forward with the development of the 5-HT_{1B} receptor knockout mice, which were found to be highly aggressive and displayed decreased anxiety in several behavioural anxiety paradigms, including the open field test and elevated plus maze (Mayorga *et al.* 2001; Zhuang *et al.* 1999).

5-HT_2 receptor family (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C})

The 5-HT_2 receptor family consists of three subtypes, showing distinct localisation in the brain. They are all single protein molecules that link with the Gq family of binding proteins that when activated stimulate phospholipase C to induce a rise in intracellular free calcium (Porter *et al.* 1990). From reviews on the therapeutic role of the 5-HT_2 receptor family, it is evident they may be involved in pathological and psychopathological conditions, (Millan 2002; Xu and Pandey 2000), as well as in normal physiological functioning of the body (Bohus *et al.* 1990; Carli and Samanin 1988; Heisler *et al.* 1998).

In this family, the 5-HT_{2A} and 5-HT_{2C} receptors have been targeted for their role in modulating anxiety states due to their localisation within the brain (Millan and Brocco, 2003). For example, 5-HT_{2C} receptor antagonists are shown to elicit anxiolytic responses in rodents when placed within the Vogel conflict test (Griebel *et al.* 1997). Additionally, knockout mice lacking the 5-HT_{2C} receptor also display reduced anxiety (Heisler *et al.* 1998). Both anxiogenic and anxiolytic responses have been observed following pharmacological targeting of the 5-HT_{2A} receptors. Research conducted by Graeff *et al.* (1996) showed that administration of 5-HT_{2A} receptor antagonists elicited anxiolytic effects in rodents. In contrast, anxiogenic effects have also been reported from administration of 5-HT_{2A} receptor antagonists, specifically in models which reflect untrained behaviours of animals (Setem *et al.* 1999). Stressors, such as restraint and chronic social stress increase the number of 5-HT_2 binding sites in the cortex (McKittrick *et al.* 1995) and an animals receptor sensitivity to 5-HT_2 agonists (Gorzalka *et al.* 1998). Hawkins *et al.* (2002) suggests that these results are consistent with the concept that both acute and chronic stressors decrease 5-HT_2 receptor activity causing receptor up-regulation and sensitivity.

1.3.2.2 5-HT and fearfulness

There is good evidence that implicates 5-HT in the regulation of the HPA stress response, such as stimulation of ACTH by 5-HT precursors and 5-HT ligands causing increases in cortisol and ACTH (Dinan 1996). Serotonin has also been found to directly stimulate the HPA axis (Dinan 1996) causing a release of CRH, ACTH and glucocorticoids (Feldman *et al.* 1987). Additionally, administration of L-5-hydroxytryptophan (5-HTP) (a 5-HT precursor) can increase plasma corticosterone levels in the rat (Gemma *et al.* 2003). Additionally, the response to a stressor on the serotonergic system can vary dependent on the type, intensity and duration of the stressor (Filipenko *et al.* 2002). Given this evidence, it is generally accepted that the serotonergic system is involved in the regulation of fear and anxiety.

The relationship between 5-HT_{1A} receptor function and anxiety was initially demonstrated by Goldberg and Finnerty (1979) who observed that Buspirone (an anxiolytic) was a partial 5-HT_{1A} receptor agonist and could be used successfully to treat anxiety disorders and psychotic patients. Buspirone has since been shown to reduce 5-HT activity in the raphe nuclei, 5-HT release and turnover and 5-HT synthesis (Blier and Ward 2003) resulting in anxiolytic effects (Tunnicliff 1991). Further evidence cited below indicates that dysfunction of the serotonergic system is prominent in the aetiology of anxiety and depression and plays a significant role in psychiatric disorders, especially when provoked or aggravated by stress.

Evidence for the involvement of 5-HT dysfunction in stress-related disorders emerges from research showing decreased activity of central 5-HT pathways was associated with a number of stress-related psychiatric disorders (e.g. anxiety, depression (Bhagwagar *et al.* 2002; Mann *et al.* 1996), aggression (Miczek *et al.* 1995) and alcoholism (Weijers *et al.* 2001)). The development of knockout mice has allowed more targeted investigation of the behavioural effects and drug responses in animals with a genetic deletion of particular 5-HT receptors. For example, the 5-HT_{1A} receptor knockout mice were found to respond with increased anxiogenic behaviours in a number of behavioural paradigms. The mice spent less time in the open arms of the elevated plus maze (EPM) and elevated zero maze (Pellow *et al.* 1985). Mice spent less time in the center of the open field test and more time spent in that area reflects decreased anxiety (Bhatnagar *et al.* 2004) and less time exploring a novel object in the novelty test (Parks *et al.* 1998) (Heisler *et al.* 1998). Findings by Sarrias (1991) illustrated a low incidence of free tryptophan and 5-HT in the plasma of human patients diagnosed with depression as

compared to normal control patients. Arab horses, which are well known to have an anxious or nervous temperament (Hausberger *et al.* 2004) were found to be more active, vocalize more and have lower plasma 5-HT concentrations in comparison to the less reactive standard bred horses (Bagshaw *et al.* 1994).

The relationship between serotonin activity and fear-related behaviour in production animals was investigated in mink (*Mustela lutreola*) that had been selected for fearful and confident behaviours, with the aim of selecting for less fearful animals to improve the welfare of the farmed mink (Malmkvist *et al.* 2003). Mink were selected on their behavioural response to approach of a handler, during handling (Trapezov's hand test) and on the mink's willingness to approach a novel object. It was hypothesized that animals with a more fearful temperament, based on these behavioural responses, would have more frequent and severe activation of the HPA axis, compared with the confident mink. Mink from the fearful line were then found to have markedly higher plasma 5-HT, although the 5-HT precursor plasma levels were not different between the two lines. This measure was then suggested to be a good indicator of animals with an anxious temperament, as the fearful mink had approximately a 4-fold higher concentration of plasma 5-HT than the confident mink line. Additionally, in an earlier study on the same lines of animals, investigations focused on chronic treatment with selection serotonin reuptake inhibitors (SSRI's), thereby increasing extracellular levels of 5-HT (Malmkvist and Hansen 2002). This resulted in an increase in the approach behaviour of the confident mink towards humans, indicating a marked reduction in fearfulness towards humans and a divergence in the selection lines. This evidence shows that the increase in 5-HT was responsible for the change in the animals inherent fearfulness (i.e. temperament), resulting in animals which exhibited reduced fearfulness.

One particularly salient study initially started by Belyaev *et al.* (1959) and subsequently continued by (Trut 1999) investigated the long term breeding of Silver foxes (*Vulpes vulpes*) for domestication. The primary trait for which the researchers selected for was lack of fear of humans. Behaviours were selected based on the animal's response to a series of fear paradigms, ranging from the approach of a human, tendency for the animals to allow hand feeding and petting and whether the animals actively sought human contact. After approximately 40 generations, very docile foxes were produced. Changes from the wild type were observed in physiology, behaviour and morphology. For example, domestic dogs exhibit

general fear responses at the age of 12 weeks, whilst wild foxes show the same response at 6 weeks, conversely the domesticated foxes in this experiment exhibited fear responses at 9 weeks compared with the wild type foxes, indicating delayed fear responses, which is related to the period of time where pups bond with their mothers. The domesticated foxes also showed reduced fear of humans, to the point of seeking out human attention. Physiological changes from the wild type included decreases in the level of corticosteroids, differential timing of hormonal development and a significant increase in 5-HT levels. Moreover, morphological changes were observed in the loss of pigmentation of the fox's coat, reductions in the size of the skull, feminization of male skulls and a change from upright ears to floppy ears.

An additional view of the 5-HT system and its role in the expression of fearfulness has been postulated by Deakin and Graeff (1991) called the dual 5-HT fear hypothesis. They suggest that 5-HT plays a dual role depending on the systems activated. Specifically, the ascending DRN pathway innervates the amygdala and frontal cortex to facilitate learned defensive behaviours, which would relate to potential threats or danger, whilst the DRN-periventricular pathway would inhibit the fight or flight response to immediate threats or danger. Therefore, the two pathways exert opposed actions on the neural mechanisms that underlie the expression of fearfulness (Graeff *et al.* 1997). Zhuang *et al.* (1999) reviewed the relevant pharmacological literature regarding the 5-HT system and suggested that the different types of responses could be attributed to the locality of the 5-HT receptors in the brain having an influence on the behavioural responses. For example, activation of the 5-HT_{1A} autoreceptor may decrease anxiety, whilst activation of the postsynaptic receptor may increase anxiety.

Further evidence to support a neurophysiological basis for the expression of fearfulness and therefore temperament is based on molecular genetics, receptor binding and pharmacological challenge studies in both rodent and human models. Research on certain candidate genes, such as the serotonin transporter, GABA, 5-HT and dopamine receptors (Bond 2001) has been particularly enlightening. Flint *et al.* (1995) mapped the location of three loci on chromosomes from mice that were found to influence the emotional reactivity of the mice when they were exposed to the EPM anxiety model. Neuroendocrine challenges also contribute to discrimination of behavioural responses to a particular ligand/treatment, which can be indicative of a particular neurotransmitter function. Other molecular tools, such as

quantitative trait loci (QTL) mapping allow the investigation of the molecular bases of psychobiological traits, such as those of emotional reactivity in mice (Turri *et al.* 1999; Castanon and Mormède 1994). Fisher *et al.* (2006) identified quantitative trait loci (QTL) linked with stress and learning response stress in Merino sheep exposed to isolation, novelty, shearing and a maze test, suggesting a genetic basis for some stress and learning responses in sheep. The advantage of using these genetic tools is that it allows more targeted and selective direction of receptors than acute pharmacological models, but in combination is more advantageous.

There is good evidence to confirm an association between the 5-HT system and fear and anxiety, but the strength of the association can vary. Benzodiazepine anxiolytics generally alleviate anxiety partly by decreasing 5-HT release. However, antidepressant drugs are mostly beneficial in the majority of anxiety disorders with chronic administration, which is likely to enhance 5-HT transmission (Blier and Ward 2003). Some studies also report dose-dependent effects, where changes in the serotonergic activity induce anxiolytic and anxiogenic-like effects in reward-punishment paradigms. In addition, anxiogenic and anxiolytic-like effects after administration of agonists and antagonists to rodents have been variable dependent on their hormonal state. This was shown by Díaz-Véliz *et al.* (1997) with administration of ketanserin (a 5-HT antagonist) administered to rats. Behaviours were observed and recorded in response to two anxiety paradigms (EPM and retention of a passive avoidance response) which found the results to differ dependent on the hormonal status of the rat. Female rats responded with anxiogenic behaviours, except for those in diestrus, which showed anxiolytic behaviours, whilst male rats showed no changes in behaviour at all. Lu and Bethea (2002) showed that sex differences could explain some of the variability in responses, as estradiol and progesterone levels in macaques decreased 5-HT_{1A} autoreceptor mRNA levels, thereby increasing 5-HT transmission. Dominguez *et al.* (2003) found sex differences in serotonergic activity using the EPM test in rats. Alternately this difference could also be attributed to reflect a greater susceptibility of the male rats to anxiogenic conditions, which is commonly seen in humans diagnosed with generalized anxiety disorder. However, Shors and Miesegaes (2002) found that performance of female rats was impaired when exposed to an acute stressor, whilst the opposite occurred in male rats when exposed to the same behavioural paradigm. One methodological issue that may contribute to the equivocal results is the duration of the treatment. 5-HT_{1A} anxiolytics and SSRI's are clinically effective only when administered

chronically, such as Buspirone's therapeutic reduction of fear, which is only evident after weeks of treatment (Malmkvist *et al.* 2003).

Although complex emotional states cannot be reduced to imbalances of a single neurotransmitter, it is clear that 5-HT plays a prominent role in the variability in fearfulness and in the aetiology of neurological disorders. The serotonergic system and the HPA axis are closely intertwined and 5-HT's innervation of the CNS helps to regulate the HPA axis during normal and stressful periods. Various stressors affect the serotonin system (5-HT levels, neurotransmission, synthesis and degradation); therefore an improved understanding of this system may help to explain the variability between animals in their response to fear eliciting challenges.

1.4 Combination between behavioural genetic tools and pharmacologic models

Within this thesis, a large part of the experimental protocol was conducted on sheep selected for divergent responses in temperament, which were originally based on differential expression of ambulation in the arena test and an agitation test score when socially isolated from conspecifics {Murphy 1999 #1390}. There appears to have been little research investigating links between particular neurophysiological pathways, behavioural responses and genetic background, particular in regards to livestock temperament. The ability to select animals based on behavioural traits that are associated with temperament opens up a large area of research, and allows us to investigate what underpins some of these behaviours, genetically and behaviourally. This procedure is already well documented in the literature and is as variable as selection for tonic immobility, defecation rate, increased or decreased sociability, maternal behaviours or vocalisation rate {O'Connor, Jay, et al. 1985 #1410}{Gallup 1979 #10650}{Hall 1938 #12550}{Lindzey, Winstor, et al. 1965 #13360}. Additionally, this is the premise behind the domestication process {Belyaev 1979 #10530}. However, the area to combine selection of specific traits in an animal, thereby resulting in behavioural changes, also allows us to explore physiologically and neurophysiologically where those changes occur between the selected animals. As mentioned previously there are a large number of tests that investigate temperament in livestock, but what they actually measure can sometimes be called into question, however many different tests are valid and do consistently measure the expected behaviour. Additionally, up to quite recently we were only able to study animal responses to

challenges by exposing them to the challenge and then observing and analyzing the subsequent behaviours.

Additionally, the use of pharmacological methods, such as administration of drug treatments that manipulate the internal milieu also occur in many different experimental protocols, with both positive and negative results in regards to measuring different aspects of temperament.

New animal models are being developed all the time to focus on particular behaviours or traits of temperament as well. As early as 1975, animal models were selected on their behavioural responses in an attempt to understand what underlies temperament, which resulted in the Maudsley reactive and non-reactive rat strains {Broadhurst 1975 #13460}. Therefore, with the consistent development of furthering of these three different methods it seems appropriate to try and combine the different tools, to help gain a better understanding of the neurobiological pathways of fearful and anxious states and an animal's individual response to differing situations, and therefore a background into what underpins temperament in livestock.

1.5 Summary

Little is known about the neurophysiological regulation of the expression of temperament and an improved understanding would be advantageous in regards in assessing how animals can cope and adapt to the changing demands of production environments. The ability to select for animals that are better able to cope with changes has multiple production benefits, but most importantly, it has ramifications for the animal itself. Additionally, how animals respond to fear is of priority as this can also have major effects on production and welfare. There appears to have been little research investigating links between particular neurophysiological pathways and behavioural responses, particular in regards to livestock temperament. Therefore investigating the GABA, 5-HT and how they may contribute to temperament differences in livestock may provide some new insights.

This research was undertaken in an attempt to better elucidate the neurophysiological regulation of temperament. The program of research was undertaken in stages; initially dose response studies for specific neurotransmitters (GABA and 5-HT) were conducted to determine neurotransmitter action and pathways (Chapter 3). Secondly, a specific fear challenge model for sheep was developed that facilitated a fear-potentiated response (Chapter

4). Thirdly, specific GABA and 5-HT receptor agonists (identified in the dose response studies) were administered to sheep from divergent temperament selection lines to examine their effects on fear potentiated challenges (Chapter 5). Finally, investigation of the role of cortisol during the HPA response, particularly regarding the negative feedback mechanism as an influence on temperament differences, was also conducted (Chapter 6). These results should improve our understanding of the mechanisms underpinning individual differences in temperament which in turn may allow us to; 1) categorise certain animals better suited to particular environments, 2) increase the fundamental understanding of the stress physiology in livestock, 3) develop new insights into the coping strategies livestock use when responding to stressors and 4) help to improve livestock welfare.

Chapter 2 General, materials and methods

2.1 Animals

The Merino ewes and wethers used in the experiments described in chapters 3 and 4 were sourced from the 'Chiswick' research station flock, managed by CSIRO Livestock Industries, Armidale, NSW. In the experiments described in chapter 5, the Merino ewes and wethers used were sourced from the 'Allandale' temperament selection lines, a resource managed by the School of Animal Biology, University of Western Australia, WA. The latter flock comprised two selection lines (calm and nervous) selected specifically for temperament and based on two temperament tests, the open field test and the isolation box test (IBT). The temperament flock originated from progeny of commercial Merino ewes of mixed ages that had been joined with commercial Merino rams from the Australian Merino Society strain. Lambs were subsequently maintained as one flock. The original flock was selected between 1990 and 1996, where ewes and rams were selected on an index of behavioural responses from the two temperament tests to establish the two lines (calm and nervous). Two hundred and sixty three ewes were tested as weaners to develop the divergent behavioural selection lines and twenty rams were selected, 10 top ranked (calm) and 10 low ranked (nervous) rams. Temperament tests were conducted yearly, two weeks after weaning. Following testing the entire weaner ewe flock were run together and the weaner ram flock were maintained together (Murphy 1999).

2.2 Behavioural tests

Restraint and isolation from conspecifics can elicit fear in many animals, including sheep (Cockram *et al.* 1994; Vandenheede *et al.* 1998), cattle (Hopster and Blokhuis 1994), rodents (Serra *et al.* 2004; Wongwitdecha and Marsden 1996) and poultry (Strobel *et al.* 2003). The behavioural response to such challenges can then be used as an indicator of fearfulness, or as defined here, temperament. The IBT (isolation box test) used in the following experiments was similar to that used by Gridnard *et al.* (2000) and Cockram *et al.* (1994) for cattle and sheep, respectively. The challenge invokes a fear response as the animal is isolated from its flockmates within a novel context for a standard one minute interval. The recorded agitation score reflects the animal's fearfulness to the situation, but also its ability to adapt to the challenge. In addition, an arena test was used in chapter 3 to assess behavioural responses.

2.2.1 Isolation box test (IBT)

The isolation box (1.5 m^3) was constructed of solid plywood sides with a slatted wooden floor. During testing the box was placed on four rubber car tires. A specialised electronic counter (agitometer (Murphy *et al.* 1994)) was attached to the side of the box and recorded the amount of noise and movement within, or degree of agitation. The box was fully enclosed, except for the top, which was covered with shade cloth (Figure 2.1).

Figure 2.1 Isolation box test with animal exiting box

The standard test consisted of isolating the animal for 1 minute within the box. A calibration unit for the boxes was designed (Blache and Ferguson, 2006 *In preparation*), as the acoustic properties of each box, as well as conditions where the boxes were assembled, varied. Therefore, calibration of each box was designed to reduce extraneous variation or bias between boxes and sites to ensure measurement consistency. In previous studies, the IBT was found to be highly repeatable (Blache and Ferguson, 2006 *In preparation*) and reliable as a measure of temperament in sheep (Murphy *et al.* 1994).

An alternate interpretation of the results from the IBT is that it is measuring sociability of the animals rather than fear. The same interpretation is possible in terms of the selection of the calm vs. nervous animals. However, within this thesis we have assumed that the measurement of the IBT is related to the fear axis and this is discussed within the thesis.

2.2.2 Arena test

The original arena test was developed by Fell and Shutt (1989) as a means to evaluate how repelled sheep were to humans after mulesing surgery. Since then, the test has been adapted

for use in quantifying livestock behaviour to various stressors, including assessment of parasite load in sheep (Fell *et al.* 1991) and ewe maternal traits (Kilgour and Szantar-Coddington 1997). The arena test involves a motivational choice for the sheep, (Kilgour 1998) by creating a conflict situation between the social attraction of the test animal to its flockmates at the end of the arena, versus the fear of the observer stationed in front of the pen of flockmates.

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

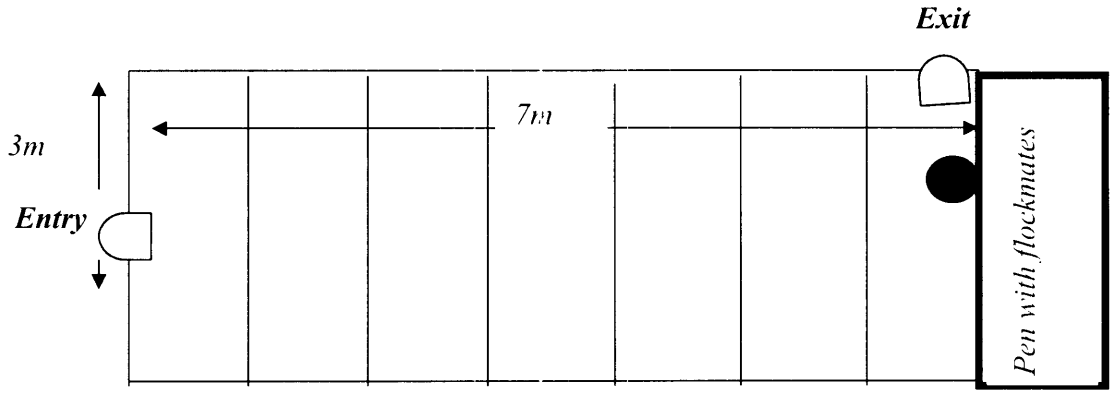

Figure 2.2: The arena test layout (black circle marks observer)

2.2.2.1 Behavioural measures

A number of behaviours were recorded during testing these included:

- Vocalisation (Vocs): number of bleats
- Zones crossed (ZX): the number of zones the animal entered
- Zone in which most time spent (ZT): the zone in which an animal spent the most time
- Closest approach (CLOSE): the zone in which the animal got closest to the observer/flockmates

- Total time in marked zone (TIME): the total time spent in each zone multiplied by the number of the zone. The higher the number, the more time the animal spent further away from the observer. This indicates a measure of the time spent in each zone and proximity to zone 1 (where observer was situated).

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

2.3 Physiological measurements

Physiological measurements taken before, during and subsequent to the fear-eliciting challenges included heart rate and blood cortisol concentration.

2.3.1 Heart rate (HR)

Polar heart rate monitors (Polar Electro Oy, Finland, www.polarusa.com) were used to measure HR. An electrode transmitter detects the HR of the animal and transmits to the receiver, which displays HR during the monitoring period. The transmitter and electrodes were attached to an adjustable fabric girth belt, which was easily fitted to each animal regardless of size (Figures 2.4 a and b). The electrodes were placed on the animal on two areas: the left dorsal side below the scapula and below the right axilla. Each site was prepared by clipping and shaving the wool and applying electrode gel (Elefix, Nihon Kohden Cooperation, Shinju-ku, Tokyo).

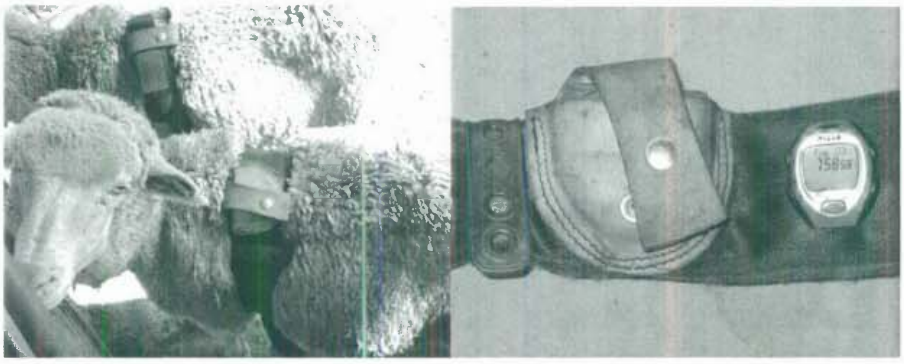

Figure 2.2 a) Merino wether fitted with heart rate monitor and belt and b) pouch and polar monitor on belt

Heart rate data was initially filtered for spurious data (>240 bpm, which accrues when electrodes lose contact with the skin) and separated into specific time intervals relevant to the commencement of the challenge treatments. These intervals were taken as eight min periods

prior- to, during- and post- commencement of the challenges (the challenges are discussed in more detail in Chapters 4 and 5) and excluded intervals where the animals were moved, handled or blood sampled.

Several different ways to analyse heart rate responses to stress have been suggested, but in this thesis differences between the mean heart rate has been the key focus. In a study by Tallet *et al.* 2006 {Tallet, Veissier, et al. 2006 #12480} the heart rate mean was used as a measure to assess how handled and non-handled lambs perceived humans. Vincent and Leahy (1997) {Vincent & Leahy 1997 #9190} measured mean HR and variability in dogs during temperament testing and suggested that the variability of HR responses to the stressors was more indicative of mental workload, as opposed to the absolute values. Geverink *et al.*, (2002) {Geverink, Schouten, et al. 2002 #2430} also used mean HR to analyse the differences between pigs in relation to different stressors. Mohr *et al.*, (2002) {Mohr, Langbein, et al. 2002 #370} showed that measurement of a plethora of different HR variability parameters would be better to show stress loads on calves and dairy cows.

2.3.2 Blood sampling

Sheep were manually restrained and blood was collected in jugular venepuncture in vacutainers (Becton Dickinson Pty Limited, 4 Research Park Drive, Macquarie University Research Park, North Ryde, NSW 2113, AU) with a 1.2 x 25 mm, 18_G needle (Becton Dickinson). Vacutainers were held on ice until they were returned to the laboratory. Samples were centrifuged (Beckman G5-6R Centrifuge) at 3500 rpm for 15 min (4°C) and serum was decanted into labelled tubes and held in duplicate at –20 °C until analysis.

2.3.2.1 Cortisol radioimmunoassay (RIA)

A commercial RIA kit (Spectria Cortisol RIA. Orion Diagnostica, Espoo, Finland) adapted and validated for ovine serum was used to determine plasma cortisol concentrations, adapted and validated for ovine plasma. Each kit contained I^{125} cortisol tracer, 100 pre-coated tubes and six cortisol standards (0, 20, 75, 500 and 2000 nmol/L lyophilized cortisol standards in human serum). The mean recovery of added cortisol to ovine plasma was 102% and the sensitivity of the assay was 10nmol/L. The stated cross-reactivities of the anti-cortisol antibody with corticosterone, cortisone, dexamethasone, prednisolone and prednisone were 0.2, < 0.1, < 0.1, 45.3, and 0.3 %, respectively. All reagents, samples and controls were

bought to room temperature. Standards supplied with the kit were prepared by adding 500 µl of phosphate buffered saline (PBS) and mixed. The 20 nmol/L standard was serially diluted to 10 and 5 nmol/L. Controls, standards and samples were gently vortexed before pipetting. Standards, samples and controls (20 µl) were pipetted into the anti-cortisol antibody coated tubes in duplicate. Tracer (500 µl) was added to all tubes and incubated for 2 hours at 37 ^{0}C. The tubes were decanted and washed in 1 ml of distilled water. Samples were left for approximately five minutes on absorbent paper to drain. Each tube was then counted for 1 minute using a gamma counter (1282 Universal GAMMA Counter, PO Box, 20101, Turku, Finland).

Serial blood samples were collected during the challenge period and two parameters were derived: the estimated peak cortisol response and the integrated cortisol response. Following a fear eliciting challenge, the peak cortisol response was estimated to occur between 15 – 25 min based on previous literature (Cook 2002). The AUC was derived using the trapezoidal rule.

It is well known that corticosterone fluctuates with two different rhythms, one being a pulsatile secretory pattern and the other a diurnal rhythmicity, synchronized by light. In general, diurnal species, such as sheep, have peak cortisol levels shortly after wakening in the morning, which decline significantly over the day, in the absence of external influences {Tiefenbacher, Lee, et al. 2003 #6920}{Lauc, Zvonar, et al. 2004 #8690} {Mormède, Andanson, et al. 2007 #13310}. This pattern is well documented in many different species, including sheep {Fulkerson & Tang 1979 #13320}. Therefore there is a possibility that the time of day a blood sample is taken, may affect cortisol levels. However, careful organisation of experimental methods, habituation of the animals to the procedure, as well as sampling the same animal at the same time can help to alleviate this problem. For example, monitoring cortisol over the habituation period can give an indication as to whether levels are very different or similar in nature during the experimental period.