Temperament and activity of the hypothalamo-pituitary-adrenal axis in sheep

Stacey Ellen Rietema
BSc Agriculture (Hons)

This thesis is presented for the degree of Doctor of Philosophy

2015

The School of Animal Biology
Faculty of Science
The University of Western Australia
Temperament is highly variable, even within a species and gender, and a more ‘reactive’ temperament has been associated with greater activity of the hypothalamic-pituitary-adrenal (HPA) axis, a key system in the response to stress, both in the presence and absence of stressors. Although the role of the HPA axis in stress is generally thought of as being protective, an excess of cortisol can be harmful to the individual as it suppresses important processes for longer than necessary to overcome the stressor. Therefore, individuals with a highly responsive temperament may be at a greater risk of compromised health and welfare due to stress, as they may more frequently experience greater concentrations of cortisol both in the absence and presence of stressors.

Work in this thesis tests whether, in sheep with genetically-based difference in temperament, hyper-reactive, or ‘nervous’, animals have a more active and responsive HPA axis than hypo-reactive, or ‘calm’, animals in the absence and presence of stimuli, and if there is a consequent effect on metabolic hormone balance. These sheep have been bred for 2 decades for their divergent behavioural responses to the stressors of isolation and human presence. These animals also have associated differences in their HPA axis responses to stressors, and are known to differ in some parameters of metabolism.

The series of experiments compares the two temperament groups, looking at, in brief, 1) the diurnal rhythm of HPA activity, and its relationship with metabolic hormone patterns; 2) the effect of repeated exposure to acute stressors on HPA axis activity in the presence and absence of stressors; 3) the responsiveness of the
adrenal gland to pituitary stimulation; and 4) the responsiveness of the pituitary-adrenal axis to hypothalamic stimulation.

Firstly, selection for temperament did not affect the diurnal rhythm of secretion of cortisol, or prolactin, a stress responsive hormone. However, ‘calm’ animals had greater concentrations of insulin during the afternoon than ‘nervous’ animals. A similar tendency was seen in the rhythm of leptin secretion. Therefore, selection for temperament did not affect resting activity of the HPA axis, but it did appear to affect insulin patterns, independently of the HPA axis.

Secondly, nervous animals do not seem to be more prone to chronic stress at the level of stressor intensity used in this study. There was no effect of the repeated stressor treatment on cortisol and insulin concentrations in the absence of stressors, or on the behavioural and cortisol response to isolation, suggesting that our stressor model was insufficient to induce chronic stress. There were no differences between the temperament groups in cortisol or insulin concentrations in the absence of stressors, but ‘nervous’ animals showed a longer cortisol response and greater behavioural response to the acute stressor than ‘calm’ animals, indicating that temperament-based differences in the HPA axis may only be apparent during a stressful stimulus.

Finally, we showed that temperament did not affect the responsiveness of the pituitary-adrenal axis to stimulation with hypothalamic peptides, or of the adrenal glands to pituitary stimulation. Therefore, it is clear that ‘nervous’ animals have a greater HPA axis response to acute stressors than calm animals, but this is not due to changes in the responsiveness of the pituitary-adrenal axis. Furthermore, selection for temperament has not affected the resting activity of the HPA axis. Instead, the difference in the HPA axis response to stressors between the temperaments might lay in the neural circuits that process perception of the
stressor and connect it to the activation of hypothalamic peptide secretion. Additionally, there is some evidence that temperament is associated with differences in concentrations of the metabolic hormone, insulin, but such effects are independent of the HPA axis, and require further study. This thesis furthers the understanding of the link between temperament and stress physiology, which can help promote health and welfare.
Declaration

The work presented in this thesis is, to the best of the candidate’s knowledge and belief, original and is the candidate’s own work, except as acknowledged in the text. The material has not been submitted, either in whole or in part, for a degree at this or another university.

On the 1st of June 2015 a version of Chapter 3 has been accepted for publication by the journal Domestic Animal Endocrinology as Rietema et al (2015) “Twenty four-hour profiles of metabolic and stress hormones in sheep selected for a calm or nervous temperament”, doi:10.1016/j.domaniend.2015.05.005. This paper was prepared for inclusion in this thesis, with contributions from Dominique Blache, Shane Maloney, Graeme Martin, Margaret Blackberry, and Penny Hawken. The accepted version has been included as an appendix to this thesis.

Signed

Stacey Rietema

Dominique Blache

10th June 2015
Acknowledgements

I wish to acknowledge those who have offered help, guidance, assistance, and friendship throughout my PhD.

To my supervisors, Dominique Blache, Graeme Martin, and Penny Hawken, I offer my biggest thanks for sharing your expertise and offering your time. I really value all of your support and advice. Special thanks go to Dominique, who came on board a few months into my candidature on the condition that he play a minor role, and ended up as coordinating supervisor. It was never my intention to trick you.

There are many people from Animal Science I must thank – for their help, as well as for all the Friday cakes. A big thanks go to Margaret Blackberry for your help and patience with the radioimmunoassays. Thank you to Zoey Durmic for being my advisory panel. Thanks to many people for helping me with my experimental work – Kirrin, Kelsie, Mikaela, Jo, Trina, Chelsea, Travis, Joy, Steph, Sam, and Cesar. Special mention to Megan and Menze.

To my Mum and Dad – thank you for everything over the years.

And thank you to John, for always being willing to making everything better, and always doing just that.
O Lord my God, when I in awesome wonder
Consider all the works Thy hands have made,
Then sings my soul, my Saviour God, to Thee:
   How great Thou art! How great Thou art!
Contents

Summary ................................................................. ii

Declaration ............................................................... v

Acknowledgements .................................................... vi

Chapter 1. ................................................................. 2

Chapter 2. ................................................................. 5

Chapter 3. ................................................................. 42

Chapter 4. ................................................................. 67

Chapter 5. ................................................................. 88

Chapter 6. ................................................................. 105

Chapter 7. ................................................................. 127

References ............................................................... 140

Appendix ................................................................. 189
Chapter 1.

General introduction

The ability to cope with and respond appropriately to stressors is important for an animal if it is to overcome and survive challenges whilst minimising harm and expenditure of resources (Moberg, 2000). However, the ability to cope with stressors varies greatly between individuals, partly due to their temperament (Boissy, 1995; Pottinger, 2000), an innate characteristic that affects how an individual perceives and responds to situations that is usually assessed by measuring behavioural reactivity to given stressors (Boissy, 1995; Reale et al., 2007). Some individuals have a highly responsive temperament that has been associated with greater activity of the hypothalamic-pituitary-adrenal (HPA) axis, in both the presence and absence of stressors (Windle et al., 1998a; Pottinger, 2000; Capitanio et al., 2004). The HPA axis is a key system in the physiological response to stress and, although its role is protective, excess HPA activity can be damaging because it influences processes such as metabolism, immunity and reproduction (Matteri et al., 2000). Therefore, if an individual can better cope with stress by virtue of their temperament, it may have advantages for general health and welfare.

Although temperament seems to affect HPA activity, it is not fully understood whether this relationship is mediated by the stimulatory and inhibitory mechanisms within the HPA axis itself, by the brain pathways involved in the perception of stressors, or by an interaction between these brain pathways and the HPA axis. Our understanding of the links between temperament and HPA axis
activity have been investigated with animal models of temperament, mainly using laboratory rodents (Pottinger, 2000). These models involve selective breeding based upon behavioural responses to selected stressors relevant to the species, thereby generating a model of fear-related temperament with a genetic basis, with minimised influence of other factors, such as early life history, recent experience, or diet (Pottinger, 2000).

The main focus of studies with the genetically derived models has been on the response of the HPA axis to acute stressors, with some work focussing on the responsiveness of the pituitary and/or adrenal glands to their stimulatory and inhibitory signals. However, there is little work on the effect of temperament on the HPA activity at rest, particularly over more than a few hours, or on HPA activity during chronic stress. Furthermore, the relationship between temperament and HPA activity has not been fully characterised within the same model – for example, description of HPA activity at rest, in response to acute and chronic stressors, plus the responsiveness of each organ of the axis to its stimulatory and inhibitory signals.

This work described in this thesis thus investigates the effects of temperament on HPA activity using the ‘UWA Temperament Flock’, a sheep model of fear-related temperament. This flock has been selected on the behavioural response of the sheep to isolation and human presence, resulting in two groups – a hypo-responsive line, labelled ‘calm’, and a hyper-responsive line, labelled ‘nervous’ (Murphy et al., 1994). In addition to offering a non-rodent perspective, thereby increasing insight into species variation in the relationship between temperament and HPA activity, this model furthers our understanding of a species that is relevant to industry.

The primary aim of the research in this thesis was to test whether selection for fear-related temperament in Merino sheep has affected the activity of the HPA axis, both at rest and during stimulation with psychosocial stressors or exogenous
hormones. A secondary aim was to test whether the relationship between fear-related temperament and HPA activity affects other biological systems that are known to be responsive to stress, in particular metabolic homeostasis (insulin, leptin and prolactin). The general hypothesis was that, compared to calm sheep, nervous sheep would show greater activity within the HPA axis, both at rest and in response to stimulation with external stressors and exogenous hormones. Furthermore, it was hypothesised that greater HPA activity in nervous animals would lead to increased secretion of leptin and prolactin, but decreased concentrations of insulin.
Chapter 2.

Review of the literature

Introduction and scope of the review

Stress is a concept used broadly by scientists and lay people alike to refer to the response to the threats and challenges experienced in life. Moberg’s (2000) definition of stress – “the biological response elicited when an individual perceives a threat to its homeostasis” – captures the variety of forms that stress can take, from physical to physiological, real or merely perceived. Because stress has the potential to be harmful, it is often viewed negatively, but, because it is unavoidable, the best an individual can do is to cope with stressor and minimise its impact (Moberg, 2000; Tilbrook et al., 2000).

However, there is large variety, even within a species and gender, in the ability of an individual to respond to and cope with stress (Boissy, 1995; Pottinger, 2000). This ability is affected by temperament, an innate part of an individual that can be thought of as a filter through which each individual assesses situations, and therefore affects the response to the situation (Boissy, 1995; Reale et al., 2007). Temperament has a genetic component, and so some individuals are genetically more or less susceptible to becoming stressed (Wigger et al., 2001; Bickell et al., 2009). The heritability of temperament traits offers the potential to utilise temperament in practical and meaningful ways. For instance, breeding for a less responsive temperament may improve animal welfare and productivity within
animal production system by generating livestock that are better able to cope with the stressors within these systems.

However, although we know that fear-related temperament affects the stress-responsiveness of an individual, what is not well understood is the effect that it has upon the biology of an individual. For example, there is evidence that fear-related temperament is linked to activity of the hypothalamic-pituitary-adrenal (HPA) axis, a primary system in the neuroendocrine response to stress (Windle et al., 1998a; Blache and Bickell, 2010; Hawken et al., 2012a), but this relationship has not been fully characterised. Because the HPA axis is a key for overcoming threats and also influences many other systems throughout the body, it is important to understand how, if at all, temperament affects the activity of the HPA axis. This review describes the relationship between temperament and the HPA axis, and comprises three parts. First, the functioning of the HPA axis is reviewed; second, the concept of animal temperament and how it is assessed is explored; finally, the relationship between temperament and the HPA axis is discussed, the existing literature on this relationship is examined, and gaps in our knowledge are identified. Most literature in the field has come from research on rodent models but, where possible, this review focuses on the sheep and the UWA temperament flock, a genetically-based temperament model used in the experimental work in this thesis.

Section A. The hypothalamic-pituitary-adrenal axis

The HPA axis, a primary element in the response to stress (Matteri et al., 2000), is a classical neuroendocrine system whereby the brain controls adrenal glucocorticoid secretion by feed-forward stimulation and inhibitory feedback. Through the glucocorticoids, the HPA axis also modulates many body processes, including the immune system, reproduction, and food intake, and it is also
responsive to other systems (Matteri et al., 2000). Complex interactions among several endocrine and neural systems tightly regulate the HPA axis so that homeostasis is maintained, avoiding the deleterious effects of excess glucocorticoid levels, and preserving the ability to respond adequately to a stressor (Matteri et al., 2000).

**Figure 1.** Structure of the HPA axis. Corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP) are released from the hypothalamus, and stimulate ACTH production in the pituitary gland. In turn, ACTH stimulates glucocorticoid secretion from the cortex of the adrenal gland. Glucocorticoids act back on the hypothalamus and, to a lesser extent, the pituitary gland to inhibit their own secretion (dashed arrows). Each hormone of the HPA axis is released at rest in pulses, which change in amplitude and frequency to form a circadian rhythm, as demonstrated by the glucocorticoid data (adapted from Spiga et al., 2007). The circadian rhythm is controlled by the master clock in the suprachiasmatic nucleus of the hypothalamus, and by the peripheral clock in the adrenal gland. Activity of the HPA axis also rises in response to stressors, stimulated by cognitive pathways of the fore-brain.
Organisation of the HPA axis

The HPA axis is organised into three levels: the hypothalamus, the anterior pituitary gland and the adrenal gland (Figure 2.1). Each organ responds to stimulatory signals received from the preceding organs, as well as negative feedback signals from the lower levels of the axis, to regulate both resting and stress-response activity (Matteri et al., 2000).

The hypothalamus

The hypothalamus is the chief controller of the HPA axis, the beginning of a hormone cascade that translates perception of a stressor into a glucocorticoid response. The hypothalamus primarily stimulates glucocorticoid production via 2 peptides, corticotrophin releasing hormone (CRH) and arginine vasopressin (AVP). CRH is secreted from parvocellular neurons situated in the paraventricular nucleus (PVN) (Whitnall, 1993). A proportion of these neurons also produce AVP (Whitnall, 1993), although in sheep only 5–15% of CRH cells produce AVP, at rest and during stress (Rivalland et al., 2005; Rivalland et al., 2007). AVP is also produced by magnocellular neurons of the PVN and supra-optic nucleus (Whitnall, 1993). In sheep, 3–10% of AVP cells also produce CRH both at rest and during stress (Rivalland et al., 2005; Rivalland et al., 2007).

At rest, CRH and AVP are secreted in a pulsatile manner (Figure 2.1), but their basal secretion is increased in response to an acute stressor (Engler et al., 1989). The two peptides act synergistically on the pituitary gland, but CRH is typically more important in governing adrenocorticotropic hormone (ACTH) release in most species, with AVP playing an accessory role (Liu et al., 1983; Rivier and Vale, 1983; Jia et al., 1991; Minton and Parsons, 1993). In sheep, a greater proportion of CRH neurons than AVP neurons are activated during isolation plus restraint (Rivalland et al., 2005), and CRH treatment elicits larger pituitary-adrenal axis responses.
than AVP (McFarlane et al., 1995). However, other studies suggest that AVP is more important in response to a metabolic or audio-visual stressor, possibly indicating stressor-dependent roles of AVP and CRH (Engler et al., 1989; Caraty et al., 1990).

*The pituitary gland*

The pituitary gland is a small gland that is functionally connected to the hypothalamus via the pituitary stalk, but is not part of the brain (Matteri et al., 2000). The main function of the pituitary gland is to maintain homeostasis of a variety of systems within the body via hormones (Matteri et al., 2000). The corticotroph cell of the anterior lobe of the pituitary gland responds to CRH and AVP stimulation by releasing adrenocorticotrophin (ACTH) into the peripheral blood stream (Jacobson, 2005).

*The adrenal cortex*

In mammals, the adrenal glands, situated above the kidneys, are responsible for producing several stress response hormones and androgens (Bentley, 1998). There is an outer cortex that produces steroid hormones and that surrounds a medulla, the site of production of catecholamines that participate in the stress response of the sympathetic nervous system (Bentley, 1998).

The adrenal cortex is the final organ in the HPA axis, and itself consists of 3 functional zones, each of differing function and structure, and producing a different group of hormones (Bentley, 1998). Moving from the outer layer towards the medulla: the *zona glomerulosa* produces mineralocorticoids (e.g., aldosterone), the *zona fasciculata* produces glucocorticoids (e.g., cortisol), and the *zona reticularis* produces androgens (Bentley, 1998). The layers are not distinct in their ability to produce a particular hormone, because there is some degree of crossover – rather,
each layer seems to differ in its ability to respond to the different stimuli (Bentley, 1998).

The cells of the zona fasciculata respond to ACTH stimulation by producing glucocorticoids, primarily cortisol in most mammals, although corticosterone is dominant in rodents because they lack 17α-hydroxylase (Dallman et al., 1987a). ACTH is also responsible for trophic support, enabling the adrenal gland to maintain its ability to produce glucocorticoids (Thomas et al., 2004). During ACTH stimulation, the adrenal cortex synthesises glucocorticoids from cholesterol, that are then released into the peripheral circulation (Bentley, 1998).

\textit{Glucocorticoids}

Glucocorticoids are present in the body at varying concentrations, so in a discussion about their activity and control, it is useful to define what ‘high’ and ‘low’ concentrations are. Any definition must be relative, as the numerical concentration of glucocorticoids varies with species and with the method used to measure the concentration. Furthermore, a distinction must be made between ‘free’ glucocorticoid concentrations, and concentrations of glucocorticoids bound by the protein corticosteroid-binding globulin (CBG). This protein has a high affinity for binding to glucocorticoids, preventing them from being able to bind to receptors, and therefore biologically inactivating them (De Kloet and Reul, 1987; De Kloet et al., 1998; Dallman, 2007). On average, less than 5 % of circulating concentrations of glucocorticoids are unbound by CBG (De Kloet et al., 1998). CBG is thought to protect against excessive concentrations of glucocorticoids, whilst maintaining a pool of readily available hormone (Bentley, 1998; Dallman, 2007). With these factors in mind, ‘high’ and ‘low’ concentrations of glucocorticoids can be broadly defined. Basal concentrations, i.e. in the absence of stress, and between pulses, can
be considered low. Barnett and Hemsworth (1990) suggest that free glucocorticoid concentrations more than 40% above basal values pose a risk to welfare when sustained, so can therefore be considered high. It must be noted that in a pulsatile system such as the HPA axis, concentrations of glucocorticoids 40% above the baseline are achieved at the peaks of pulses (Windle et al., 1998a; Hawken et al., 2013).

Once released into the peripheral circulation, glucocorticoids exert their effects on the body via two types of receptor: glucocorticoid receptors (GRs), and mineralocorticoid receptors (MRs), also termed glucocorticoid types I and II receptors, respectively. Both GRs and MRs are members of the superfamily of steroid receptors that are ligand-inducible transcription factors (De Kloet et al., 1998; Heitzer et al., 2007). Glucocorticoids have a 10-fold greater affinity for MRs than GRs, and so MRs are mostly occupied even when circulating glucocorticoid levels are low (de Kloet et al., 2005; Joels and Baram, 2009). GRs are more fully occupied during stress and the peak in the circadian rhythm of glucocorticoid release (Jacobson, 2005). MRs exist in discrete locations within the brain, with a low density within the hypothalamus (Dallman, 2007). In peripheral tissue, MRs are protected from being activated by glucocorticoids by a co-located enzyme, 11-β-dehydrogenase isozyme 2 (11β-HSD2), that renders the glucocorticoid inactive (Seckl, 1997; De Kloet et al., 1998; de Kloet et al., 2005; Jacobson, 2005). Brain cells do not contain 11β-HSD2, and as glucocorticoids circulate at greater concentrations and diffuse into the brain with more ease than aldosterone, brain MRs are primarily occupied by glucocorticoids (Dallman, 2007).

GRs are expressed in nearly every cell of the body in differing forms and have varying effects in different parts of the body (Stahn and Buttgereit, 2008). The unbound GR resides in the cytosol and, at binding, moves rapidly to the nucleus. Once bound, the GR complex primarily regulates transactivation of anti-
inflammatory genes and transrepression of pro-inflammatory genes (Stahn and Buttgereit, 2008). The rapid effects of glucocorticoids are mediated by direct and indirect non-genomic actions by interacting with cellular membranes, via proteins released from the bound cytosolic GR complex, and via the membrane-bound activated GR complex (Brann et al., 1990). The HPA axis and its associated activities can be manipulated with GR agonists, such as dexamethasone, and antagonists, such as mifepristone.

**Negative feedback**

The final product of the HPA axis, the glucocorticoids, ultimately regulates its own secretion by exerting inhibitory feedback on the higher organs of the axis (Figure 2.1) (Dallman et al., 1987b; Jacobson, 2005). This negative feedback ultimately decreases glucocorticoid production and prevents the problems associated with excess glucocorticoid levels. However, the wide range of physiological glucocorticoid concentrations presents a challenge for appropriately regulating ultradian pulses, circadian rhythms, and stress responses, whilst maintaining a responsive HPA axis under both healthy and chronically stressed conditions. To meet this challenge, the HPA axis utilises two types of receptors with differing affinities for glucocorticoids, the MRs and GRs, discussed above, and controls feedback by varying time delays (Dallman, 2007).

Negative feedback by the glucocorticoids is primarily exerted at the brain level, through both MRs and GRs (De Kloet and Reul, 1987; De Kloet et al., 1998; Dallman, 2007) and actions on the neuronal pathways that control the release of CRH and AVP, possibly by reducing stimulatory input into the PVN (Dallman et al., 1987b; Dallman, 2007). There is not a single site of feedback activity that inhibits all PVN neuronal responses to stressors, but rather a range of sites, as different neurons are activated by different stressors (Dallman, 2007).
At lower circulating concentrations of glucocorticoids, feedback is mediated by the MRs (Dallman et al., 1987b; De Kloet and Reul, 1987; Dallman, 2007). Occupancy of the MRs in the brain is necessary to maintain low circulating levels of ACTH during the trough of the circadian rhythm, whereas during the peak of the circadian rhythm, and in response to a stressor, occupancy of both MRs and GRs is necessary to inhibit ACTH secretion (De Kloet et al., 1998; Young et al., 1998; Dallman, 2007).

Negative feedback is also exerted at the level of the corticotrophs in the pituitary gland, which contains GRs but not MRs (Dallman, 2007), by reducing the responsiveness to CRH, but maintains responsiveness to AVP (Dallman, 2007). Because they have a low affinity for glucocorticoids, GRs only mediate negative feedback at high concentrations of glucocorticoids (Dallman, 2007). In addition, the pituitary corticotrophs contain large amounts of high-affinity CBG that binds glucocorticoids, rendering them inactive and unable to act upon GRs (De Kloet and Reul, 1987; De Kloet et al., 1998; Dallman, 2007). Therefore, the pituitary gland is relatively insensitive to negative feedback from glucocorticoids, becoming the final site of inhibitory control of the HPA axis, able to only respond to the highest glucocorticoid concentrations (Dallman, 2007).

Negative feedback occurs at three speeds, referred to as ‘slow’, ‘intermediate’ (or ‘delayed’), and ‘fast’ (Dallman and Yates, 1969; Dallman, 2007). Slow feedback is the result of days or weeks of chronic glucocorticoid exposure, as found in chronic stress situations, and will be discussed in the section devoted to chronic stress. Fast and intermediate feedback control stimulated glucocorticoid secretion, as found in the acute stress response and the circadian rhythm. Fast feedback occurs over seconds to minutes, and does not require protein synthesis, but acts on the neurons via the cell membrane (Dallman, 2007; Stahn and Buttgereit, 2008). Glucocorticoid treatment inhibits stimulation of CRH neurons within minutes, a
process that is not affected by inhibition of RNA or protein synthesis, showing that
genomic effects are not involved (Sayers and Sayers, 1947; Yates et al., 1961;
Dallman, 2007). Furthermore, fast feedback acts at about 25% of maximal
inhibition, meaning that the HPA axis is still able to respond to further stressors
(Keller-Wood and Dallman, 1984; Dallman, 2007).

Intermediate feedback begins approximately 30 min after glucocorticoid
concentrations rise, and lasts for hours (Dallman, 2007). The level of inhibition
induced depends on the magnitude of the prior glucocorticoid exposure, the time
since exposure, and the total dose of glucocorticoid (Sayers and Sayers, 1947; Yates
et al., 1961; Takebe et al., 1971; Keller-Wood and Dallman, 1984). Intermediate
feedback returns the HPA axis to pre-stress levels, preventing the axis from
excessive responses to subsequent stimulations (Dallman, 2007).

**Circadian rhythm of the HPA axis**

The HPA axis is constantly active at a basal level, even during non-stressed
periods. Secretory activity presents a circadian rhythm, characterised by a basal
level of secretion overlaid by ultradian pulses (Figure 2.1; Veldhuis et al., 1989).
Generally, glucocorticoid release closely follows that of ACTH, and ACTH shows a
circadian rhythm similar to that of cortisol, but with lower amplitude (Carnes et
al., 1988; Bornstein et al., 2008; Dickmeis, 2009). In all mammalian species
studied, including humans, rats, monkeys, cattle and sheep, pulses form a
circadian rhythm by increasing and decreasing in amplitude and/or frequency
throughout the day (Figure 2.1; Fulkerson and Tang, 1979; Carnes et al., 1988;
Veldhuis et al., 1989; Lefcourt et al., 1993; Windle et al., 1998b; Kolber et al.,
2008). The timing of the peak and nadir of the circadian rhythm depends on a
variety of factors, including species, time of awakening, genetics, eating habits and
age, as well as disturbances to normal rhythms and environment (Liddle, 1966;
Hays et al., 1975; Trenkle, 1978; Turner, 1984; Simonetta et al., 1991; Linkowski et al., 1993). Generally, the peak is associated with the activity phase of the individual, occurring in the early morning for diurnal animals and early evening for nocturnal animals (Dickmeis, 2009). Studies in sheep have yielded varying findings on the nature of a circadian rhythm in glucocorticoid concentrations. In Merino ewes and Desert bighorn sheep, the peak in glucocorticoid concentrations occurred in the early morning, around 0100–0200 h, and the trough in the afternoon hours (Fulkerson and Tang, 1979; Turner, 1984). In contrast, McNatty et al. (1972) report that anoestrous New Zealand Romney ewes displayed a peak around 0900 h, whereas Bassett (1974) found no evidence of a circadian rhythm in Border Leicester x Merino wethers. This variation could be due to breed or environmental differences, but many of these studies do not statistically analyse pulse characteristics such as amplitude, which could contribute to the disagreements in the findings.

**Control of the circadian rhythm of the HPA axis**

The circadian rhythm in glucocorticoid levels is controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus, the body’s master clock that controls the peripheral clocks (Moore and Eichler, 1972; Jacobson, 2005; Haus, 2007; Dickmeis, 2009; Nader et al., 2010). The activity of the SCN is autonomous, but responds to the light/dark cycle, and other zeitgebers such as feeding habits also contribute to pattern generation by altering peripheral clocks (Damiola et al., 2000; Stephan, 2002). Lesions of the SCN cause a loss of rhythm in ACTH and cortisol secretion, demonstrating significant input into the circadian pattern of the HPA axis (Moore and Eichler, 1972; Szafarczyk et al., 1979; Dickmeis, 2009). The SCN input is thought to be both stimulatory and inhibitory, as lesions of the SCN in rats is found to cause both a rise in the trough of the cycle, and, in adrenalectomised rats
supplemented with corticosterone, a loss of the peak (Cascio et al., 1987; Buijs et al., 1993a).

The SCN exerts its control over glucocorticoid secretion through two mechanisms: indirectly via the HPA axis and directly by action on the adrenal gland via the autonomic nervous system (Dickmeis, 2009). In the first mechanism, the SCN is thought to affect release of AVP and CRH via its direct projections to the PVN and surrounding brain areas (Berk and Finkelstein, 1981; Sawchenko et al., 1984b, a; Buijs et al., 1993b; Engeland and Arnhold, 2005). In addition, there is evidence suggesting the SCN has a direct neural connection with the adrenal glands (Oster et al., 2006; Dickmeis, 2009; Nader et al., 2010). Glucocorticoid concentrations still cycle even in the absence of an ACTH cycle, but this cycle is lost when the adrenal glands are denervated (Meier, 1976; Ottenweller and Meier, 1982). There is also evidence for a neural SCN-adrenal gland connection that operates via the autonomic nervous system (Buijs et al., 1999; Ueyama et al., 1999; Engeland and Arnhold, 2005; Ishida et al., 2005; Ulrich-Lai et al., 2006), conveying information about light from the SCN to the adrenal gland, inducing increased adrenal nerve activity and changes in glucocorticoid release that do not reflect ACTH patterns (Niijima et al., 1992; Niijima et al., 1993; Buijs et al., 1999; Ishida et al., 2005). It is not yet known whether these light signals are important in regulating the normal circadian rhythm (Dickmeis, 2009).

The SCN may also mediate the circadian rhythm of glucocorticoid release by directly acting on the adrenal gland, via the autonomic nervous system, to increase the responsiveness of the cortex to ACTH stimulation (Engeland and Arnhold, 2005; Ulrich-Lai et al., 2006; Dickmeis, 2009). The adrenal gland displays changing responsiveness to stimulation throughout the day (Dallman et al., 1978). Removal of the SCN leads to a loss of this rhythm, and denervation of the adrenal glands alters the rhythm, and so it is likely that the SCN controls the circadian changes in
the responsiveness of the adrenal gland to ACTH stimulation (Dijkstra et al., 1996; Sage et al., 2002; Ulrich-Lai et al., 2006).

In addition to the SCN, the peripheral clock of the adrenal gland also controls the daily rhythm of glucocorticoid release (Andrews and Folk, 1964; Nader et al., 2010). The clock itself is under SCN control and is very sensitive to external cues (Valenzuela et al., 2008). Adrenal cultures from mice lacking functional molecular adrenal clocks do not show a diurnal rhythm in responsiveness to ACTH stimulation, a result confirmed by in vivo experiments (Oster et al., 2006). Transplants of normal adrenal glands into the clock-less mice restored the glucocorticoid rhythm, indicating that adrenal clocks contribute to the rhythm in ACTH responsiveness of the adrenal gland (Oster et al., 2006).

Although found in a wide range of species, the purpose of the circadian rhythm of HPA activity is not clear. It is hypothesised that the trough period of the cycle helps avoid the problems associated with prolonged glucocorticoid exposure (Jacobson, 2005). Furthermore, circadian rhythms in the HPA axis appear to control the sleep/wake cycle, but it is possible that sleeping and activity patterns also affect HPA activity (Buckley and Schatzberg, 2005; Dickmeis, 2009). The purpose of the rhythm might not yet be defined, but it is clear that the rhythm is important, as a variety of alterations from the norm are associated with a range of disorders such as depression, obsessive compulsive disorder, posttraumatic stress disorder, insomnia, obesity, and changes in other hormone systems (Walton et al., 1980; Monteleone et al., 1994; Yehuda et al., 1996; Leitch et al., 2003; Mattsson et al., 2003; Buckley and Schatzberg, 2005; Keller et al., 2006).

Role of pulsatility

As mentioned previously, glucocorticoid release occurs in an ultradian pattern of discrete pulses (Fulkerson and Tang, 1979; Veldhuis et al., 1989; Windle et al.,
Pulses consist of alternating periods of HPA activation and inhibition, whereby circulating glucocorticoid concentrations rise quickly, before returning to a low nadir point. During inhibition, the HPA axis appears to be in a refractory period, where it does not respond to mild stressors (Windle et al., 1998a).

Pulsatility is an important characteristic for the proper functioning of the HPA axis. In fact, it has been recently demonstrated that pulsatile administration of ACTH is necessary to activate glucocorticoid secretion and the synthesis of steroidogenic enzymes (Spiga et al., 2011). It is thought that the pulsatile nature of glucocorticoid secretion helps prevent the saturation of the receptors that can cause desensitization, as found in gonadotrophin-releasing hormone (GnRH; Young et al., 2004). Pulsatile release of glucocorticoids, therefore, may help maintain and optimise responsiveness of the HPA axis to stimulation (Lightman et al., 2000; Lightman et al., 2008; Lightman and Conway-Campbell, 2010). Additionally, Young et al. (2004) hypothesised that the size and frequency of glucocorticoid pulses may communicate information to tissues via changes in the balance of activation of receptors over the duration of the pulse. The authors proposed that as glucocorticoid levels rapidly change from low to high concentrations, the change in the ratio of activation of GRs and MRs may play a role in mediating the effect of glucocorticoids (Young et al., 2004). This hypothesis is partially supported by a study that shows cortisol pulsatility encodes a biologically meaningful signal that has effects on cell gene expression and phenotype, if not on GR expression or activation (McMaster et al., 2011). Therefore, pulsatility in the HPA axis is important, but its purpose requires further study.

The mechanism for regulation HPA pulsatility is also as yet unknown. Although it is popularly thought that there is a pulse generator at the uppermost levels of the HPA axis, there is as yet no strong evidence for its existence (Walker et al., 2010). CRH is often thought to be the driver of pulsatility, but disconnection of the
hypothalamus from the pituitary gland does not cause a loss of glucocorticoid or ACTH pulsatility, although the normal stress response is blocked (Engler et al., 1990). Therefore, pulsatile CRH secretion is not required for glucocorticoid pulsatility.

Recently it has been proposed that pulses are generated by time delays in the feed-forward and feedback mechanisms of the HPA axis (Lightman and Conway-Campbell, 2010; Walker et al., 2010; Walker et al., 2012). This hypothesis was supported by mathematical modelling experiments and in follow up in vivo work in rats (Walker et al., 2010; Walker et al., 2012). Constant levels of CRH can activate the pituitary-adrenal axis to produce pulses of glucocorticoids (Walker et al., 2010; Walker et al., 2012). Without a requirement for pulsatile CRH, it is possible that rapid negative feedback by glucocorticoids on the pituitary corticotrophs is the primary regulating factor in glucocorticoid pulsatility (Walker et al., 2010; Walker et al., 2012).

The cause and significance of glucocorticoid pulsatility is not fully understood but, as with the circadian rhythm, it is clearly necessary for the proper functioning of an individual because changes such as a lack of a trough in pulse amplitude are associated with disease (e.g., arthritis; (Windle et al., 2001). Of course, alterations in the pulsatility of glucocorticoids are likely to be linked with, if not a cause of, changes in the circadian rhythm.

The stress response

When presented with a stressor, the body activates a range of physiological and behavioural changes so it can cope with or remove the source of stress (Matteri et al., 2000) . In the HPA axis, a neuroendocrine response to stimulation by stressors results in an increase in hormonal output at each level of the axis, causing changes such as temporary suppression of reproductive systems and diversion of energy
away from growth processes (Matteri et al., 2000; Tilbrook et al., 2002; Tilbrook et al., 2008).

**Acute stress response**

Acute stressors are defined as lasting for periods of seconds to a few hours (Turner et al., 2005) and the HPA axis responds to them with a rapid and sustained release of glucocorticoids into the peripheral bloodstream. Plasma concentrations of glucocorticoids remain high for a period that depends on the duration and severity of the stressor, and then decrease to pre-stressor levels as the negative feedback systems restore balance.

Although acute stressors invoke a stress response, short durations of stress are rarely harmful to the animal’s welfare or biological functioning (Moberg, 2000). High concentrations of cortisol are anti-anabolic and suppress the immune system and growth, but, in the short term, these effects maximise resource availability and optimise the ability to overcome the stressor and restore homeostasis.

**Chronic stress**

Chronic stress can result from constant exposure to a single stressor, also called a chronic stressor, such as pain or extreme cold, or from exposure to a series of acute stressors or acute stressors encountered during a subclinical long-term stressor, such as exposure to low doses of toxins (Moberg, 2000). In this situation, endocrine stress responses can fail to restore a state of healthy homeostasis and remain activated, leading to a significant drain on body reserves and suppressed or impaired bodily functions (Moberg, 2000).

Chronic stress can alter the activity of the HPA axis at rest and also the response to stressors. Thus, compared to control animals, chronically stressed animals have greater mean glucocorticoid concentrations at rest, particularly during the trough
of the circadian rhythm (Becker et al., 1985; Barnett et al., 1987; Janssens et al., 1995b; van der Staay et al., 2010; Wagner et al., 2011), and larger glucocorticoid responses to acute stress (Bhatnagar and Dallman, 1998; Bhatnagar and Vining, 2003; Wagner et al., 2011), that could be due to increased adrenocortical responsiveness (Janssens et al., 1994; Janssens et al., 1995a). The increased circulating concentrations of glucocorticoids can suppress a variety of essential biological processes and therefore lead to a range of pathological states, including reduced growth, muscle wastage, impaired fertility and suppressed immunity (Tilbrook et al., 2002; Hawthorne et al., 2003). Furthermore, chronic stress can lead to glucocorticoid resistance (Avitsur et al., 2001; Miller et al., 2002; Cohen et al., 2012) that, in turn, can lead to further increases in glucocorticoid production, and therefore associated problems such as hyperandrogenism (Chrousos et al., 1993).

It must be noted that not all studies agree with this position. For example, with chronic stress, starlings showed a decrease in basal and stress-responsive concentrations of glucocorticoids (Rich and Romero, 2005), mice showed an increase in glucocorticoid concentrations in the trough of the rhythm but a decrease in peak concentrations (Sterlemann et al., 2008), and pigs showed a decrease in glucocorticoid concentrations at a peak of the circadian rhythm with no change in the trough, leading to a blunted circadian rhythm (de Jong et al., 2000). These disagreements in the literature could be explained by differences among species, genotypes or genders, in the type or frequency of the stressor, or in the overall duration of the stressor model. Furthermore, inadequate sampling frequency for assessment of glucocorticoid patterns could lead to conflicting findings, particularly when examining the circadian rhythm, due to the pulsatile nature of glucocorticoid secretion. For example, amongst the studies conducted in pigs, sampling frequencies ranged from one sample after slaughter from 5 animals per
experimental group (van der Staay et al., 2010), to twice daily for 20 weeks from 4–9 pigs per group (Janssens et al., 1995b), to hourly sampling for 24 h from 12 animals per group (de Jong et al., 2000). None of these protocols are sufficiently rigorous for pulse analysis.

The effect of behaviour on the stress response of the HPA axis

Animals that display behavioural coping mechanisms often have lower glucocorticoid responses to stressors than those that do not or are unable to express these behaviours (Koolhaas et al., 1999). For example, rats that respond to an electrified prod in their cage with proactive behaviour, i.e. burying the probe with cage bedding, show smaller glucocorticoid responses to shock than rats that react passively by freezing (Deboer et al., 1990; Korte et al., 1992). Similarly, food-deprived pigs subjected to a session of intermittent feeding showed a decrease in concentrations of glucocorticoids over the session if allowed to engage in chain-pulling, compared to no change if not allowed access to a chain (Dantzer and Mormede, 1981; Dantzer et al., 1987). However, pigs that were mass-fed and allowed access to a chain showed an increase in glucocorticoid concentrations over a session, indicating that the effect of behaviour on HPA axis activity is not straightforward, and may be influenced by the stressor (Dantzer et al., 1987). Although this concept is somewhat outside the scope of this thesis, it is important to be mindful of this relationship in a study of the link activity of the HPA axis to temperament, as inferred by behavioural responses to stressors.

Interactions of glucocorticoids with other endocrine systems

The role of the HPA axis and its product, the glucocorticoids, can be broadly described as control of energy partitioning, and prioritising of energy use to maintain and overcome threats to homeostasis in the body (Bentley, 1998; Jacobson, 2005). To achieve these outcomes, glucocorticoids induce changes in a
range of tissue groups that control reproduction, metabolism, and immunity, and alter the balance of other endocrine systems within the body. In this thesis, in addition to glucocorticoids, we pay attention to prolactin, another stress responsive hormone, and to insulin and leptin, hormones associated with metabolism, because of the effect glucocorticoids have on metabolic processes.

Prolactin

Prolactin is a stress-responsive hormone that can affect immunity and behaviour in many species, making it of interest in the study of the HPA axis and temperament (Lamming et al., 1974; Fava and Guaraldi, 1987; Van de Kar and Blair, 1999; Freeman et al., 2000; Torner and Neumann, 2002; Yayou et al., 2010; Lennartsson and Jonsdottir, 2011). Prolactin is a peptide primarily produced from the lactotroph cells of the anterior pituitary gland, and is under predominately inhibitory control from the hypothalamus via dopamine (Freeman et al., 2000). It is released in response to a range of stress stimuli, including physical, psychological, and internal homeostatic disturbances (Freeman et al., 2000; Lightman et al., 2008; Lennartsson and Jonsdottir, 2011). The mechanism by which prolactin responds to stress is unclear, but appears to involve stimulating factors such as prolactin-releasing-peptide (PrRP) (Gala, 1990; Hinuma et al., 1998; Matsumoto et al., 1999). PrRP is currently thought to integrate a range of neuroendocrine responses to stress – for example, it is also reported to stimulate CRH neurons, causing ACTH release, demonstrating commonalities in the regulation of both prolactin and the HPA axis (Matsumoto et al., 2000; Maruyama et al., 2001; Onaka et al., 2010). Furthermore, the magnitude of glucocorticoid and ACTH responses to stressors is positively correlated to the magnitude of the prolactin response (Lennartsson and Jonsdottir, 2011). This correlation supports the hypothesis that prolactin plays a protective role against damage during stress, possibly by immunoenhancement
effects, as, presumably, the larger the glucocorticoid stress response, the greater
the need for protection (Van de Kar and Blair, 1999; Freeman et al., 2000).

In addition to being under the same regulatory mechanisms, prolactin and the
glucocorticoids regulate each other. For example: i) adrenalectomised animals have
greater basal and stimulated prolactin levels than intact animals (Ben-David et al.,
1971; Harms et al., 1975; van der Schoot and de Greef, 1983); ii) treatment with
exogenous glucocorticoids can reverse the effect of adrenalectomy upon prolactin
secretion, and decrease both basal and stressed concentrations of prolactin in
entire animals (Harms et al., 1975; Brann et al., 1990; Freeman et al., 2000); iii)
high concentrations of prolactin, as found during pregnancy and lactation,
attenuate the HPA axis response to stress (Torner and Neumann, 2002). Therefore,
prolactin and the glucocorticoids can suppress each other’s release.

Insulin

Acute increases in glucocorticoids result in suppression of the insulin response to
nutrient stimulation, presumably to maximise availability of glucose (Kalhan and
Adam, 1975; Billaudel and Sutter, 1979; Pierluissi et al., 1986; Lambillotte et al.,
1997; Sutter-Dub, 2002). This response can be detected within minutes and occurs
only during stimulation of insulin secretion (Billaudel and Sutter, 1979, 1982b;
Barseghian et al., 1984; Pierluissi et al., 1986; Sutter-Dub, 2002). Glucocorticoids
also reduce the synthesis of pro-insulin and insulin and therefore the tissue content
of these peptides within the pancreas in the short-term (Billaudel and Sutter, 1977,
1982a). However, the insulin response to glucocorticoids appears to be biphasic –
after the initial suppression of secretion, the increased concentrations of glucose in
the blood stimulate insulin secretion, thus restoring homeostasis (Sutter et al.,
1968; Sutter-Dub, 2002).
Leptin

The HPA axis and glucocorticoids seem to have a complex relationship with leptin. Glucocorticoids stimulate leptin release and the expression of mRNA for the *obese* gene, as demonstrated by the effects of dexamethasone on humans *in vivo* and on human and rodent adipose tissue *in vitro* (De Vos et al., 1995; Murakami et al., 1995; Slieker et al., 1996; Masuzaki et al., 1997). These observations suggest that glucocorticoids act directly on adipose tissue, and that leptin secretion would increase during stress (Sandoval and Davis, 2003). In patients with Cushing’s syndrome, an excess of glucocorticoids, concentrations of leptin are greater than in non-obese and obese controls, at a given percentage of body fat (Masuzaki et al., 1997).

In contrast, other studies have shown no change in leptin concentrations during acute challenges of HPA axis, despite increases in glucocorticoid concentrations, demonstrating that the relationship is not absolute (Wand and Schumann, 1998; Nye et al., 2000). One of the confounding factors is that, although the HPA axis is thought to stimulate leptin release, the sympathetic nervous system (SNS), which is also activated during stress, is thought to inhibit leptin release (Sandoval and Davis, 2003). This conflict between the systems may explain the contradictory findings, and also cloud the biological significance of the relationships among stress, glucocorticoids, and leptin.

Section B. Temperament

What is temperament?

Individuals vary widely in their response to the same situation (Windle et al., 1998a; Pottinger, 2000; Beausoleil et al., 2008), even within species, gender, breed
and background, and for a variety of traits, amongst humans and animals (Bekoff, 1977; Hemsworth et al., 1990; Abel, 1991; Hessing et al., 1994; Murphy et al., 1994; Boissy and Bouissou, 1995; Stohr et al., 2000; Gunnar et al., 2003; Davis et al., 2005). An individual, however, may consistently display the same pattern of reaction across time for several situations, and this individual level of stress-responsiveness is largely affected by temperament (Boissy, 1995; Pottinger, 2000). Temperament is defined by Saunders comprehensive veterinary dictionary as “the peculiar behavioural character and mental cast of an animal” (Abutarbush, 2008), and by the Oxford English dictionary as “a person’s or animal’s nature, especially as it permanently affects their behaviour” (Stevenson). Temperament is an innate characteristic of an individual and can be thought of as a filter through which each individual assesses situations that then determines the response to the situation (Boissy, 1995; Reale et al., 2007).

With regards to stress, fearfulness, or emotional reactivity, is an important aspect of temperament because it describes an individual’s characteristic reaction to stressors such as novelty or surprise (Boissy, 1995). This fear-related aspect of temperament is particularly interesting in the assessment of the ‘robustness’ of animals, and for increasing their resilience to stressors, particularly in situations where humans influence or control animal environments, such as farming.

**Measuring temperament**

Fear-related temperament is often inferred from behavioural responses to a challenge, with neuroendocrine responses used to a lesser extent (Ramos and Mormede, 1998). This is done for a variety of reasons: i) the use of standardised tests helps reduce subjectivity and the effects of inter- and intra-assessor variation in assessing temperament (Mills, 1998; Ramos and Mormede, 1998; Forkman et al., 2007; Vettes et al., 2013); ii) behavioural tests are the only option for assessing
temperament in animals because they are unable to verbally communicate to us their emotions; iii) behavioural assessment is non-invasive and relatively simple, so is better for the test subject and the outcome because it reduces potential influence of sampling methods.

To this end, a suite of standardised tests have been developed to allow assessment of fear-related temperament. Generally, these tests place an individual in a challenging situation, involving stressors such as novelty, isolation, restraint, or unpredictability (Ramos and Mormede, 1998; Forkman et al., 2007). During the tests, a range of variables relevant to the species and trait being tested, such as movement, vocalisations, or grooming behaviours, are quantified. For example: for rats and mice, probably the most studied species in this regard, one of the most common and longest standing tests for these species is the open field test (Hall, 1934; Broadhurst, 1969; Ramos and Mormede, 1998). The animal is placed for a fixed amount of time in a large, brightly lit arena surrounded by walls that prevent escape (Hall, 1934; Lister, 1990). The open field test challenges the animal because of its novelty as well as the fear that rats and mice have towards being in illuminated, open spaces (Hall, 1934; Lister, 1990). High defecation rates and low ambulation are considered to reflect greater fearfulness (Hall, 1934; Lister, 1990).

A variety of tests has been established for assessment of fear-related temperament in sheep, typically using isolation and novelty as stressors. For instance, most studies use variations of the isolation box test and/or the arena test (described in detail below), both of which use isolation from peers within a novel setting (Dodd et al., 2012). Human presence is also a significant stressor for sheep, and is used in many variations of the arena test (Dodd et al., 2012). Locomotor and vocal activity are typically quantified in these tests as measures of temperament, with greater levels of locomotor and vocal activity generally considered to reflect greater levels of fear (Romeyer and Bouissou, 1992; Vandenheede et al., 1998; Vierin and
Bouissou, 2003). However, the interpretation of these behaviours may not be straightforward – for instance, both high activity (escape attempts) and low activity (immobility due to fear) could be interpreted to reflect a high level of fearfulness, or high activity could be due to fear, or to exploration due to curiosity (Forkman et al., 2007; Dodd et al., 2012).

**Heritability of temperament**

Fear-related temperament is influenced by genetic, epigenetic, and environmental factors (Pottinger, 2000; Reif and Lesch, 2003). The prevailing influence varies with trait and species. For example, cross-fostering studies in mice demonstrate a strong influence of the behaviour of the rearing mother on the temperament of the offspring (Francis et al., 2003). In humans, it has been demonstrated that the *in utero* environment can also influence the temperament of the offspring (Davis et al., 2005). In contrast, in rats and Merino sheep there is little impact of the environment and the rearing mother on fear-related temperament, and genetic make-up exerts the strongest influence (Wigger et al., 2001; Bickell et al., 2009). In Merino sheep, fear-related temperament is moderately heritable (0.2 – 0.4; Blache and Bickell, 2010; Plush et al., 2011), so some individuals are innately inclined to be more or less reactive to a stressor because of their genotype.

**Section C. Interaction between temperament and the HPA axis**

**Temperament and the HPA axis**

Temperament is usually inferred from the behavioural response to stressors, but it appears that fear-related temperament can also influence HPA activity, both in response to stressors, and in the absence of stressors. For example, rats that are behaviourally hypo-reactive when exposed to a novel environment or noise stress...
show smaller HPA axis responses to the stressor than their hyper-reactive counterparts (Windle et al., 1998a; Marquez et al., 2006). A similar relationship seems to exist in humans, as shy children tend to have greater plasma concentrations of cortisol in a social situation or when feeling rejected by their peers than children who are more out-going (Dettling et al., 1999; Gunnar et al., 2003). Furthermore, non-human primates in a subordinate position in a group hierarchy have more inhibited temperaments and greater concentrations of cortisol, particularly when faced with challenges, than dominant individuals (Abbott et al., 2003). Therefore, it seems that individuals with more reactive temperaments show larger HPA responses to stressors than their less reactive counterparts.

Temperament has also been associated with changes in the activity of the HPA axis in the absence of stressors. For example, two strains of rat show divergent behavioural responses to a range of stressors, including white noise stress and an open field test, with the Lewis rats being hypo-reactive compared to the Fischer line (Sternberg et al., 1992; Windle et al., 1998a). The circadian rhythm of glucocorticoid concentrations is much less distinct in Fischer rats because they do not display a decrease in the amplitude of pulses in the morning hours, as seen in Lewis rats (Windle et al., 1998a). In Rhesus macaques, high excitability was associated with lower afternoon glucocorticoid concentrations, whilst low confidence traits were linked with lower morning glucocorticoid concentrations and a lack of circadian decline in the afternoon (Capitanio et al., 2004). Therefore, there appears to be a link between temperament and the circadian rhythm in HPA activity, but there are currently few studies on the nature and mechanisms of this relationship.

Although temperament appears to influence HPA activity, it is not clear which level of the axis is affected. Furthermore, the mechanisms may vary between
temperament models because the basis for assessing and selecting temperament varies, and so the genetic component of temperament is likely to differ across temperament models. That said, it is likely that temperament affects HPA activity by actions either in the brain processing of the perception of a stressor, and/or in the responsiveness of the HPA axis to feed forward stimulation or negative feedback (Figure 2.2). In the first case, more stress-responsive individuals may perceive a given stressor to be more stressful than a less responsive counterpart, and so signal a larger response of the HPA axis. For example, in mice and humans, a more reactive temperament has been associated with greater activity within the amygdala, a portion of the brain involved in decision-making and emotional reactions (Davidson et al., 2000; Stein et al., 2007; Muigg et al., 2009; Tasan et al., 2011). Alternatively, differences in activity of the AVP and CRH pathways have been associated with temperament traits in rats, mice, humans, cattle and Rhesus macaques (Sternberg et al., 1992; Keck et al., 2002; Smoller et al., 2003; Murgatroyd et al., 2004; Salome et al., 2004; Wigger et al., 2004; Smoller et al., 2005; Barr et al., 2008; Muigg et al., 2009; Pugh et al., 2011; Sheikh et al., 2013).

Temperament could also interact directly with the HPA axis. If the HPA axis is more or less sensitive or responsive to its own stimulatory and inhibitory signals, this could alter both the stress response and circadian rhythm of the HPA axis. Studies in a variety of species have found relationships between temperament and the responsiveness of the pituitary and adrenal glands to stimulation, indicating that pituitary-adrenal axis responsiveness may be responsible for effects of temperament on HPA responsiveness to stressors. For example, in humans, novelty-seeking traits were inversely related to the glucocorticoid response to CRH stimulation (Tyrka et al., 2006). In addition, the relatively hyper-reactive Fischer rat has a larger ACTH response to 0.5 µg CRH, and a more rapid, but not larger overall, response to 2 µg CRH than the hypo-reactive Lewis rats (Spinedi et al., 2008).
Furthermore, sheep that had smaller cortisol responses to ACTH treatment were deemed to be less fearful when faced with human presence and an open field test compared to sheep with large cortisol responses (Lee et al., 2014b). In each case, the less fearful temperament shows smaller pituitary-adrenal responses to exogenous hormone treatment, suggesting that the ability of the pituitary or adrenal glands could explain the divergence between temperament groups in the magnitude of HPA responses to acute stressors. Therefore, fear-related temperament may affect the responsiveness of the pituitary-adrenal glands, but the nature of this relationship might change with species and temperament model.

Overall, behavioural responsiveness to stressors is associated with differences in HPA activity. However, although many tests have been developed to induce fear-related behaviour and rate the level of stress the animal is experiencing, few models have been developed to examine the direct effect of temperament on HPA activity. This distinction is important because temperament is only one of the factors that can affect the individual’s response to stress. Additional factors such as recent experience, maternal influence, early-life history and age can also affect the response to stressors. With respect to early life history, post-natal handling of rats for the first 21 d of life significantly reduces the behavioural and HPA responses to stressors for up to 10 months post-treatment (Ferré et al., 1995; Nunez et al., 1996). With respect to genetic factors, Lewis and Fischer rats display differing behavioural responses to stressors, with correlated differences in HPA axis activity in both the absence and presence of stimuli (Sternberg et al., 1992; Spinedi et al., 1994; Windle et al., 1998a). However, these animals have not been specifically or selectively bred for temperament, only on their ability to mount an appropriate inflammatory response that directly involves glucocorticoids, thus possibly confounding the relationship between temperament and HPA activity.
Figure 2. Possible sites for temperament to impact upon activity of the HPA axis, as indicated by the large grey arrows.

(Sternberg et al., 1989a; Sternberg et al., 1989b; Spinedi et al., 1994; Windle et al., 1998a). Therefore, relying on results from individuals classified by temperament upon the results of a single test, or bred on other criteria is not sufficient for determining the relationship between temperament and the HPA axis. Instead, we need to use models with a known genetic basis for temperament and also ensure minimal external influences, if we are to characterise the true relationship between temperament and HPA activity, and thus reveal the processes that underpin the relationship.

**Animal models of temperament**

To this end, various animal models have been set up to analyse the effect that stress responsiveness has upon the biology of the individual. These models focus on the unlearnt behavioural response to a challenging situation, interpreted as
reflecting the temperament of the individual. Individuals showing extremes in the level of the response to the stressor(s) are then chosen for breeding to generate hyper- and hypo-reactive lines, where temperament has a genetic basis. Animal models of temperament in a variety of species are outlined below, including any findings on the relationship between temperament and the HPA axis.

The high and low anxiety-related behaviour model of rats and mice

The high/low (HAB/LAB) anxiety-related behaviour models are mice and rats that have been selected for their divergence in anxiety-related behaviours during an elevated plus maze (EPM; (Liebsch et al., 1998b; Salome et al., 2002; Kromer et al., 2005; Kessler et al., 2007). LAB animals spend more time in the open arms of the EPM than HAB animals, a behaviour that is interpreted as being less anxious because rats and mice tend to avoid open areas (Liebsch et al., 1998b; Kromer et al., 2005). These animals also differ in their behavioural response to other stressors: HAB mice are less active during forced exposure to the open arms of the EPM (Muigg et al., 2009), HAB rats are less active in the open field test (Liebsch et al., 1998b), and HAB rats and mice are less active in the black-white (or light-dark) box test, and have fewer entries into and spend less time in the light, white compartment, an environment that is aversive, than their LAB counterparts (Henniger et al., 2000; Salome et al., 2002; Kromer et al., 2005). HAB rats and mice also spend more time immobile in the forced swim and tail suspension tests than their LAB counterparts (Liebsch et al., 1998b; Salome et al., 2002; Kromer et al., 2005), although reverse results have been observed in the forced-swim test in rats (Salome et al., 2002). In summary, HAB animals behave more anxiously than LB animals to a range of stressors.

The divergence in behavioural responsiveness to stress between the HAB/LAB rats is also reflected in the HPA axis response to some stressors. Adult male rats from
the HAB line had a greater ACTH and glucocorticoid response to exposure to the open arm of the elevated plus maze than LAB rats, although both lines had similar basal concentrations of both hormones (Landgraf et al., 1999). In comparison, some analyses found pregnant HAB rats had greater basal concentrations of ACTH and glucocorticoids than pregnant LAB rats, but this was not observed consistently throughout the series of tests (Neumann et al., 1998). Furthermore, in virgin females and pregnant females, HAB/LAB rats did not differ in the ACTH and glucocorticoid response to exposure to the open arm of the elevated plus maze for 5 minutes followed by a forced swim test, or to CRH treatment (Neumann et al., 1998).

The divergence in the response of the HPA axis to stress seen in the male rats may be driven by central release of the ACTH secretagogue, AVP, as the more anxious HAB rats have higher rates of mRNA expression for AVP and release of AVP within the hypothalamic paraventricular nucleus (PVN) after exposure to the open arms of this maze than the LAB rats (Wigger et al., 2004). Secretion of AVP into the circulation from the posterior pituitary gland is similar in the HAB and LAB lines, as is expression of CRH mRNA in the brain, although they do differ in the bed nucleus of the stria terminalis (Wigger et al., 2004). Furthermore, functional polymorphisms in the regulatory regions of the AVP gene have been associated with the 2 phenotypes: a polymorphism in the promoter region of the AVP gene in HAB rats leads to over-expression and release of AVP, whereas, in LAB mice, there is a polymorphism in the AVP gene that seems to lead to a deficit in bioavailable AVP, and probably underlies the hypo-anxiety of LAB mice (Landgraf et al., 2007). In summary, temperament appears to affect HPA activity in some situations in the HAB/LAB model, and this relationship is likely to involve AVP (Landgraf, 2003).
The Maudsley reactive (MR) and non-reactive (MNR) rat

The Maudsley rats have been divergently selected on their behaviour in an open field test (Broadhurst, 1975). The frequency of defecation when exposed to the unfamiliar environment is interpreted as reflecting emotional reactivity, with MR rats defecating more frequently during the test than MNR rats (Denenberg, 1969; Broadhurst, 1975). MR rats also display high levels of freezing during the open field test, whilst MNR rats show more exploratory behaviour, which is also interpreted as reflecting emotional reactivity (Denenberg, 1969; Broadhurst, 1975). The behavioural response to the open field test is highly heritable (> 0.9) for defecation, and moderately heritable (0.4 - 0.8) for ambulation (0.4 - 0.8; Broadhurst, 1969).

MR and MNR rats did not differ in glucocorticoid concentrations before or after exposure to the open field test, the selection stressor for this model, or to forced swimming, an intense stressor, or in the ACTH or glucocorticoid response to foot shock (Abel, 1991; Blizard and Adams, 2002). A later study found that MNR rats had a smaller ACTH response to restraint stress than MR rats, but both groups showed a similar glucocorticoid response, that was also similar to unselected Wistar rats (Kosti et al., 2006). MR rats were found to have greater levels of gene expression in the adrenal glands for neuropeptide Y (NPY), a stress-responsive peptide, than MNR rats, indicating that adrenal NPY is responsible for blunting adrenocortical responses to ACTH in the MR line (Kosti et al., 2006). Therefore, the relationship between temperament and HPA activity is unclear in Maudsley rats, and does not appear to affect the production of glucocorticoids in either strain compared to non-selected rats.
Model of fear response in Japanese quail

Japanese quail (*Coturnix coturnix japonica*) have been selectively bred for the duration of tonic immobility (TI), an unlearnt fear response, after an immobilisation stressor (Mills and Faure, 1991). Birds that show long-term immobility (LTI) are deemed to be more fearful than those that move more quickly (STI), and this trait has an estimated heritability of 0.2-0.4 (Mills and Faure, 1991; Faure et al., 2006). STI birds appear to be generally less fearful, as they freeze less and vocalise and move more in an open field, emerge earlier from a ‘hole-in-the-wall’ box (Jones et al., 1991), are easier to catch than LTI birds (Mills and Faure, 2000), and are less fearful of a novel environment compared to LTI birds, although both genotypes show similar responses to a novel object (Richard et al., 2008; Saint-Dizier et al., 2008; Richard et al., 2010).

However, the glucocorticoid response to a 2-min restraint stress or to restraint in a crush cage is greater in STI birds than LTI birds, but the genotypes respond similarly to repeated induction of TI and to 1-min restraint stress (Hazard et al., 2005; Hazard et al., 2008a; Hazard et al., 2008b). Glucocorticoid concentrations are similar for the STI and LTI birds in the absence of stressors (Hazard et al., 2005; Hazard et al., 2008a; Hazard et al., 2008b). The difference between the genotypes in glucocorticoid response to stress might be due to adrenocortical responsiveness because, overall, STI birds had larger glucocorticoid responses to ACTH treatment than LTI birds, although the nature of the relationship varied with gender and age, particularly in LTI birds (Hazard et al., 2005; Hazard et al., 2008b). Therefore, it appears that quail that appear less behaviourally disturbed by stressors can have larger responses of the HPA axis to the stressors than quail that appear more disturbed.
In another model of temperament in quail, chicks were selected for 5 generations for high and low locomotor activity in a novel environment, interpreted as low and high levels of fearfulness, respectively (Jones et al., 1982). These animals were then shown to express different behaviour when faced with four other stressors (emergence from a box; open field test; response to a bell; tonic immobility), with the more active birds appearing the less fearful in each test than less active birds (Jones et al., 1982). However, to our knowledge, HPA activity has not been assessed in these animals.

In summary, there is a demonstrated relationship between temperament and HPA activity in three of the four temperament models discussed above. However, in none of them has the relationship been fully characterised – for example, none of the models have examined the circadian rhythm of activity in the HPA axis. In this thesis, we propose to further develop an animal model of temperament, the UWA temperament flock, to advance the understanding of the relationship between temperament and the HPA axis, by characterising further the activity of the HPA axis.

The UWA Temperament Flock

The UWA Temperament Flock is a model of fear-related temperament in sheep, based on measurable differences in behavioural reactivity. The flock has been bred since 1993 for over 20 generations for either high or low activity in two tests: the isolation box test and the arena test (Murphy et al., 1994). For ease of reference, the hyper-reactive animals are labelled ‘nervous’, and the hypo-reactive animals called ‘calm’. These temperaments have a strong genetic basis, with a low to moderate (0.2-0.4) heritability (0.2 - 0.4; Bickell et al., 2009; Blache and Bickell, 2010).
The divergence in behavioural reactivity to the selection stressors appears to be associated with reactivity of the HPA axis because nervous animals have larger cortisol responses to isolation than calm animals (Blache and Bickell, 2010; Hawken et al., 2012a).

Testing

Sheep are subjected to two tests that measure their behavioural responses to stressors of human presence and isolation from peers (Murphy et al., 1994). These tests differentiate between animals with divergent levels of social motivation, fear of humans, and reactivity to visual isolation from peers, resulting in two classes of animals that have different fear-related temperaments. Testing occurs at weaning, between 14–16 weeks of age (Bickell et al., 2009).

Isolation test

In the isolation test, an individual lamb is placed in a solid plywood box (1.5 m$^3$) for 1 minute, out of visual contact with peers. Vibrations from locomotor activity and vocalisations are recorded using a calibrated digital meter, to return an arbitrary measurement of activity. This test attempts to measure the animal’s fear of isolation, a major stressor for sheep, and its adaptation to the stressor (Blache and Ferguson, 2005). Greater scores in the behavioural response to social isolation are thought to indicate a greater level of fear (Romeyer and Bouissou, 1992; Vandenheede et al., 1998).

Arena test

This is an open field test that challenges individuals with a conflict of choice (Beausoleil et al., 2008). A single lamb is let into the arena (7 × 3.3 × 1.8 m; length × width × height), where a static human stands between it and a group of peers.
The animal has a conflict of desires – to remain distanced from the human, or to join its peers. Locomotor activity across marked zones is recorded.

High scores in locomotor and vocalisation behaviour suggest the animal is experiencing a high degree of nervousness and disturbance due to the presence of a predator (human). However, it may also represent high social motivation (Romeyer and Bouissou, 1992). It has also been argued that, in sheep, immobilisation may reflect equal or greater levels of fear than high activity in some situations, including in the presence of a predator, and so this test alone yields ambiguous results for interpreting temperament (Romeyer and Bouissou, 1992; Vandenheede et al., 1998). The solution is to combine the tests.

Selection score

The results of the two tests are combined and used to rank each individual within its line, using the following selection score:

\[
\text{Selection index} = 100 + \left[ \frac{i - \bar{x}}{\text{SD}} \right] + \left[ \frac{\text{TOTAL CROSS}_1 - \text{TOTAL CROSS}_2}{\text{TOTAL CROSS}_2} \right]
\]

where \( i \) = individual score, \( x \) = mean, and \( \text{SD} \) = the standard deviation of the mean.

Calm individuals have a lower score, whereas more nervous individuals have greater scores.

HPA axis activity in the UWA Temperament Flock

The HPA axis has not yet been studied in detail in the UWA temperament flock, although there have been some observations indicating a difference between the two lines. Nervous animals show a glucocorticoid response to exposure to short-term isolation (5-10 min) approximately 1.5-fold larger than that of calm animals (Blache and Bickell, 2010; Hawken et al., 2012a). On initial exposure to isolation plus a novel object, calm and nervous animals showed similar glucocorticoid
responses but, on repeat exposure, nervous animals had a greater glucocorticoid response than calm animals (Blache and Bickell, 2010). Nervous animals also appeared to anticipate the exposure to the novel object at the third exposure, whereas calm animals did not (Blache and Bickell, 2010).

During prolonged exposure to isolation, nervous animals presented greater mean and basal plasma concentrations of glucocorticoid in the third hour of exposure than calm animals, but not in the first, second or fourth hours (Hawken et al., 2013). Temperament also affected the response to the anxiolytic agent, lavender oil (Lavendula augustifolia), with the oil decreasing glucocorticoid concentrations and agitation levels in the calm animals, but increasing the responses in nervous animals (Hawken et al., 2012a). In contrast, there was no difference between the temperament groups in the cortisol response to a 10 min exposure to the arena test described above (Beausoleil et al., 2008). Therefore, genetic selection for temperament in Merino sheep has affected the HPA response to some stressors, but it is not known how or where this interaction occurs, nor whether there are differences in the circadian activity.

**Conclusion**

The current literature suggests a relationship between temperament and the activity of the HPA axis that may affect the robustness of the individual, but there are only a few temperament models for exploring this relationship and, within those models, the relationship has not yet been fully characterised. The UWA Temperament Flock is a promising model for exploring the effect that fear-related temperament has upon the activity of the HPA axis, and determining how this relationship is mediated. The aim of this thesis is to contribute to the progression
of this understanding using the UWA flock, by examining the effect of fear-related temperament on:

1) The resting activity of the HPA axis;
2) The propensity to develop a state of chronic stress;
3) The responsiveness of the pituitary gland and adrenal glands to endocrine stimulation;
4) Systems that are responsive to stress and changes in HPA axis activity, i.e. prolactin, insulin, and leptin.
Chapter 3.

Twenty four-hour profiles of metabolic and stress hormones in sheep selected for a calm or nervous temperament

Abstract

Even in the absence of stressors, temperament is associated with changes in the concentration of stress-responsive hormones and, possibly because of such changes, temperament can affect metabolism. Here we tested whether, in sheep bred for temperament for 14 generations, ‘nervous’ females have greater concentrations of stress-responsive hormones in the absence of stressors than ‘calm’ females, and whether these differences are associated with changes in the concentrations of metabolic hormones. In resting ‘calm’ (n = 8) and ‘nervous’ (n = 8) sheep, concentrations of cortisol, prolactin, leptin, and insulin were measured in blood plasma sampled via jugular catheter every 20 min for 24 h. The animals were individually penned, habituated to their housing and human handling over 7 wk, and fed before sampling began. Diurnal variation was evident for all hormones, but a 24-h cortisol pattern was detected in only 7 individuals. There was no effect of temperament on the concentrations of cortisol or prolactin (P > 0.05), but ‘calm’ animals had greater concentrations of insulin in the early afternoon than ‘nervous’ animals (14.5 ± 1.1 vs. 10.0 ± 1.6 μg/mL; P < 0.05), and a similar tendency was seen for leptin (P = 0.092). We conclude that selection for temperament affects the
concentration of metabolic hormones in the absence of stressors, but this effect is independent of stress-responsive hormones.

Introduction

Temperament determines, at least in part, how an individual responds to stressful situations and can vary widely among species and between genders (Boissy, 1995; Pottinger, 2000). It can change the pattern of secretion of the stress-responsive hormones, cortisol and prolactin, in both the absence and presence of stressors (Fava and Guaraldi, 1987; Pottinger, 2000; Charmandari et al., 2005) but can also reportedly affect metabolism. Hafez and Lindsay (1965) suggested that ‘nervousness’ increases the energetic cost of living, and several studies have shown a direct relationship between temperament and various indicators of metabolism. For example, sheep that have been genetically selected to be ‘nervous’ had a lower net feed intake (Amdi et al., 2010) and, had lower core body temperatures when fed below maintenance requirements than their ‘calm’ counterparts (Henry et al., 2010). This apparent relationship between temperament and metabolism is supported by studies in cattle, showing that calmer animals had greater growth rates, and lower plasma concentrations of glucose, lactate and non-esterified fatty acids at rest than animals that are more reactive to stressors (Burrow and Dillon, 1997; Cafe et al., 2011a; Cafe et al., 2011b) but the mechanism driving this relationship is unclear.

Cortisol and prolactin are secreted in pulses and there is a diurnal pattern in the blood concentration (Veldhuis et al., 1989; Van Cauter, 1990; Spiga et al., 2014) that, at least in the case of cortisol, appears to be affected by temperament. For example, Fischer rats are genetically more reactive to stress and have no diurnal variation in cortisol secretion, resulting in greater overall concentrations of cortisol
than the less reactive, Lewis rats, in the absence of stressors (Windle et al., 1998a). Similarly, in Rhesus macaques, personality traits indicative of temperament directly affected the diurnal pattern of cortisol secretion, in the absence of stressors (Capitanio et al., 2004). Specifically, animals classed as highly excitable had lower concentrations of cortisol in the afternoon (the trough of the rhythm), and animals exhibiting low confidence had lower concentrations of cortisol in the morning and lacked a decline in concentrations of cortisol in the afternoon (Capitanio et al., 2004). Whilst most of the studies cited above suggest that temperament can affect the resting activity of the HPA axis, no study, to our knowledge, has investigated the pattern of cortisol secretion in animals selectively bred for their temperament.

Prolactin has also been linked to temperament and stress-responsive behaviour, but most studies have looked at the response to stimuli, rather than resting concentrations (Manuck et al., 1998; Gerra et al., 1999; Gerra et al., 2000; Yayou et al., 2010). The magnitude of the prolactin response to stimuli is inversely correlated with the temperament traits of aggression and harm avoidance, and positively correlated with novelty and sensation-seeking traits, as well as the behavioural response to stress (humans: Manuck et al., 1998; Gerra et al., 1999; Gerra et al., 2000; cattle: Yayou et al., 2010). In humans, prolactin responses to stimuli are positively correlated with the cortisol response, so it is possible that the temperament of an individual could also affect the diurnal pattern of prolactin secretion (Freeman et al., 2000; Gerra et al., 2000; Lennartsson and Jonsdottir, 2011).

Endogenous differences in the diurnal pattern of stress-responsive hormones could help to explain the apparent effects of temperament on metabolism because cortisol directly effects the concentration of the two primary metabolic hormones, insulin and leptin. Glucocorticoids stimulate leptin secretion (Masuzaki et al., 1997; Sandoval and Davis, 2003) and acute increases in cortisol concentrations suppress
insulin secretion in response to nutrient intake (Pierluissi et al., 1986; Lambillotte et al., 1997). The role of prolactin as a regulator of metabolism is still being elucidated but, in rodents, depending on conditions, prolactin is reported to stimulate and potentiate leptin secretion (rats: Gualillo et al., 1999; mice: Viengchareun et al., 2004; review: Ben-Jonathan et al., 2006), although a suppressive effect has also been reported (rats: Mastronardi et al., 2000; mice: Ling and Billig, 2001; review: Ben-Jonathan et al., 2006). Prolactin appears to stimulate insulin secretion, as it induces transcription of the insulin gene in rat and human pancreatic cells in vitro and in mice deficient in prolactin receptors pancreatic islets are smaller, less dense and with lower insulin content, and they have an attenuated response to glucose challenge (rats: Crepaldi et al., 1997; rats and humans: Fleenor and Freemark, 2001; mice: Freemark et al., 2002; review: Ben-Jonathan et al., 2006). It is thus clear that the stress-responsive hormones, cortisol and prolactin, can affect the secretion of metabolic hormones, but it is not known whether an increased responsiveness to stressors can increase the concentrations of the stress-responsive hormones in a non-stressed state, and thereby lead to changes in metabolic endocrine balance.

To further our understanding of the relationship between temperament, the resting state of the hypothalamic-pituitary-adrenal (HPA) axis and metabolic hormones, we measured the concentrations of cortisol, prolactin, insulin and leptin, in sheep selectively bred for different temperaments. The ‘calm’ and ‘nervous’ sheep used in this study have been selected for over 14 generations for hypo- or hyper-reactivity to isolation and human presence; a phenotypic trait that is heritable and has a genetic basis (Blache and Bickell, 2011b). To detect any innate differences in the diurnal pattern of secretion of stress and metabolic hormones, the animals were sampled in the absence of any additional stressors (i.e. at rest). Specifically, we hypothesised that compared to ‘calm’ animals, the ‘nervous’ animals will have:
1) greater plasma cortisol concentrations and less pronounced variation in the amplitude of pulses over 24 h; 2) greater plasma concentrations of prolactin; and 3) greater plasma concentrations of leptin, with leptin values highest after the peak in the patterns of cortisol and prolactin concentrations; and 4) lower plasma concentrations of insulin.

**Methods**

**Animals**

This experiment was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and was approved by the Animal Ethics Committee of The University of Western Australia (RA/3/100/333).

The UWA temperament flock comprises two lines of Merino sheep that have been bi-directionally selected for 14 generations for extreme behavioural reactions to the stressors of social isolation and human presence. Locomotor activity and vocalisations are measured in two tests, as described by Murphy (1999). In brief, the first test is an isolation challenge, whereby animals are placed singularly in a solid plywood box (1.7 m x 1.2 m x 1.2 m), which prevents visual contact with conspecifics. Locomotor activity and high intensity vocalisations are measured by a digital agitation meter fitted on the side of the box, previously calibrated for low, medium, and high activity. The meter measures an accumulation of vibrations, and so it can measure high pitch vocalisations and high locomotive activity at the same time. The meter is placed on the long side of the box, at 2/3rd of its length and 4/5th of its height along. The second test is a novel arena test in which individuals are faced with the conflict of having to pass a human to be with peers. Mobility and vocalisations are recorded from both tests to calculate a temperament score, and
hypo-reactive sheep are termed ‘calm’ whilst hyper-reactive sheep are termed ‘nervous’ (Beausoleil et al., 2008; Blache and Bickell, 2011b). Males that show the most extreme score from each line are used to breed the subsequent generation of the temperament flock.

For the present experiment, 2-year-old ewes were selected from the calm (n = 8) and nervous (n = 8) lines of the UWA temperament flock. These animals had divergent scores in the arena test (CROSS: calm 6.3 ± 2.16 v nervous 18.4 ± 4.58 crosses; BLEAT: calm 5.3 ± 3.36 v nervous 39.4 ± 13.70) and isolation box text (BOX: calm 24.5 ± 10.13 v nervous 71.8 ± 7.70) conducted at weaning. Furthermore these animals were subjected to the isolation box test for 1 min, 2 d before commencing habituation to the animal house. The isolation test is a valid test for the emotional state of the animals because it forms part of the selection criteria for the flock, and the behavioural response has been shown to be robust and repeatable over the life of the animal (Blache and Ferguson, 2005). During the test, an animal was placed in a completely enclosed plywood box (1.5 m³) for 1 min, out of visual contact with peers (Murphy et al., 1994; Blache and Ferguson, 2005). The animal's movement and vocalisations were measured as described above.

For the duration of the experiment, animals were individually penned indoors, in 1 room, for 7 wk, beginning on November 16 (mid-non-breeding season), at the University of Western Australia (31° 58⁰ S). Photoperiod was 12L:12D with lights on at 0600 h. The animals were subjected to minimal human contact and were under constant video surveillance. Pen-cleaning and feeding followed a regular schedule, with the animals fed before 0700 h daily. Animals were fed a diet of hammer-milled oaten chaff with 20% lupin seed and 2.5% mineral mixture (Siromin, Narrogin Mineral Stockmix, Narrogin, WA, Australia) providing 9.2 MJ of metabolisable energy and 13% crude protein per kg DM. Each animal was fed
enough to maintain bodyweight. Feed intake was monitored, and all animals finished their daily ration within 90 minutes.

**Sampling**

After 38 d of habituation to the housing, the animals were fitted with an indwelling jugular cannula with an extension to the shoulder. The following morning, the animals were fed at 0600 h. We decided to feed the animals prior to the commencement of sampling, because although feeding stimulates secretion of leptin and insulin, missing a feed is a stressor, and not feeding the animals can potentially lead to hypoglycaemia, which can trigger cortisol and prolactin responses, and disrupt the normal circadian rhythm of these hormones (Copinschi et al., 1975; Nathan et al., 1979).

Blood sampling commenced at 0700 h and continued every 20 min for 24 h. Lights were kept on for sampling at night. Heparin (50 i.u. per 50 uL of saline; heparin sodium BP, Pfizer Australia, Sydney, NSW) was used to prevent blood samples from coagulating, and heparinised saline (5000 i.u. heparin sodium BP per litre) was used to flush cannulae between samples to keep cannulae patent. Samples were centrifuged at 2000 g for 10 min so that plasma could be separated, and stored in plastic tubes at −20°C until assay.

**Hormone analysis**

**Cortisol**

Plasma cortisol concentrations were measured in duplicate 100 µl aliquots after extraction with methylene chloride:ethanol (99:1 v/v) using a radioimmunoassay based on separation with dextran-coated charcoal (Abraham et al., 1972). The limit of detection was 0.20 ng/ml. Six replicates of two control samples were included in the assay to estimate the intra-assay coefficients of variation of 7.9 % at
Prolactin

Plasma prolactin was measured with a homologous double antibody RIA (Miller et al., 1995). All samples were processed in a single assay. The samples were assayed in duplicate 10 µl aliquots and the limit of detection was 0.26 ng/ml. The assay included six replicates of three control samples which were used to estimate the intra-assay coefficients of variation of 6.7 % at 0.5 ng/ml, 8.1 % at 1.0 ng/ml and 3.8 % at 2.0 ng/ml.

Insulin

Plasma insulin was assayed in duplicate by a double-antibody radioimmunoassay (Tindal et al., 1978). All samples were processed in a single assay and the limit of detection was 0.78 µU/ml. The assay included six replicates of three control samples which were used to estimate the intra-assay coefficients of variation of 6.7 % at 2.5 µU/ml, 5.2 % at 3.7 µU/ml and 5.6 % at 11.1 µU/ml.

Leptin

Plasma leptin was measured in duplicate by a double-antibody radioimmunoassay developed for sheep in our laboratory and described in detail by Blache et al. (2000). All samples were processed in a single assay and the limit of detection was 0.10 ng/ml. The assay included six replicates of three control samples which were used to estimate the intra-assay coefficients of variation of 1.9 % at 0.42 ng/ml, 6.8 % at 0.91 ng/ml and 4.9 % at 1.4 ng/ml.
Data analysis

Pulses of cortisol and prolactin secretion were detected with MUNRO, an adaptation of the PULSAR program (Merriam and Wachter, 1982), that was developed for the Apple Macintosh computer (‘Munro’, Zaristow Software, West Morham, Haddington, East Lothian, UK). This program identifies pulses using ‘G’ parameters, the number of standard deviations by which a peak must exceed the baseline to be accepted as a pulse, with the value of the parameter adjusted for the number of samples (1-5) comprising the putative pulse. For the present study, the G parameters were 3.98, 2.4, 1.68, 1.25, and 0.93 for G1-G5, respectively. To calculate the relevant standard deviation, MUNRO uses Baxter parameters that define the parabolic relationship between hormone concentration and standard deviation. For cortisol, the Baxter parameters were -0.751 (B1, the y-intercept), 0.3404 (B2, the x coefficient), and -0.00008 (B3, the x^2 coefficient); for prolactin, they were 0.6872 (B1), 0.0156 (B2), and 0.0003 (B3). MUNRO identifies pulses in the data set, and returns the timing, amplitude, area under the pulse curve, and nadir for each pulse.

The hormone data were arbitrarily divided into four equal time periods of 6 h: (0700–1300 h; 1300–1900 h; 1900–0100 h; 0100–0700 h) to allow analysis of variation in pulse characteristics over the 24-h day (Windle et al., 1998a). This approach was based on a previous method for description of diurnal variation in sheep (Fulkerson and Tang, 1979). The time periods encompass and separate phases in the cycle (peak, trough), and are long enough to include sufficient pulses (about 3) to enable statistical analysis.

All statistical tests were conducted using Genstat (Twelfth Edition, VSN International Pty Ltd, UK). Prior to statistical analysis, all data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using
Bartlett’s test. Where data were not normally distributed, or where variance was not homogenous across treatments, the data were transformed as described below. All data are presented as untransformed values for ease of interpretation.

For cortisol and prolactin, we compared the following pulse characteristics between time periods and between temperaments: amplitude, area under the pulse curve, nadir, baseline, mean concentrations, and pulse frequency. Pulse frequency was calculated as the number of pulses per h. Baseline concentrations for cortisol and prolactin were determined for each individual animal by selecting the 7 lowest values in each 6 h time period, and averaging these (Martin et al., 1983). Over a 24-h period, the lowest 28 points were used. Insulin and leptin concentrations were not pulsatile and so we analysed only the mean concentrations.

Pulse amplitude, area under the pulse curve, nadir, baseline, and mean concentrations for cortisol and prolactin, and mean concentrations of insulin and leptin were initially compared over time and between temperaments using repeated measures ANOVA. Differences were considered significant if $P \leq 0.05$, and a tendency if $P \leq 0.10$, and so $P \leq 0.10$ reported as exact values. Where significant differences were found, paired 2-tail t-tests were used to compare pulse characteristics among time periods, and Student’s t-tests were used to compare characteristics between temperaments in the same time period. For both cortisol and prolactin, values for the pulse amplitude, area under the pulse curve, nadir, baseline and mean concentration were transformed logarithmically (base 10) before analysis to correct for variance being proportional to the mean. Total number of pulses over 24 h was compared between temperament groups using Student’s t-test.

Pulse frequency data were not normally distributed and so were compared among time periods using Friedman’s test and between temperaments using the Mann-
Whitney U test. Where Friedman’s test showed significance, time period pairs were compared using Wilcoxon Matched-Pairs tests.

To analyse the diurnal variation in hormone patterns, all hormone profiles were fitted with a cosine curve using cosinor analysis, restricted to a 24 h period (Refinetti, 2006). Where the fit of the curve was significant, the mesor, amplitude, and timing of the acrophase (in degrees) of the curve were returned. Not all hormone profiles returned a significant cosine curve, restricting the sample size to 7 animals for cortisol, 12 for prolactin, 15 for insulin, and 13 for leptin (Table 3.1). The acrophase, mesor, and amplitude of the curves were compared between temperaments using 2-tailed t-tests.

Because only 7 out of 16 cortisol profiles returned a significant cosinor pattern, and this outcome was not associated with temperament, it appeared that there were 2 groups of animals, those with and those without diurnal variation. Therefore, the cortisol dataset was re-analysed with the relevant statistical tests, as described above, but including the presence or absence of a rhythm as a factor.

Scores from the isolation box test were compared between the temperaments using Student’s t-test.

Results

Isolation box test

Nervous animals had significantly greater agitation scores from the isolation box test than calm animals (nervous $54 \pm 9.1$ vs calm $21 \pm 5.2$ (arbitrary units); $P = 0.007$).
Cortisol

All of the animals showed discrete pulses of cortisol secretion over 24 h, with pulses peaking up to 35 ng/mL (Figure 3.1). The total number of pulses over 24 h (mean ± S.E. shown throughout; calm 13.4 ± 0.5 vs. nervous 12.8 ± 0.6; P > 0.10) was similar between temperaments.

There was a significant effect of time on mean concentrations (P = 0.043), and baseline (P = 0.033), but not on area under the pulse curve, or nadir (all P > 0.10; Figure 3.2). Pulse frequency varied with time in both temperaments (calm: P = 0.016, nervous: P = 0.043; Figure 3.2). Pulse amplitude varied greatly, both within (c.v. 69 ± 4.5 %) and between (c.v. 39 %) individuals and, although each animal appeared to have a pattern of larger and smaller peaks, the patterns were not consistent among individuals (Figure 3.1) and so there was no difference in pulse amplitude among time periods (P > 0.10; Figure 3.2). There was no effect of temperament or an interaction between time and temperament on any characteristic of cortisol secretion.

Specifically, pulse frequency was decreased between 0700 – 1300 h compared to 1300 – 1900 h (P < 0.001) and 1900 – 0100 h (P = 0.039), and decreased between 0100 – 0700 h compared to 1300 – 1900 h (P = 0.003; Figure 3.2), regardless of temperament. Baseline concentrations of cortisol were greater between 0100 – 0700 h than between 0700 – 1300 h (P = 0.0013), 1300 – 1900 h (P = 0.017), and 1900 – 0100 h (P = 0.025; Figure 3.2).

Mean concentrations of cortisol were greater between 0100 – 0700 h than between 0700 – 1300 h (P = 0.043) and 1300 – 1900 h (P = 0.024; Figure 3.2).

Cosinor analysis of cortisol profiles returned a significant curve for 7 out of 16 animals (Table 3.1). Within these animals, calm animals had a lower minimum
than nervous animals (P = 0.034), but there was no difference between temperaments in mesor, amplitude, acrophase, or maximum.

The cortisol dataset was re-analysed including the presence or absence of a diurnal rhythm as a factor in the ANOVA model. There was an effect of an interaction between time, temperament and presence of a diurnal rhythm on mean cortisol concentrations (P = 0.028) and a tendency for an effect on area under the pulse curve (P = 0.051; Figure 3.2). There was an effect of an interaction between temperament and presence of a diurnal rhythm on mean cortisol concentrations (P = 0.006), pulse amplitude (P = 0.048), and pulse nadir (P = 0.003). There was an effect of time on mean cortisol concentrations (P = 0.023) and baseline concentrations (P = 0.009). There was a tendency for an effect of temperament on pulse nadir, with nervous animals having slightly greater concentrations than calm animals (P = 0.075).

Between 0700 – 1300 h calm animals with a diurnal rhythm (DR) had lower mean cortisol concentrations than calm animals with no diurnal rhythm (NDR; P = 0.005), or any nervous animals (nervous DR: P = 0.044, nervous NDR: P = 0.043; Figure 3.2). Calm DR animals also had lower mean cortisol concentrations compared to calm NDR (P = 0.010) and nervous DR animals (P = 0.002) between 1300 – 1900 h. Regardless of time period, calm DR animals had lower mean cortisol concentrations than calm NDR (P = 0.002) and nervous DR animals (P < 0.001); nervous NDR animals had lower mean cortisol concentrations than calm NDR (P = 0.024) and nervous DR (P = 0.002) animals; and calm NDR tended to have lower mean concentrations than nervous NR (P = 0.062). Regardless of temperament and presence of a diurnal rhythm, mean cortisol concentrations were greater between 0100 – 0700 h than 0700 – 1300 h (P = 0.043) and 1300 – 1900 h (P = 0.024).
Figure 3.1. Examples of 24-h profiles in the plasma concentrations of stress and metabolic hormones in 4 ewes that had been genetically selected for either a calm or nervous temperament. Cortisol and prolactin pulses, as determined by the analysis program Munro, are marked by an asterisk. Profiles were chosen randomly.
Characteristics of cortisol secretion over 24 h in anoestrous Merino ewes bred for calm (n = 8) or nervous (n = 8) temperament that lack (black bars) or display a diurnal rhythm (white bars) in cortisol secretion as determined by cosinor analysis. Bars indicate significant difference between groups within temperament and time period. For other significant differences, please refer to text (all values are mean ± S.E).

Regardless of time, nervous DR had greater pulse amplitudes than calm DR (P = 0.037) and nervous NDR (P = 0.021), and tended to be greater than calm NDR (P = 0.063). Furthermore, calm DR had lower pulse nadirs than calm NDR (P < 0.001), nervous DR (P < 0.001) and NDR (P = 0.018), regardless of time period. Nervous DR had greater pulse nadirs than calm NDR (P = 0.029) and nervous DR (P = 0.004). Presence of a rhythm did not affect pulse frequency (P > 0.10).

**Prolactin**

All of the animals showed discrete pulses of prolactin secretion over 24 h (Figure 3.1), and there was no difference between the temperament lines in the number of pulses detected over 24 h (calm 12.9 ± 0.5 vs. nervous 12.3 ± 0.9, P > 0.10).
Table 3.1. Coefficients of a cosinor model fitted to 24 h plasma hormone profiles of calm and nervous ewes. Values shown are the mean ± S.E for only animals that showed a significant rhythm in plasma hormone concentration, as determined by cosinor analysis.

<table>
<thead>
<tr>
<th></th>
<th>Mesor (ng/mL)</th>
<th>Amplitude (ng/mL)</th>
<th>Acrophase (Degrees ± min)</th>
<th>Maximum (ng/mL)</th>
<th>Minimum (ng/mL)</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Cortisol</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calm</td>
<td>6.1 ± 0.32</td>
<td>2.6 ± 0.43</td>
<td>227 ± 12 (2206 ± 48 min)</td>
<td>8.7 ± 0.75</td>
<td>3.5 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td>Nervous</td>
<td>7.7 ± 1.49</td>
<td>3.0 ± 0.71</td>
<td>173 ± 53 (1833 ± 213 min)</td>
<td>10.6 ± 2.19</td>
<td>4.7 ± 0.81 #</td>
<td>4</td>
</tr>
<tr>
<td><strong>Prolactin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calm</td>
<td>58 ± 3.4</td>
<td>27 ± 5.2</td>
<td>229 ± 19 (2215 ± 78 min)</td>
<td>85 ± 7.2</td>
<td>31 ± 4.9</td>
<td>6</td>
</tr>
<tr>
<td>Nervous</td>
<td>60 ± 7.6</td>
<td>31 ± 14.3</td>
<td>199 ± 18 * (2014 ± 73 min)</td>
<td>91 ± 21.5</td>
<td>29 ± 7.7</td>
<td>6</td>
</tr>
<tr>
<td><strong>Insulin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calm</td>
<td>10.7 ± 0.96</td>
<td>3.3 ± 0.35</td>
<td>111 ± 7 (1424 ± 27 min)</td>
<td>14.0 ± 1.14</td>
<td>7.40 ± 0.89</td>
<td>8</td>
</tr>
<tr>
<td>Nervous</td>
<td>9.8 ± 0.97</td>
<td>2.5 ± 0.44 #</td>
<td>128 ± 30 (1534 ± 120 min)</td>
<td>12.3 ± 1.14 *</td>
<td>7.27 ± 1.00</td>
<td>7</td>
</tr>
<tr>
<td><strong>Leptin</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calm</td>
<td>1.9 ± 0.06</td>
<td>0.2 ± 0.02</td>
<td>125 ± 7 (1520 ± 29 min)</td>
<td>2.0 ± 0.07</td>
<td>1.7 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td>Nervous</td>
<td>1.8 ± 0.14</td>
<td>0.2 ± 0.03</td>
<td>111 ± 11 * (1425 ± 44 min)</td>
<td>1.9 ± 0.13</td>
<td>1.6 ± 0.14</td>
<td>7</td>
</tr>
</tbody>
</table>

# Different from calm, P < 0.05
* Tendency to be different from calm, P < 0.10

There was a significant effect of time on the baseline (P = 0.021), mean (P = 0.006) and nadir (P = 0.023) concentrations of prolactin and a tendency for an effect on area under the pulse curve (P = 0.053). As for the cortisol profiles, the amplitude of prolactin pulses appeared to vary throughout the day for individual animals but was highly variable both within (c.v. 77 ± 6.5 %) and between (c.v. 63 %) animals (Figure 3.1 and 3.3), and no effect of time was found on pulse amplitude (P > 0.10). There was no effect of temperament on the baseline, mean concentration, nadir, or pulse amplitude, or area under the pulse curve concentrations of prolactin (all P > 0.10). There was no effect of an interaction between temperament and time on the baseline, mean concentration, nadir, or pulse amplitude, or area under the pulse curve concentrations of prolactin (all P > 0.10). Nervous animals had a decreased pulse frequency between 1900 – 0100 h compared to calm animals (P = 0.011;
Figure 3.3), although neither temperament showed diurnal variation in pulse frequency (both \( P > 0.10 \); Figure 3.3). Mean concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h \( (P = 0.013) \), 1900 – 0100 h \( (P = 0.0078) \), and 0100 – 0700 h \( (P = 0.016; \) Figure 3.3). Similarly, baseline concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h \( (P = 0.0042) \), 1900 – 0100 h \( (P = 0.024) \), and 0100 – 0700 h \( (P = 0.019; \) Figure 3). Also, nadir concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h \( (P = 0.028) \), 1900 – 0100 h \( (P = 0.0023) \), and 0100 – 0700 h \( (P = 0.033; \) Figure 3.3).

Specifically, the mean, baseline, and nadir concentrations of prolactin were lower than any other time period in the morning \( (0700 – 1300 \) h; Figure 3.3; At least \( P < 0.05 \)), with pulse area tending to do the same.

Cosinor analysis of prolactin concentrations yielded significant curves for 12 out of 16 animals (Table 3.1). The acrophase was advanced in the nervous animals compared to the calm animals by around 2 h \( (P = 0.047) \). However, there was no difference in the mesor, amplitude, minimum, or maximum between temperaments (all \( P > 0.10; \) Table 3.1).

**Leptin**

There was an overall effect of time on plasma concentrations of leptin \( (P < 0.001; \) Figures 3.1 and 3.4) but no effect of temperament \( (P = 0.191) \) and a tendency for an interaction between temperament and time \( (P = 0.092) \). Specifically, plasma concentrations of leptin were highest between 0700 – 1900 h, and lowest between 0100 – 0700 h \( (all \ P < 0.001; \) Figure 3.4).

Cosinor analysis showed that 13 animals (calm: \( n = 6 \); nervous \( n = 7 \), Table 3.1) had a significant diurnal rhythm in leptin secretion. Within these animals, there was a
Figure 3.3. Characteristics of plasma prolactin pulses over 24 hours in anoestrous Merino ewes bred for calm (white bars) or nervous (black bars) temperament. Averages of time periods shown in grey (all values are mean ± S.E; n = 16).

Figure 3.4. Plasma leptin and insulin concentrations over 24 h in anoestrous Merino ewes bred for a calm (white bars) or nervous (black bars) temperament. Averages of time periods shown in grey (all values are mean ± S.E; n = 16).
tendency for nervous animals to have an advanced acrophase compared to calm animals (P = 0.052; Table 3.1). Temperament did not affect the mesor, amplitude, minimum, or maximum of the fitted cosine curves (all P > 0.10; Table 3.1).

**Insulin**

There was an effect of time (P < 0.001) but not temperament (P = 0.110) on plasma concentrations of insulin (Figure 3.4). However, there was an interaction between temperament and time (P = 0.033). Plasma concentrations of insulin were greater during 0700 – 1300 h than between 1900 – 0100 h (P = 0.002) and 0100 – 0700 h (P < 0.001). Similarly, plasma concentrations of insulin were greater during 0700 – 1300 h than between 1900 – 0100 h (P < 0.001) and 0100 – 0700 h (P < 0.001).

Mean plasma concentrations of insulin were greater in calm than nervous animals between 1300 – 1900 h (P = 0.038) and tended to be greater between 0700 – 1300 h (P = 0.063; Figure 3.4).

Cosinor analysis of insulin concentrations yielded significant curves for 15 out of 16 animals (calm: n = 8; nervous n = 7, Table 3.1). Within these animals, cosinor curves fitted to calm animals had a greater amplitude than in the nervous animals (P = 0.019; Table 3.1), and tended to have a greater maximum (P = 0.063; Table 3.1).

Temperament had no effect on the mesor, acrophase, or minimum of the cosinor curve of plasma concentrations of insulin (all P > 0.10; Table 3.1).

**Discussion**

Temperament did not affect the pulse characteristics, mean concentrations, or the expression of a diurnal variation of cortisol or prolactin, but nervous animals had decreased concentrations of insulin in the afternoon hours, resulting in a smaller
diurnal variation, than in calm animals. There was a similar tendency for leptin concentrations. We therefore conclude that, in the absence of stressors, selection for temperament does not affect the activity of the HPA axis or prolactin secretion, but does lead to differences in the concentrations of insulin, indicating possible effects on the regulation of glucose homeostasis and perhaps adipose homeostasis.

The lack of a difference between calm and nervous sheep in the pattern or magnitude of cortisol secretion indicates that selection for behavioural reactivity to the stressors of isolation and human presence does not affect the resting activity of the HPA axis. The minimum of the cosinor curve describing the pattern of cortisol concentrations was decreased in calm animals compared to nervous animals, however, this result must be interpreted with caution, as it is based on a subset of animals (n = 7), and was not reflected in the baseline or pulse nadir concentrations of cortisol in the full set of animals. Our findings are consistent with a rodent model of anxiety, where high anxiety (HAB) rats secrete more glucocorticoids and ACTH when faced with their selection stressor than low anxiety (LAB) rats, but have similar concentrations of corticosterone and ACTH at rest (Landgraf et al., 1999). Our experiment was conducted in anoestrous ewes, and because of the interactions between sex steroids and the HPA axis we acknowledge that we may have had different findings if our experiment was conducted in oestrous-cycling ewes or males (Tilbrook et al., 2000). It is possible that stage of the oestrous cycle can affect the diurnal activity of the HPA axis, and for this reason, we chose to maximize our ability to detect a difference in HPA axis activity by eliminating potentially confounding effects and conducting the experiment in anoestrous ewes.

We conclude that in anoestrous ewes selected for temperament the resting activity of the HPA axis is not affected by the mechanisms that drive the divergence in cortisol secretion in response to the stressors of isolation and novelty (Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013).
A diurnal pattern of cortisol secretion was detected in less than half of the animals, and was independent of temperament. Although it is widely accepted that cortisol is secreted in a diurnal pattern in a number of species (Refinetti, 2006), it is not unusual for individuals within a population to not display a diurnal rhythm in cortisol secretion (Smyth et al., 1997; Ice et al., 2004). Within sheep, the existence of a diurnal rhythm itself is questionable because the studies that detected a rhythm used only 3 animals. Because the animals were kept in a single room under identical conditions, were of similar age and weight, and the experiment was conducted in the middle of the non-breeding season, thus eliminating any effects of the oestrous cycle (Becker et al., 1985), it is difficult to attribute our findings to any factor. Interestingly, in horses the absence of routine and/or hyper-reactivity of an individual to the sampling protocol was found to likely to result in enhanced cortisol secretion that masks the daily rhythm (Irvine and Alexander, 1994). Calm and nervous sheep have similar cortisol responses to some novel stressors (Hawken et al., 2013), and so the reactivity of the individual animals to the change of routine on the day of sampling could have affected their diurnal pattern of cortisol secretion. The incidence and biological significance of a diurnal rhythm in cortisol secretion in sheep requires further investigation.

Interestingly, analysis of the cortisol data, including the presence of a diurnal rhythm as a factor into the statistical model, suggests that temperament can affect the HPA axis activity but only in animals that present a diurnal rhythm. This observation reflects data from South African Merino sheep that had been selected for high multiple-rearing ability and that are less behaviourally responsive during an arena test, indicating a ‘calm’ temperament (Cloete et al., 2005). In these sheep, the magnitude of the cortisol response to an insulin challenge is affected by an interaction between phenotype and a polymorphism in a gene coding for a steroidogenic enzyme, CYP17, that controls cortisol production (Hough et al.,
Thus, the subtle differences between subsets of temperament groups in the current study could be due to polymorphisms affecting adrenal steroidogenesis. The small sample size restricts interpretation, but these observations suggest a need for further investigation into the relationship between temperament and the HPA axis.

The diurnal variation that we observed in the pulse characteristics of cortisol, across all animals, contrasted with observations in other species in that there was no variation in pulse amplitude (Veldhuis et al., 1989; Windle et al., 1998a). Instead, variation was seen in the pulse frequency and the baseline of the cortisol profile. Changes in pulse amplitude throughout the day might not be typical for sheep, but pulse frequency or baseline may play a more important role in diurnal variation. Few studies have directly examined the baseline values for cortisol concentration, but Young et al. (2001) found that pulse modelling fits human cortisol profiles poorly unless a changing baseline is factored in. Diurnal variation in cortisol pulse frequency has been seen in humans (Veldhuis et al., 1989) and rats (Windle et al., 1998a; Windle et al., 2001), and is deemed an important contributor to the diurnal variation in mean cortisol concentrations in male rats (Windle et al., 2001). It is known that pulsatile and constant process of cortisol release reflect different gene transcription responses, and that a minimum frequency of pulses is necessary to maintain mature mRNA concentrations (Dallman, 2007). From our studies, we cannot conclude whether pulse frequency or baseline play any meaningful role in the diurnal variation of HPA axis activity.

Plasma concentrations of insulin were decreased in nervous animals compared to calm animals only in the afternoon, at the peak of the pattern, indicating an interaction between temperament and the diurnal variation in the secretion of insulin. The variation in insulin concentrations over the day may reflect a true circadian rhythm as, in monogastric animals, there appears to be a circadian
rhythm in insulin secretion even when blood glucose concentrations are constant (Boden et al., 1996; Peschke and Peschke, 1998); however, the decline in insulin concentrations over the day may also simply reflect that the animals were fed 1 hour prior to sampling (Bassett, 1974). Differences in insulin concentrations between temperaments could be related to the apparent difference between temperaments in the minimum of the cortisol rhythm, but, as discussed above, the finding for cortisol is based on a subset of animals, and so must be interpreted with caution. Nevertheless, our findings are supported by other studies of temperament in ruminants. For instance, calm bulls have greater concentrations of insulin than ‘temperamental’ bulls, both before and in response to an endotoxin challenge (Burdick Sanchez et al., 2014), whilst ‘temperamental’ heifers presented a greater insulin response to cannulation than calm heifers (Bradbury et al., 2011). Our findings suggest that hyper-reactivity to stress is associated with a greater energy requirement that, in turn, leads to greater circulating concentrations of blood glucose and therefore decreasing concentrations of insulin. This concept is supported by studies in cattle, where calm animals have greater growth rates and carcass weights than ‘temperamental’ animals (Burrow and Dillon, 1997; Cafe et al., 2011a). Interestingly, temperament differences in plasma concentrations of metabolites in response to handling were not correlated with HPA axis activity (Cafe et al., 2011a). Overall, it appears that divergence between temperament groups in metabolic factors is not mediated solely by the HPA axis, but more likely to involve other systems, such as the sympatho-adrenal-medullary system.

However, the relationship between temperament and metabolism in the calm and nervous sheep is far from simple and appears to be dependent on environmental factors such as nutrition. For example, nervous sheep fed at maintenance produce less colostrum than calm sheep; however, when animals were supplemented with barley, this difference disappeared, indicating that there may be a nutrition x
genotype interaction in the control of the metabolism of calm and nervous sheep (Hawken et al., 2012b). Similarly, diet determined whether there were differences in core body temperature or adipose tissue temperatures in response to feeding in calm and nervous sheep (Henry et al., 2010). In summary, the relationship between temperament and metabolism is highly complex, but the observation that nervous female sheep fed at maintenance have decreased concentrations of insulin in the afternoon compared to calm sheep offers an insight into the mechanism behind the relationship between temperament and metabolism.

Contrary to our hypothesis, plasma concentrations of prolactin were not related to temperament, an observation that is coherent with the lack of difference between the temperament groups in cortisol secretion because the two hormones are regulated by some of the same mechanisms (Naylor et al., 1990; Maruyama et al., 2001; Mogi et al., 2008; Kaewwongse et al., 2011). Therefore, it is possible that, as with cortisol, prolactin secretion will differ between the temperament groups only during exposure to a stressor; however, no study to date has looked at prolactin concentrations in these sheep. This concept is supported by a study in heifers where behavioural reactivity to stressors was correlated with the prolactin response to those stressors, but not with concentrations of prolactin at rest (Kasuya et al., 2010). In contrast to our findings, in both the HAB/LAB and Maudsley reactive and non-reactive models, the line of rats that appear more disturbed by stressors have greater plasma concentrations of prolactin at rest than rats that are less disturbed, in the absence of any differences in corticosterone or ACTH (Blizard et al., 1977; Abel, 1991; Landgraf et al., 1999). These discrepancies may reflect differences between species and in protocols for selection and assessment of temperament. We therefore need to test whether prolactin concentrations change in parallel with cortisol concentrations in the calm and nervous sheep subjected to their selection stressors.
In summary, selection for fear-related temperament affects the magnitude of the response of HPA axis to stressors of sheep, but does not alter HPA activity in the absence of stressors in anoestrous ewes. However, selection for temperament was associated with changes in insulin secretion, through an unknown process possibly independent of the HPA axis. This effect could explain the differences in metabolism reported for calm and nervous animals.
Chapter 4.

Does temperament affect the susceptibility of sheep to developing chronic stress?

Abstract

Continued or repeated exposure to stressors can induce a state of chronic stress in which the hypothalamic-pituitary-adrenal (HPA) axis becomes hyperactive, with flow-on effects on other processes such as metabolism and immunity. Because the perceived intensity of stressors affects the development of chronic stress, individuals who are genetically predisposed to being ‘nervous’, or hyper-responsive to stressors, may be more susceptible to becoming chronically stressed than hypo-responsive, or ‘calm’ peers. We hypothesised that nervous sheep would have a more active HPA axis and greater circulating concentrations of insulin after repeated exposure to acute stressors than calm animals or nervous animals not exposed to stressors. Calm and nervous castrated male sheep (n = 32) were either exposed to 15 different acute stressors or kept at pasture (non-stressed control) over 3 wk. On the last day of stressor exposure, stressed (n = 8) and control (n = 8) animals were individually penned and, on the following day, blood was sampled every 10 min for 6 h to measure cortisol and insulin concentrations in the absence of stressors. Animals (n = 30) were also tested for their behavioural and endocrine response to an acute isolation stressor. There was no effect of the chronic stress treatment on behavioural reactivity to a stressor, HPA axis activity or insulin concentrations. Nervous animals had a longer cortisol response and greater behavioural response
to the acute stressor than calm animals, regardless of treatment. Our chronic stress model might not have been sufficiently intense to induce chronic stress, or our measurements were not suited to the detection of chronic stress.

**Introduction**

The stress response is a multi-faceted response that enables an individual to cope with and remove threats. The behavioural response aims to physically escape or remove the stressor, whereas the physiological response of the hypothalamic-pituitary-adrenal (HPA) axis maximises availability of biological resources to essential functions while restricting non-essential processes (Moberg, 2000). These responses are highly effective when dealing with acute stressors. However, problems arise when the stressors are prolonged or repeated frequently, resulting in a chronic stress that is associated with changes in both the behavioural and HPA responses to stressors. Individuals often become habituated to a specific stressor after multiple exposures (Weiss et al., 1975; Dal-Zotto et al., 2000; Dallman et al., 2002) but can nevertheless show increased behavioural and HPA responses to other stressors (Ottenweller et al., 1989; Van Dijken et al., 1992; Bhatnagar and Dallman, 1998; Ladewig, 2000; Wagner et al., 2011). During chronic stress, the HPA axis can also be hyperactive in the absence of stressors (Janssens et al., 1995b; van der Staay et al., 2010). For example, compared to control animals, chronically stressed pigs behave more fearfully in response to a novel object test (Wemelsfelder et al., 2000) and have elevated concentrations of glucocorticoids in the absence of stressors (Janssens et al., 1995b; van der Staay et al., 2010). Similarly, compared to controls, chronically stressed rats have greater concentrations of glucocorticoids in the absence of stressors, behave more fearfully in response to stressors (Ottenweller et al., 1989), and have increased
adrenocorticotrophin and glucocorticoid responses to stressors (Bhatnagar and Dallman, 1998).

Hyper-activity of the HPA axis induced by chronic stress can result in prolonged suppression of important processes such as metabolism, reproduction and immunity (Elsasser et al., 2000; Moberg, 2000; Tilbrook et al., 2002). For example, pigs exposed to unpleasant human handling have greater concentrations of glucocorticoids, slower growth rates, lower pregnancy rates in females, and smaller testes and delayed development of mating behaviour in males, compared to pigs exposed to pleasant human handling (Hemsworth et al., 1981, 1986). In particular, changes in measures of metabolism are frequently associated with chronic stress (Elsasser et al., 2000). For example, compared to control animals, chronically stressed rats gain less weight (Bhatnagar and Vining, 2003) and show increased symptoms of insulin resistance (Fu et al., 2009). In humans, chronic stress is associated with different patterns of fat deposition (Raikkonen et al., 1996; Dallman et al., 2005), symptoms of insulin resistance, including greater concentrations of insulin and glucose (Raikkonen et al., 1996), and higher incidences of metabolic syndrome (Chandola et al., 2006).

Individual susceptibility to the development of chronic stress might depend on the temperament of an individual. Fear-related temperament assesses the responsiveness of individuals to fear-eliciting stimuli (Boissy, 1995) and individuals of a more responsive temperament may be more at risk of developing chronic stress and associated problems than their less responsive counterparts because they are more fearful and highly disturbed by stressors. This seems to be the case in quail. Calandreau et al. (2011) exposed non-selected quail and quail bred for high or low emotional reactivity to a series of acute stressors. After exposure, quail with high emotional reactivity showed decreased behavioural activity in an open field test and decreased basal glucocorticoid concentrations, interpreted as a marker for
stress in this species, compared to non-exposed birds from the same line. However, exposure to the series of stressors had no effect on behaviour or glucocorticoid concentrations in quail with lower or intermediate emotional reactivity. Similarly, in mice, selection for low anxiety appears to protect individuals from developing chronic stress compared to mice that had been unselected or selected for high anxiety (Fuchsl et al., 2014).

In this study, we tested whether fear-related temperament affects the susceptibility to development of chronic stress in sheep. We used sheep selectively bred for a ‘calm’ or ‘nervous’ temperament based on their behavioural responsiveness to two stressors: human presence and isolation. The nervous animals are relatively hyper-reactive, having a greater behavioural and cortisol responses to acute stressors than their hypo-reactive calm counterparts (Blache and Bickell, 2011b; Hawken et al., 2012a). We hypothesise that 1) after exposure to a series of acute stressors, all animals will become mildly chronically stressed, and have greater baseline cortisol and insulin concentrations than animals not exposed to the stressors; 2) of the animals exposed to the stressors, nervous sheep will have greater baseline cortisol and insulin concentrations than calm sheep; and 3) animals exposed to the series of acute stressors will have greater behavioural and cortisol responses to an acute stressor than control animals, and nervous individuals will be more responsive than calm animals to the same treatment.

Methods

Animals

This experiment was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (8th Edition, 2013
and was approved by the Animal Ethics Committee of The University of Western Australia under RA/3/100/995. 

Castrated, 18-month-old male sheep (n = 32) were selected from the 'UWA Temperament Flock', a flock comprising two lines that have been bi-directionally, selectively bred for over 20 generations for extreme hyper- or hypo-reactivity to the stressors, isolation and human presence (Putu, 1988; Murphy et al., 1994). Briefly, animals are subjected to two tests at weaning. The first test is an open field test in which animals are presented with a conflict of choice – join their peers or remain distant from the human. During the second test, animals are placed in a solid box (1.2 x 1.2 x 1.7 m), visually isolating them from peers. In both tests, locomotor activity and vocalisations are recorded and used to produce a temperament score (Beausoleil et al., 2008; Blache and Bickell, 2011b). Animals with a low test score (i.e., low vocalisation intensity and locomotor activity) are termed ‘calm’, whereas the most reactive animals are termed ‘nervous’.

**Experimental procedures**

The experiment comprised three phases In Phase 1, sheep were either exposed to 15 acute stressors over 3-week period (repeated stressor model) or kept undisturbed at pasture (control) at Allandale Farm, the University of Western Australia, Wundowie (31°47’ S). Animals exposed to the series of acute stressors were kept at pasture, and brought in for the stressor exposure. In Phase 2, the animals were housed in individual pens in a shed under natural lighting for assessment of the plasma cortisol and insulin concentrations at rest (i.e., in the absence of stressors). In Phase 3, the behavioural and cortisol response of the sheep to an acute stressor was measured in calm and nervous sheep that had or had not been subjected to the chronic stress paradigm of Phase 1.

**Phase 1: Series of acute stressors**
Calm and nervous animals were assigned to control and treatment groups that were balanced for body mass (calm, control: 36 ± 2.2 kg, n = 8; calm, treatment: 37 ± 2.0 kg, n = 8; nervous, control: 37 ± 1.9 kg, n = 8; nervous, treatment: 36 ± 2.0 kg, n = 8). Animals were subjected to a series of 15 acute stressors over 3 weeks in an attempt to induce chronic stress. Treatment animals were divided into 2 groups that began the series of acute stressors 1 week apart. The stressors were chosen from standard farm procedures known to be stressful to sheep (timetable and references shown in Table 4.1).

*Individual restraint*

Individual animals were restrained against yard sides using a harness for 30 min. During the 30 min, animal handlers monitored animals from a distance.

*Isolation and ‘blower’*

Individual sheep were placed inside a solid plywood box (1.7 m x 1.2 m x 1.2 m), out of visual contact with peers. Animals remained there for 30 min. After 15 min, a panel on the box was opened, and a ‘blower’ situated 1.5 m from the panel in the box was switched on for the remaining 15 min. The blower was directed upwards, and was fitted with long plastic streamers that waved and also made a noise.

*Weighing*

Animals were led into a weigh crate that closes to keep the animal on the scales, and were held there for 2 min before being let out.

*Sheep-tipping crate*

Animals were put into a wool-tipping crate designed to hold a single animal and tip it sideways, to an almost horizontal position, to enable sampling of wool from the
mid-side. Animals were restrained in the crate and then held in the horizontal position for 2 min.

*Truck transportation*

Animals (n = 8) were transported for 90 min in the back of a vehicle fitted with an enclosed cage and rubber matting on the floor to prevent slipping. During the trip, driving speeds varied, and the vehicle stopped 3 times during the journey.

*Yarding and drafting*

Animals (n = 16) were moved around yards and drafted through a race for 90 min. During this time, animals were moved through the yards in directions that were unfamiliar to them, and were held for short amounts of time (5-10 min) in different areas of the yards. Animals were also drafted into differing, randomised groups four times, and these new groups were held in separate yards. During the 90 min protocol, the handler remained near the animals, had frequent gentle physical contact with them, and spoke loudly.

*Sham shearing*

Individual animals were laid back on their haunches and a covered clippers were run over their entire body for 5 min to imitate shearing. The clippers were switched on throughout the procedure to give the sounds and vibrations of shearing, but the blade was covered to prevent wool removal.

*Drench*

Animals were individually restrained and administered an oral dose of 20 mL water with a drench gun (Drench-matic 23mL; Henke-Sass Wolf, Germany). Animals were held in a single file race while the procedure was performed on each individual.
Group restraint and dog

The animals (n = 16) were held in a single pen (approximately 1.8 x 1.8 m) that was too small for the animals to move around easily. The animals were monitored for 120 min. After 110 min, a trained sheep dog was led around the outside perimeter of the pen for 10 min, stopping at each side of the pen twice for 1 min each time.

Foot bath

Animals were walked through a single file race containing a foot bath filled with approximately 3 cm of water. Animals were restrained in the foot bath to ensure they stepped into it and remained there for 1 min.

Random noise and indoor housing

Animals were held in 2 groups (n = 8 per group) in indoor housing for 4 h. During this period, an alarm that emitted 4 different alarm sounds up to 120 dB in volume was activated for a random duration (1 – 15 s) at random intervals (1 s – 25 min). During this period, handlers remained outside of visual contact with sheep, and remotely operated the alarm.

Novel stock-handler

An unfamiliar or ‘novel’ person performed the Yarding and drafting routine as described above. The novel handler remained in close proximity with the animals throughout the procedure, occasionally touched them, and spoke loudly. Familiar handlers remained at a distance from the sheep, and did not work with the animals.
Table 4.1. Schedule of acute stressors applied to castrated male sheep (n = 16) with a genetically-based calm or nervous temperament to induce a state of chronic stress.

<table>
<thead>
<tr>
<th>Day</th>
<th>Procedure</th>
<th>Time (min)</th>
<th>Procedure</th>
<th>Time (min)</th>
<th>Evidence of stressfulness to sheep</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Individual restraint</td>
<td>30</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Isolation + blower</td>
<td>15+15</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Weighing</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Wool tipping crate</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Truck transportation</td>
<td>90</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Yarding and drafting</td>
<td>90</td>
<td>Yarding and drafting</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>Sham shearing</td>
<td>2</td>
<td>Sham shearing</td>
<td>2</td>
<td>Hargreaves and Hutson, 1990b</td>
</tr>
<tr>
<td></td>
<td>Drench</td>
<td>1</td>
<td>Drench</td>
<td>1</td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td>13</td>
<td>Group restraint &amp; dog</td>
<td>110+10</td>
<td>Group restraint &amp; dog</td>
<td>110+10</td>
<td>Harlow et al., 1987; Komesaroff et al., 1998</td>
</tr>
<tr>
<td>15</td>
<td>Foot bath</td>
<td>1</td>
<td>Foot bath</td>
<td>1</td>
<td>Doyle et al., 2011</td>
</tr>
<tr>
<td>16</td>
<td>Random noise + indoor housing</td>
<td>240</td>
<td>Random noise + indoor housing</td>
<td>240</td>
<td>Harlow et al., 1987</td>
</tr>
<tr>
<td>19</td>
<td>Novel stock-handler</td>
<td>90</td>
<td>Novel stock-handler</td>
<td>90</td>
<td>Fulkerson and Jamieson, 1982b; Hargreaves and Hutson, 1990a</td>
</tr>
<tr>
<td>20</td>
<td>Mixing with unfamiliar sheep</td>
<td>120</td>
<td>Mixing with unfamiliar sheep</td>
<td>120</td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td></td>
<td>Pretend blood sample</td>
<td>2</td>
<td>Pretend blood sample</td>
<td>2</td>
<td>Baldock and Sibly, 1990 (Baldock and Sibly, 1990)</td>
</tr>
<tr>
<td>21</td>
<td>Laparoscopy crate</td>
<td>2</td>
<td>Laparoscopy crate</td>
<td>2</td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td>22</td>
<td>Pulse bleed</td>
<td></td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td>23</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td>24</td>
<td>Acute stress test</td>
<td></td>
<td></td>
<td></td>
<td>Parrott et al., 1994; Blache and Bickell, 2011</td>
</tr>
<tr>
<td></td>
<td>Isolation + blower</td>
<td>15+15</td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td></td>
<td>Weighing</td>
<td>2</td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td></td>
<td>Wool tipping crate</td>
<td>2</td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990</td>
</tr>
<tr>
<td>27</td>
<td>Truck transportation</td>
<td>90</td>
<td></td>
<td></td>
<td>Baldock and Sibly, 1990; Parrott et al., 1994</td>
</tr>
<tr>
<td>28</td>
<td>Pulse bleed</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>30</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Mixing with unfamiliar peers

Experimental animals (n = 16) were mixed with 70 unfamiliar ewes for a period of 120 min, to induce a social stressor by breaking up established social orders within the group. During this time, animals were drafted into smaller groups, and moved through yards, similar to the yarding and drafting pattern described above.

Sham blood sample

Animals were restrained by a single handler, whilst a second person pretended to blood sample the animal from the jugular vein. No needle was used, but the animal was held and touched as if a real blood sample was being drawn, and the procedure took 2 min. Animals were held in a single file race whilst the procedure was carried out on all animals.

Laparoscopy cradle

Animals were placed in a laparoscopy cradle, and held in the backwards tilted position for 2 min, before being released.

Phase 2: Plasma concentrations of cortisol and insulin at rest

At the end of the 3-week period, a subset of animals from each group (calm, control: n = 4; calm, treatment: n = 4; nervous, control: n = 4; nervous, treatment: n = 4) was brought into an enclosed shed, placed into individual pens (3 m²), and received an indwelling jugular catheter. To minimise the stress associated with the sampling procedure so we could quantify the effects of chronic stress on hormone secretion at rest, we took several measures: i) all animals had fence-line, visual contact with their peers, minimising social stress; ii) blood sampling was conducted from outside the pen via an extension to jugular cannula attached to a suspended wire approximately 1.80 m above the ground, thus minimising human contact; iii) the outer perimeter of the pens was surrounded by hessian cloth to reduce the
visibility of humans during blood sampling procedure; iv) the animals were sham
bled for 1 h before the start of sample collection to acclimatise them to the
procedure.

Blood was sampled (3 mL) every 10 min for 6 h (0900 – 1500 h) via the jugular
cannula and centrifuged immediately for 10 min at 2000 g. Heparin (50 i.u. per 50
uL of saline; heparin sodium BP, Pfizer Australia, Sydney, NSW) was used to
prevent blood samples from coagulating, and heparinised saline (5000 i.u. heparin
sodium BP per litre) was used to flush cannulae between samples to keep cannulae
patent. Plasma was removed, stored in plastic tubes and frozen at –20°C until
radioimmunoassay.

Phase 3: Effects of the series of acute stressors on the behavioural and
endocrine responses to acute stress

2 days after the cessation of the chronic stress paradigm, all animals were placed
in an isolation box, as described under the section titled “Animals” above, for 10
min to induce acute stress (Table 1). Testing began at 0800 h and was complete
within 3 h. Blood was sampled at box entry (t = 0) and exit (10 min post entry
time), and 30 and 60 min after box exit. Samples were processed and stored as
described above. Activity of the animals was measured over the 10 min of isolation
using an agitation meter, as described under the section titled “Animals” above.

Hormone analysis

Cortisol

All plasma samples were analysed for cortisol with a radioimmunoassay kit
(Diasorin Australia Ltd NSW) that had been modified and validated for sheep
(Beausoleil et al., 2008). The detection limit was 1.25 ng/mL. Quality control
samples (13.0 ng/mL and 28.3 ng/mL) were used to calculate inter-assay (12.8 % and 8.9 %) and intra-assay (7.6 % and 8.7 %) variation.

**Insulin**

Insulin concentrations were measured in hourly plasma samples throughout Phase 2 using a double-antibody radioimmunoassay (Tindal et al., 1978). All samples were processed in a single assay and the limit of detection was 0.78 μU/mL. Six replicates of three control samples containing 3.2, 5.2 and 11.9 μU/mL were included in the assay and were used to estimate the intra-assay coefficients of variation (8.6 %, 5.1 % and 6.0 %).

**Statistical analysis**

**Phase 2: Hormonal profiles of calm and nervous sheep at rest**

All data analysis was conducted in Genstat (Twelfth Edition, VSN International Pty Ltd, UK). Differences were considered significant if P ≤ 0.05, and a tendency if P ≤ 0.10, and so P ≤ 0.10 reported as exact values. All data were assessed for normality using the Shapiro-Wilk test and homogeneity of variance with Bartlett’s test. Where data were not normally distributed or did not have homogenous variance between treatments, the data were transformed as described below. All data is presented as untransformed values for ease of interpretation.

**Cortisol data**

Cortisol data were analysed with MUNRO, an adaptation of the PULSAR program, developed by Merriam and Wachter (1982), for the Apple Macintosh computer (‘Munro’, Zaristow Software, West Morham, Haddington, East Lothian, UK). This program identifies pulses, and uses ‘G’ and Baxter parameters to determine true pulses. The ‘G’ parameters are the number of standard deviations by which a peak must exceed the baseline to be accepted as a pulse, relative to the number of
sampling points within the pulse (1-5). The Baxter parameters describe assay variation using the parabolic relationship between hormone concentration and standard deviation about that concentration. The G parameters used were 3.98, 2.4, 1.68, 1.25 and 0.93 for G1-G5 respectively and the Baxter parameters were –0.0329 (B1, the y-intercept), 0.0733 (B2, the x coefficient), and –0.000464 (B3, the x² coefficient).

The mean concentration, amplitude, pulse frequency (number of pulses per h), nadir, and area under the curve (AUC) of each pulse were determined for each animal. Baseline cortisol concentrations were calculated as the mean of the 10 lowest points on the profile (Martin et al., 1983). Pulse amplitude and area under the curve underwent square-root transformation and data for average concentration and nadir concentration were transformed logarithmically (base 10).

All measures of cortisol secretion were compared between temperament and stress treatments using 2-way ANOVA, and where this indicated a significant difference, a Student’s t-test was used to compare groups. Pulse frequency data were non-normal and variance not homogenous, and so temperament and stress treatments were compared with the Mann-Whitney U test.

*Insulin*

The insulin data were analysed for effects of temperament, stress treatment, and time using repeated measures ANOVA. Prior to analysis, the data were transformed logarithmically (base 10) to meet the assumptions of a parametric test.

**Phase 3: Endocrine and behavioural responses to an acute stressor**

*Cortisol*
The cortisol response to the acute stressor was analysed using a repeated measures ANOVA, with temperament and stress treatment as treatment factors and day as a blocking factor. Final group numbers of animals with a full set of data were as follows: calm control n = 7; calm stressed n = 9; nervous control n = 8; nervous stressed n = 7. The amplitude of the response was calculated as the 10 min measure (immediately after box exit) minus the 0 min measure (immediately prior to box entry). Area under the curve (AUC) was determined as the total area under the curve minus the area below the baseline, considered to be the cortisol concentration immediately prior to box entry, as previously described (Pruessner et al., 2003). Amplitude and AUC were analysed using ANOVA with temperament and stress treatment as treatment factors and day as a blocking factor. Where the ANOVA indicated a significant difference, a Student’s t-test was used to compare groups.

*Isolation box scores*

The isolation box agitation scores were rank-transformed prior to ANOVA, with temperament and stress treatment as factors and day of testing as a blocking factor. Body mass directly affects the agitation score, and so was included as a covariate. Where significant effect of 1 or more factors was found, Student’s t-test was used to further analyse the data.

*Correlation between cortisol and behavioural responses*

The correlation between the amplitude and AUC of the cortisol response to the acute stressor and the agitation scores was analysed using Spearman’s rank correlation coefficient.
Results

Phase 2: Effect of the series of acute stressors on cortisol and insulin concentrations at rest

Cortisol and insulin

There was no effect of exposure to the series of acute stressors, temperament, or any interaction between temperament and exposure to the series of acute stressors on mean concentrations of cortisol, pulse amplitude, nadir, baseline, pulse frequency, or area under the pulse curve in the absence of stressors (all $P > 0.10$; Figure 4.1). Similarly, there was no effect of time, exposure to the series of acute stressors, temperament, nor was there any effect of an interaction between these factors on plasma concentrations of insulin (calm: control $10.1 \pm 1.83 \mu U/mL$, stressed $7.8 \pm 0.92 \mu U/mL$; nervous: control: $9.1 \pm 1.56 \mu U/mL$, stressed $9.6 \pm 0.92 \mu U/mL$; all $P > 0.10$).

Phase 3: Effect of the exposure to the series of acute stressors on the cortisol and behavioural response to an acute stressor

Behaviour

The behavioural response to the acute stressor was affected by temperament ($P < 0.001$) but not by interaction between temperament and exposure to the series of acute stressors ($P > 0.10$; Figure 4.2). Nervous animals had a larger agitation score in the isolation box test than calm animals ($P < 0.001$; Figure 4.2).

Cortisol

The cortisol response to the acute stressor was not affected by exposure to the series of acute stressors, and there was no interaction between temperament and
exposure to the series of acute stressors (P > 0.10; Figure 4.2). There was an effect of time (P < 0.015) and an interaction between temperament and time on plasma concentrations of cortisol in response to the acute stressor (P = 0.047; Figure 4.2). There was an overall effect of temperament on the amplitude (P = 0.039) and area under the curve (P = 0.043) of the cortisol response to the acute stressor.

Figure 4.1. Resting plasma cortisol pulse characteristics over 6 h in castrated male sheep (n = 16) with either a calm or nervous temperament, that had either been exposed to a 3 wk series of acute stressors designed to mimic farm procedures (black bars), or had remained in the paddock for that time period (control; white bars). All measurements are shown as mean ± S.E.

Specifically, the amplitude and AUC of the cortisol response to the stressor was almost twice as high in nervous animals (nervous: amplitude 16.2 ± 2.85 ng/mL, AUC 560 ± 100 μg/mL) as in calm animals (calm: amplitude 8.8 ± 1.85 ng/mL; P = 0.033, AUC 270 ± 85 μg/mL; P = 0.039). Calm animals also had lower mean cortisol concentrations 30 min after box exit than nervous animals (P = 0.044), although there was no difference between temperaments at box exit (P > 0.10). Regardless of temperament or treatment, concentrations of cortisol were greater at box exit than at prior to box entry or at 30 and 60 min after box exit (P < 0.001; Figure 4.2). The
Figure 4.2. Behavioural and cortisol responses to an acute isolation stressor in calm (squares) and nervous (circles) castrated male sheep that had been exposed to a 3-week series of acute stressors based on standard farm procedures (dark shapes), or had remained in the paddock for that time (control; open shapes). Asterisks denote in a) difference from same group in calm animals, and in b) difference between temperament groups at that time point. Group numbers were as follows: calm, control n = 7; calm, stressed n = 9; nervous, control n = 8; nervous, stressed n = 7. All values are mean ± S.E.

Amplitude and AUC of the cortisol response to the isolation box test were correlated to the agitation score (amplitude: r=0.55, P < 0.001; AUC r = 0.37, P = 0.047).

Discussion

Exposure to the series of acute stressors had no residual effects on behaviour or on cortisol or insulin secretion in either calm or nervous sheep, and so we reject our hypotheses. It appears that our series of acute stressors was not effective at inducing mild chronic stress in mature sheep of either temperament, as determined by the measurements that we used. This outcome suggests that that either the stressors were not sufficient to induce chronic stress in the calm and nervous sheep, that the sheep habituated to the regime, or that sheep are relatively resistant to the development of chronic stress.
The inability of the series of acute stressors to induce even a mild chronic stress, as indicated by increased HPA activity, was somewhat surprising because the stressors we used have all been shown to be stressful for sheep (Table 4.1). We aimed to induce a state of mild chronic stress rather than a more severe state of stress that could mask subtle differences. However, the absence of any indication of chronic stress in animals of either temperament indicated that either the intensity of the series of acute stressors was too low or that the animals became habituated to the regime. Some of the stressors, such as shearing and proximity to dogs, are quite severe for sheep (Komesaroff et al., 1998) but others, such as drenching and yarding, may have been too brief to contribute to the development of chronic stress (Hargreaves and Hutson, 1990b). It is also possible that a 3-week series of stressors was not long enough to induce a state of mild chronic stress or that the inter-stressor interval of up to 3 d was too long, allowing the animals to recover adequately from one stressor before the next, rather than accumulating the effects (Moberg, 2000). In comparison, in a previous study with lambs, it was found that daily exposure to acute stressors for 6 weeks led to more fearful behaviour in response to novelty and human presence, and to lower cortisol concentrations in response to confinement (Destrez et al., 2013). However, exposure to stressors did not affect the behavioural response to a suddenness test or the cortisol response to novelty and suddenness tests or an ACTH challenge. Therefore, it is probable that we would have seen indicators of chronic stress if the series of stressors had been longer with more intense and/or more frequent stressors.

Alternatively, the sheep may have become habituated to the stressor regime because most of the stressors took place in the same physical setting, and the primary stressors (novelty, close human presence) were associated with several of the challenges. Thus, weighing, sham shearing, drenching, and foot bathing may have been perceived by the sheep as similar, rather than novel, stressors.
Therefore, although we used different stressors throughout the series to reduce the likelihood of habituation, these similarities might have effectively led to habituation, reducing the perceived intensity of the stressor and inhibiting the development of chronic stress (Natelson et al., 1988; Pitman et al., 1990). This concept is supported by previous work in sheep where lambs exposed to a series of acute stressors for 4 weeks did not differ from control lambs in their behavioural response to stressors, which the authors concluded could be due to habituation of the treated lambs to novelty (Doyle et al., 2011). Therefore, in the current study, it is possible that that animals rapidly habituated to the stressors and did not develop mild chronic stress at any point, or that indicators of chronic stress may have been present early in the series and then disappeared due to habituation.

We wanted to induce a state of mild chronic stress in the calm and nervous sheep in an attempt to reveal any effect of temperament on the susceptibility to becoming stressed. However, the lack of any effect of the series of acute stressors on any measure for either temperament suggests that sheep are relatively resistant to developing chronic stress. This concept is supported by previous work in sheep, where Doyle et al. (2011) found no effect of exposure to a series of acute stressors over 4 weeks on cortisol concentrations at rest or in response to an ACTH challenge, or on behavioural reactivity to novelty, suddenness, or discrepancy from expectation tests. Cardiac activity indicated that treated lambs recovered more quickly from the discrepancy from expectation test than control lambs, but this was not seen in the novelty or suddenness tests (Doyle et al., 2011). Similarly, Destrez et al. (2013) found that approximately daily exposure to a series of acute stressors over 6 weeks did not affect the cortisol response to novelty and suddenness tests, or to an ACTH challenge, but that treated lambs had lower cortisol concentrations in response to confinement than controls. However, compared to control animals, treated lambs did behave more fearfully in response to the novelty and human
tests, and had lower heart rates in response to human presence and a lower white blood cell count (Destrez et al., 2013). These outcomes suggest two conclusions. First, in sheep it seems the HPA axis is highly resilient to the development of chronic stress. Sheep are prey animals with few defence mechanisms besides flight (Boissy, 1995), so being highly responsive to acute stressors but highly resilient to chronic stress is likely to be beneficial for their survival. Furthermore, it is likely that domestication has resulted in selection for sheep that are able to thrive and survive under human interactions, and so today’s sheep are highly resilient to the kinds of stressors used in this study. Second, behaviour and cardiac activity may be better criteria for assessing chronic stress in sheep than activity of the HPA axis.

Temperament did not affect the cortisol or insulin response of the animals to the series of acute stressors, indicating that nervous sheep are not more susceptible to suffering from chronic stress than calm sheep, at least with the level of stressor exposure used in this study. However, interpretation of this outcome is difficult because the model did not appear to be sufficient to induce chronic stress in either temperament. Previous studies in quail and mice show that a highly responsive temperament can increase susceptibility to developing chronic stress, whilst a hypo-responsive temperament can decrease susceptibility (Calandreau et al., 2011; Fuchsl et al., 2014). Temperament may similarly affect the development of chronic stress in sheep under sufficiently stressful situations.

In summary, the series of acute stressors used in the current study did not induce chronic stress, as indicated by concentrations of cortisol and insulin at rest, or the behavioural and cortisol responses to an acute isolation stressor, in either calm or nervous sheep. It appears that the series of acute stressors was insufficiently intense to induce chronic stress, or that the sheep became habituated to the procedures. Temperament did not have any effect on the endocrine or behavioural indicators of chronic stress at rest or in response to an acute stressor. However,
differences between the calm and nervous sheep may be evident under a more intense regime of stressors, and a more intense series of stressors seems to be required to induce chronic stress in sheep.
Chapter 5.

The effect of temperament in the responsiveness of the HPA axis I:

The adrenal gland

Abstract

As the end organ in the hypothalamic-pituitary-adrenal (HPA) axis, the responsiveness of the adrenal gland to stimulation is important for coping with stressors. Temperament, a measure of behavioural response to stress, has been associated with differences in responsiveness of the HPA axis to stressors. In this study, we investigated whether changes in the responsiveness of the adrenal gland to pituitary stimulation were related to temperament. Castrated male Merino sheep (n = 16) that had been bred for either ‘calm’ or ‘nervous’ temperament were treated with dexamethasone (0.125 mg/kg BW, i.v.) to suppress endogenous production of adrenocorticotropic hormone (ACTH), then given 4 doses of exogenous ACTH (0.0125, 0.05, 0.2 and 0.8 i.u.), at 90 min intervals, beginning 90 min after dexamethasone treatment. After each ACTH treatment, blood was sampled via jugular cannula every 5 min for 30 min, and then every 10 min for the next 60 min, to measure the cortisol response to treatment. The magnitude of the cortisol response was directly related to the dose of ACTH, but there was no effect of temperament. We conclude that, in Merino sheep, selection for temperament did not affect the capacity of the adrenal gland to produce cortisol in response to stimulation with ACTH.
Introduction

The cortex of the adrenal gland is responsible for the synthesis and secretion of the key stress hormone, cortisol, under the control of the hypothalamic-pituitary axis (Bentley, 1998; Matteri et al., 2000). During stress, the hypothalamic-pituitary axis signals the adrenal gland to synthesise and secrete extra cortisol into circulation, leading to a repartitioning of resources throughout the body that maximizes the ability to overcome the threat (Bentley, 1998; Matteri et al., 2000; Charmandari et al., 2005). The effects of cortisol are powerful and widespread, affecting processes such as metabolism, immune function and reproduction, and, whilst these changes are beneficial in the short term, in the long term they can become detrimental to health and welfare (Bentley, 1998; Matteri et al., 2000; Charmandari et al., 2005). Therefore, hyper- or hypo- responsiveness of the adrenal gland to pituitary stimulation may cause alterations in normal biological function for longer or shorter periods than are necessary to overcome the threat, compromising the health and welfare of the individual (Charmandari et al., 2005).

Variation in the activity of the adrenal gland is associated with temperament, a measure of the behavioural response to stress. Individuals who are more behaviourally responsive, or ‘nervous’, when faced with a stressor display larger adrenal responses to stressors than their less behaviourally responsive, or ‘calm’, counterparts, as seen in humans, rats, cattle and sheep (Gunnar et al., 1989; Sternberg et al., 1992; Windle et al., 1998a; Curley Jr. et al., 2006; Marquez et al., 2006; Curley et al., 2008; Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013). This variation in adrenal response could be due to variation in any of the components of the axis, including the adrenal gland, within which there could be variation in the response to stimulation by ACTH from the pituitary gland. Temperament has been associated with responsiveness of the adrenal in two separate models in sheep. In one model, intact female sheep that displayed smaller 89
cortisol responses to ACTH treatment were more active during an open field test and less fearful in response to a human presence, compared to more sheep that had larger cortisol responses to ACTH treatment (Lee et al., 2014b). In a second model, genetic variation in the responsiveness of the adrenal axis has been observed in sheep selected for divergence in the ability to rear multiple lambs. Sheep with good multiple-rearing ability are less behaviourally responsive during an arena test and have a smaller cortisol response to the physiological stressor of an insulin challenge than animals selected for poor multiple-rearing ability (Cloete et al., 2005; Van der Walt et al., 2009; Hough, 2012). In addition, the magnitude of the adrenal response was associated with polymorphism in a gene coding for the steroidogenic enzyme cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), which is involved in cortisol production in the adrenal gland (Bentley, 1998; Hough, 2012). These findings suggest that selection for temperament may affect the ability of the adrenal gland to respond to pituitary stimulation, and this may drive the divergence in the HPA axis response to stressors. However, this concept has not been tested in a model specifically based on temperament.

To study the relationship between temperament and adrenocortical responsiveness to ACTH, we have used Merino sheep selectively bred for either a calm or nervous temperament based on their behavioural response to isolation and human presence (Blache and Bickell, 2011b). The behaviourally hypo-responsive calm sheep also have smaller cortisol responses to stressors than the relatively hyper-responsive nervous sheep, which may be due to differences in adrenocortical responsiveness to pituitary stimulation (Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013). Therefore, we tested whether sheep selected for a nervous temperament have a larger cortisol response to exogenous ACTH than sheep selected for calm temperament. We pre-treated animals with dexamethasone to inhibit endogenous 90
ACTH activity and used small doses of ACTH to maximise our ability to observe any subtle differences in adrenal sensitivity between the temperament groups.

Methods

Animals

This experiment was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and was approved by the Animal Ethics Committee of The University of Western Australia under RA 33/100/1036.

The experiment was conducted at the University of Western Australia (31° 56’ S) with 20-month-old male sheep (n = 20) that had been castrated before puberty (wethers). Animals came from the ‘calm’ and ‘nervous’ lines of the ‘UWA temperament flock’ (see below), and temperament groups were balanced for weight (calm: 53.0 ± 1.51 kg BW, nervous: 52.2 ± 1.97 kg BW). They were individually penned indoors and then habituated to housing and human contact for 21 d before experimentation. Animals were subjected to natural lighting during this time (approximately 11L:13D).

UWA temperament flock

The UWA temperament flock comprises Merino sheep that have been bi-directionally selected over the past 20 generations for their behavioural reaction to the stressors of isolation and human presence (Beausoleil et al., 2008; Blache and Bickell, 2011b). In brief: the animals are subjected to 2 tests at the time of weaning; the first test is an isolation challenge, where individual animals are placed inside a solid plywood box (1.7 m x 1.2 m x 1.2 m) that prevents visual contact with peers; the box is fitted with a calibrated meter that measures the
vibrations resulting from the animal’s movements and high-intensity vocalisations. The second test is an arena test that presents animals with the conflict of having to pass an immobile human to be with peers. The animal is placed at one end of an arena, marked out with zones, and other sheep are penned at the opposite end, with the human in between. The animal must choose whether to remain alone, or come close to the human in order to join its peers. The movement of the animal across the marked zones is recorded. The data from both tests are used to calculate a temperament score that is used to classify relatively hypo-active sheep as ‘calm’ and hyper-active sheep as ‘nervous’ (see Beausoleil et al., 2008; Blache and Bickell, 2011b). Males with the most extreme scores from both lines are used to breed the subsequent generation of the temperament flock.

**Preliminary tests**

To determine the appropriateness of the selected doses of dexamethasone and ACTH, two calm and two nervous animals (not used in the main experiment) were fitted with jugular cannulae. One day later, they were used to confirm that 0.125 mg/kg (i.v.) dexamethasone (Dexason, Ilium, Australia) would suppress cortisol secretion within 60 min post-injection, and for at least 8 h. Blood was sampled hourly for 8 h, beginning immediately before dexamethasone treatment. After a 2 d delay, the selected ACTH (ACTH, Polypeptide, France) treatments were assessed for their ability to elicit a cortisol response that would last 20 - 70 min. The animals again received dexamethasone (0.125 mg/kg, i.v.) and, after 90 min, each animal received each of four intravenous doses of ACTH (0.5, 1, 2.5 and 5 i.u.) at 90 min intervals. Initial ACTH doses were selected to induce small cortisol responses, and were based upon previous studies (Beaven et al., 1964; Fulkerson and Jamieson, 1982; Turner et al., 2002; Knott et al., 2008).
Main experiment

Animals (n = 16) were pre-treated with dexamethasone (0.125 mg/kg, i.v.), 90 min before ACTH treatment. Four doses of ACTH (0.0125, 0.05, 0.2, and 0.8 i.u.) were given to each animal via jugular cannula, at 90 min intervals. The doses were given in one of 4 differing orders, and 4 different animals (n = 2 per line) were treated on each day of the 4 d experiment. Doses were chosen to yield small cortisol responses lasting less than 70 min from results of preliminary tests and those reported in previous studies (Beaven et al., 1964; Fulkerson and Jamieson, 1982; Turner et al., 2002; Knott et al., 2008). Most dose rates from the preliminary study (1, 2.5, and 5 i.u.) yielded cortisol responses that lasted longer than 70 min, and so smaller doses were selected for the main study.

Blood was sampled at 0, 30, 60 and 90 min after dexamethasone administration. Immediately after the 90 min sample, an ACTH bolus injected and blood was sampled every 5 min for 30 min, and then every 10 min for the next 60 min. The next ACTH injection was then administered after the last sample and the sampling schedule was repeated as described above for the next 90 min. All 4 doses were administered during 1 day so the total sampling lasted 6 h from the first ACTH injection. Heparin (50 i.u. per 50 uL of saline; heparin sodium BP, Pfizer Australia, Sydney, NSW) was used to prevent blood samples from coagulating, and heparinised saline (5000 i.u. heparin sodium BP per litre) was used to flush cannulae between samples to keep cannulae patent.

Hormone analysis

Immediately after collection, blood samples were centrifuged for 10 min at 2000 g. Plasma was drawn off and stored at −20°C in plastic tubes until radioimmunoassay for cortisol. Plasma cortisol concentrations were measured using commercial kits (Diasorin Australia Ltd. NSW) using a modified method that had been validated
for sheep, as described by Beausoleil et al (2008). The limit of detection of the assay was 1.1 ng/mL. Quality control samples (13.5 ng/mL and 32.2 ng/mL) were used to calculate inter- (5.0 % and 5.6 %) and intra-assay (8.2 % and 7.9 %) variation.

**Statistical analysis**

The amplitude, delay to peak, rate of increase, area under the total response curve (AUCT) and area under the total response curve to peak (AUCP) of the cortisol response to ACTH administration were calculated for statistical analysis. The amplitude was calculated as the maximum cortisol concentration minus the cortisol concentration at the time of treatment, and the rate of increase was calculated as amplitude divided by the time taken to reach to maximum value. Area under the curve was calculated relative to the baseline as described by Pruessner et al. (2003). All statistical analyses were done using Genstat (Twelfth Edition, VSN International Pty Ltd, UK). Differences were considered significant if P ≤ 0.05, and a tendency if P ≤ 0.10, and so P ≤ 0.10 reported as exact values. Prior to analysis, all data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Bartlett’s test. Where data were not normally distributed, or where variance was not homogenous across treatments, data were transformed as described below. All data are presented as untransformed values for ease of interpretation. Delay to peak, AUCP, and the rate of increase underwent logarithmic (base 10) transformation to correct for right-skewness and because variance was proportional to the mean.

The effect of dexamethasone on cortisol concentrations prior to ACTH treatment was analysed using repeated measures ANOVA, with temperament as a treatment factor, order and day of receiving treatment as blocking factors, and bodyweight as a covariate.
The amplitude, time to peak, rate of increase, and AUCP of the response to ACTH treatment were compared using a split-plot ANOVA. For each test, dose rate and temperament were the treatment factors, day of receiving each treatment was used as a blocking factor, animal used as whole plots, order of treatment used as subplots, and bodyweight used as a covariate. Order and day of receiving treatment did not significantly affect the cortisol response to any treatment (P > 0.05). Where significant differences between dose or temperament group were detected with the ANOVA, data was further analysed using Student’s t-test.

For area under the total response curve, no transformation could establish normality and homogenous variance, so the data were analysed using non-parametric tests. In this case, AUCT was compared between treatments using Friedman’s non-parametric ANOVA and, where significant differences found, Wilcoxon matched-pairs test was used for post-hoc analyses. Temperament groups were compared within treatments with the Mann-Whitney U test.

The amplitude, AUCT and AUCP of the cortisol response to treatment were expected to increase with dose rate, in a typical dose-responsive manner (Fulkerson and Jamieson, 1982). To determine whether temperament had any effect on the dose-response, regression analysis was performed on the amplitude, AUCT and AUCP of the response. The data for each aspect of the curve was plotted against the natural logarithm of the dose rate, and fitted with a regression line for each animal to find the slope of the line. The slope data was compared between temperament groups using a Student’s t-test.

The cortisol response to the largest dose of ACTH (0.8 i.u.) lasted longer than 90 min, and so interfered with the response of the following treatment. This affected the response to 1 dose of ACTH each for 12 animals. To determine whether this affected the outcome of the study, these measurements were removed, and the
statistical tests repeated as described above. Final group numbers for this analysis were n = 12 for 0.0125, 0.05 and 0.2 i.u., and n = 16 for 0.8 i.u., with equal numbers of calm and nervous animals in each.

Results

Preliminary tests

Dexamethasone treatment reduced concentrations of cortisol by 81 ± 5.3 % by 60 min post-injection (P = 0.005), and by 82 ± 5.1 % by 90 min (P = 0.004), compared to pre-treatment concentrations. Concentrations of cortisol were decreased by 91 ± 9.3 % of pre-treatment concentrations at 8 h post-treatment (P = 0.006). Temperament did not affect the cortisol response to dexamethasone at any time point (all P > 0.10).

All ACTH doses elicited a cortisol response (amplitude: 0.5 i.u.: 23.4 ± 4.86 ng/mL, 1 i.u.: 23.7 ± 3.68 ng/mL, 2.5 i.u.: 38.8 ± 3.80 ng/mL, 5 i.u.: 44.5 ± 2.44 ng/mL). The cortisol responses to 1, 2.5 and 5 i.u. ACTH lasted longer than 70 min (concentrations at 70 min post-treatment: 1 i.u.: 10.5 ± 1.52 ng/mL, 2.5 i.u.: 28.3 ± 7.90 ng/mL, 5 i.u.: 31.1 ± 6.20 ng/mL). The cortisol response to 0.5 i.u. ACTH had returned to baseline concentrations by 70 min (concentrations at 70 min post-treatment: 1 i.u.: 3.5 ± 0.43 ng/mL).

Main experiment

Dexamethasone treatment reduced circulating concentrations of cortisol by 81 ± 9.2 % at 90 min after administration, compared to pre-treatment values (P < 0.001), with no difference between temperament groups (mean ± S.E. shown throughout; calm: before dexamethasone treatment 4.63 ± 1.54 ng/mL, after 0.3 ± 0.28 ng/mL; nervous: before 6.80 ± 1.92 ng/mL, after 1.9 ± 0.97 ng/mL; P = 0.146).
There was an effect of ACTH dose on the amplitude (P < 0.001), delay to peak (P < 0.001), AUCP (P < 0.001) and AUCT (P < 0.001) of the cortisol response. Generally, as the dose of ACTH increased, larger and longer cortisol responses were elicited. Specifically, the amplitude of the cortisol response increased significantly as the ACTH dose was increased from 0.0125 i.u. to 0.05 i.u. and then to 0.2 i.u., regardless of temperament. For the dose of 0.8 i.u. ACTH, the amplitude was not significantly greater than with 0.2 i.u. ACTH (P = 0.085; Figure 5.1).

The AUCT and AUCP to peak also generally increased significantly with dose, with the only exception being the change in AUCP between 0.05 and 0.2 i.u. ACTH (P = 0.085; Figure 5.1, Table 5.1). The time to peak cortisol concentration was shortest for 0.0125 i.u. and longest for 0.8 i.u., with similar intermediate values observed for 0.05 i.u. and 0.2 i.u. (Figure 5.1). The rate of increase was fastest for 0.2 i.u. (all P < 0.05 compared to other doses rates), but similar for all other doses (P > 0.10; Figure 5.1).

There was neither an effect of temperament nor an interaction between dose and temperament on any aspect of the cortisol response (i.e. amplitude, time to peak, rate of increase, AUCT and AUCP) to ACTH treatment (all P > 0.10; Table 5.1, Figure 5.1).

Removal of data from treatments immediately following the 0.8 i.u. ACTH treatment from analysis showed that the elevated baseline at the end of the 0.8 i.u. treatment did not affect any of the outcomes reported above, although there tended to be an effect of an interaction between dose and temperament on rate of increase (P = 0.070).

There was no effect of temperament on the slope of the dose response for the amplitude, AUCT, or AUCP of the cortisol responses to ACTH treatment (all P > 0.10; Table 5.1).
Figure 5.1. Plasma cortisol concentrations in calm (n = 8; solid lines) or nervous (n = 8; dashed lines) castrated male Merino sheep following an intravenous injection of ACTH. The animals had been pretreated with dexamethasone (0.125 mg/kg) to suppress endogenously-controlled secretion. Concentrations are shown relative to pre-treatment baseline. Mean concentrations, and mean S.E. bar, averaged across time points, shown for each temperament (calm – left S.E. bar, capped ends, nervous – right S.E. bar, no end caps).

Discussion

Our hypothesis that nervous sheep would show a larger cortisol response to exogenous ACTH than calm sheep was rejected, at least for the doses of ACTH that were used in this study. We therefore conclude that greater cortisol response to social stressors shown by nervous sheep than by calm sheep (Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013) is not due to differences between
Table 5.1. Characteristics of the cortisol response in calm (n = 8) or nervous (n = 8) castrated male Merino sheep treated with increasing doses of ACTH(0.0125 – 0.8 i.u.) and slope of the dose response.

<table>
<thead>
<tr>
<th>ACTH dose (i.u.)</th>
<th>Amplitude (ng/mL)</th>
<th>Time to peak (min)</th>
<th>Rate of increase (ng/mL/min)</th>
<th>AUCT (ng/mL)</th>
<th>AUCP (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Calm</td>
<td>Nervous</td>
<td>Calm</td>
<td>Nervous</td>
<td>Calm</td>
</tr>
<tr>
<td>0.0125</td>
<td>20.2 ± 4.25</td>
<td>22.0 ± 3.66</td>
<td>12 ± 0.9</td>
<td>14 ± 1.8</td>
<td>1.7 ± 0.31</td>
</tr>
<tr>
<td>0.05</td>
<td>31.2 ± 6.03</td>
<td>37.3 ± 6.46</td>
<td>14 ± 1.8</td>
<td>26 ± 5.9</td>
<td>2.2 ± 0.29</td>
</tr>
<tr>
<td>0.2</td>
<td>55.4 ± 3.97</td>
<td>45.1 ± 3.66</td>
<td>24 ± 3.9</td>
<td>19 ± 3.1</td>
<td>2.7 ± 0.46</td>
</tr>
<tr>
<td>0.8</td>
<td>53.9 ± 3.58</td>
<td>63.2 ± 4.75</td>
<td>49 ± 6.4</td>
<td>34 ± 3.9</td>
<td>1.4 ± 0.34</td>
</tr>
<tr>
<td>Slope</td>
<td>20.4 ± 3.21</td>
<td>19.9 ± 3.18</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
the genetic lines in the responsiveness of their adrenal cortex to pituitary stimulation by ACTH. Having eliminated this possibility, we propose that the effect of temperament in the responsiveness of the HPA axis to social stressors must be expressed at brain or pituitary levels.

The lack of a relationship between temperament and responsiveness to ACTH is both supported and contradicted by the literature. Our findings are supported by a previous study with the same temperament model, but using an intra-muscular treatment with a single dose of long-acting ACTH in ovariectomised female sheep, in which there was also no difference between calm and nervous animals (Henry et al., 2010). The present study thus extends on this earlier work by adding male animals and by using a dose-response with doses of ACTH that are physiologically relevant (see below). A similar outcome was reported for female and castrated male cattle in which the magnitude of the cortisol response to ACTH treatment was not related to temperament (Van Reenen et al., 2005; Cafe et al., 2011a; Sutherland et al., 2012). In male cats also, the cortisol response to ACTH was not related to the behavioural response to a spray bath (Iki et al., 2011).

With respect to the literature that disagrees with our findings, differences in the experimental design may account for the variation in findings. For example, female cattle with low behavioural responsiveness, as determined by a relatively slow flight speed, presented larger areas under the cortisol response curve, but similar amplitudes of response to ACTH stimulation, than their more responsive counterparts (Curley et al., 2008). However, in that study, the more responsive cattle had greater concentrations of cortisol before ACTH treatment than the calmer cattle, suggesting an effect of human handling (Curley et al., 2008). This may have affected the capacity of the adrenal gland to respond to further stimulation, as well as making the responses of the more reactive animals seem smaller and shorter as their responses were super-imposed upon greater baseline
values (Curley et al., 2008). In another study, again with only a single dose of ACTH, intact female sheep that displayed smaller cortisol responses were more active during an open field test and less fearful in response to a human presence, compared to more responsive sheep (Lee et al., 2014b). Ovary-intact female sheep are reported to present greater responsiveness than gonadectomised male or female sheep, or intact male sheep, and so the difference in findings between Lee et al. (2014b) and our study may reflect a gender-dependent interactions between temperament and adrenocortical responsiveness (van Lier et al., 2003). This can only be verified by direct experimental comparison of sheep in each reproductive status.

On the other hand, the measurement and assessment of temperament, and the definition of temperament models, also varies widely among laboratories, and this could also explain the different outcomes, even within a species and gender, as reported for female cattle (Van Reenen et al., 2005; Curley et al., 2008; Sutherland et al., 2012). In our laboratory, we define “temperament” in relation to the behavioural responsiveness of sheep to social stressors (isolation and human presence). The model is very robust because we have selectively bred for behavioural reactivity (locomotor activity and vocalisations) over 20 generations and the data clearly demonstrate highly repeatability (r = 0.40 - 0.76; Blache and Ferguson, 2005), moderate heritability (h = 0.45; Blache and Ferguson, 2005) and minimal interference by non-genetic factors (Bickell et al., 2009). In the other models, the criteria for temperament scoring are not as consistent and the basis for selection varies. These differences, detailed in Table 5.2, could account for the disagreements. For example, the male cattle model was based on 14 tests of flight speed and 17 tests of crush score (Cafe et al., 2011a; Cafe et al., 2011b), whilst the female cattle model was based on 1 test of flight speed (Curley et al., 2008), although this test has previously been shown to yield consistent results for
individuals over time (Curley Jr. et al., 2006; Müller and von Keyserlingk, 2006). In addition, the female sheep model was selected based on adrenal responsiveness to ACTH, within only 1 generation, and the temperament of the low and high responders to ACTH was evaluated using only one test for isolation and one for human presence (Lee et al., 2014b).

In addition, none of the studies outlined in Table 5.2 utilised models that were based on temperament with a known genetic basis, so they all could be influenced by uncontrolled factors, such as early life history, recent experience, maternal influence, or social rank. Each method has merit, but our findings using a genetic model of temperament with high repeatability and moderate heritability (Blache and Bickell 2011) provide an important insight into the mechanism through which the genetic component of temperament affects the physiological response of individuals to a stressor.

Another inconsistency among laboratories is the dose of ACTH used to challenge the adrenal cortex. We observed peak cortisol concentrations of up to 80 ng/mL, similar to those seen in calm and nervous sheep in response to stressors (Hawken et al., 2012a; Hawken et al., 2013), so the doses we used were physiologically relevant and also reflected the level of pituitary stimulation elicited by exposure to an acute stressor. However, in other studies, where there was evidence of a relationship between temperament and adrenocortical responsiveness, the doses of ACTH were 4-6-fold greater than our maximum dose of 0.8 i.u. (Table 5.2; Curley et al., 2008; Lee et al., 2014b). Although it is possible also in our model that temperament may affect the
Table 5.2. Comparison of studies on the relationship between temperament, as determined by behavioural reactivity to stressors, and adrenocortical responsiveness.

<table>
<thead>
<tr>
<th>Study</th>
<th>Species</th>
<th>Sex</th>
<th>Age at testing</th>
<th>Temperament test</th>
<th>Selection method</th>
<th>Dose of ACTH</th>
<th>Response to ACTH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cafe et al., 2011</td>
<td>Cattle</td>
<td>Castrated male</td>
<td>1 y</td>
<td>FS, CS</td>
<td>14 FS, 17 CS</td>
<td>2.5 μg/kg BW, i.m.</td>
<td>No difference</td>
</tr>
<tr>
<td>Curley Jr et al., 2008</td>
<td>Cattle</td>
<td>Female</td>
<td>2 y</td>
<td>FS</td>
<td>1 test</td>
<td>0.1 i.u./kg BW, i.v.</td>
<td>MF &gt; LF</td>
</tr>
<tr>
<td>Sutherland et al 2012</td>
<td>Cattle</td>
<td>Female</td>
<td>5 y</td>
<td>FS</td>
<td>1 test</td>
<td>0.05 mg, i.v.</td>
<td>No difference</td>
</tr>
<tr>
<td>Van Reenen et al 2005</td>
<td>Cattle</td>
<td>Female</td>
<td>3, 13, 26 wk</td>
<td>Novel environment, novel object, OF</td>
<td>1 repeat of each test at each age</td>
<td>0.016 μg/kg BW, i.v.</td>
<td>No correlation</td>
</tr>
<tr>
<td>Lee et al., 2014</td>
<td>Sheep</td>
<td>Female</td>
<td>3 – 5 y</td>
<td>OF, AT</td>
<td>Response to ACTH</td>
<td>0.2 μg/kg BW, i.v.</td>
<td>LF &gt; MF</td>
</tr>
<tr>
<td>Henry et al 2012</td>
<td>Sheep</td>
<td>Ovariectomised female</td>
<td>3 – 5 y</td>
<td>Isolation, AT</td>
<td>Selectively bred for temperament</td>
<td>0.5 mg i.m. *</td>
<td>No difference</td>
</tr>
<tr>
<td>Iki et al 2011</td>
<td>Cats</td>
<td>Castrated male</td>
<td>2.75 y</td>
<td>Spray bath</td>
<td>1 test</td>
<td>0.125 mg, i.m.</td>
<td>No correlation</td>
</tr>
</tbody>
</table>

FS – flight speed, CS – crush score, OF – open field test, AT- arena test, LF – less fearful, MF – more fearful  * Used a long acting form of ACTH (Synacthen Depot)
response of the adrenal gland to greater dose rates of ACTH, the cortisol responses to these dose rates may not be physiologically relevant.

The divergence in the cortisol response of calm and nervous sheep to stressors is probably mediated by differences in the responsiveness of the hypothalamic or pituitary components of the HPA axis, rather than the adrenal cortex (Chapter 3 of this thesis; Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013). For example, nervous sheep may be hyper-responsive to hypothalamic peptides arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH) leading to increased secretion of ACTH and, in turn, cortisol, in response to stressors. This concept is supported by studies of temperament in rats and cattle. In both of these models, more reactive animals show larger glucocorticoid responses to stressors, and larger pituitary responses to exogenous AVP (cattle), and CRH (rats) than less reactive animals (cattle (Curley Jr. et al., 2006; Curley Jr. et al., 2010); rats (Spinedi et al., 1994; Windle et al., 1998a)). Furthermore, the hyper-reactivity of the HPA axis of high-anxiety rats to their selection stressor (open arm of the elevated plus maze) is primarily driven by increased expression and release of AVP from the paraventricular nucleus (PVN) of the hypothalamus, with CRH playing a minor role (Wigger et al., 2004). In the absence of any differences in adrenocortical responsiveness to ACTH in calm and nervous sheep, it is clear that we need to test these aspects of the HPA axis.

In summary, the divergent cortisol responses of calm and nervous sheep to social stressors are not due to differences in the responsiveness of the adrenal cortex to ACTH. We propose that the divergent cortisol responses of calm and nervous sheep to social stressors may be due to differences in the responsiveness of the pituitary gland to AVP and/or CRH, and/or in the expression or activation of AVP or CRH neurons in the hypothalamus.
Chapter 6.

The effect of temperament in the responsiveness of the HPA axis II: The pituitary-adrenal axis

Abstract

Temperament affects the magnitude of the response of the hypothalamic-pituitary-adrenal (HPA) axis to stressors, possibly because of differences in the responsiveness of the anterior pituitary gland and adrenal cortex to hypothalamic stimulation. Here, we tested whether animals of ‘nervous’ temperament are more responsive to stimulation of the pituitary-adrenal system than animals of ‘calm’ temperament. We measured the plasma ACTH and cortisol response of ‘calm’ and ‘nervous’ castrated male Merino sheep (n = 16) to treatment with saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW, i.v.), corticotrophin releasing hormone (CRH, 0.5 µg/kg BW, i.v.), or the combined AVP and CRH treatments. Treatments were administered in a Latin Square design. Blood was sampled through a jugular cannula from individually-penned animals that had been habituated to their environment for at least 3 weeks. There was no effect of temperament on the pituitary or adrenal response to treatment with AVP or CRH alone or combined. We conclude that the difference between the temperament groups in the HPA axis response to stressors such as isolation is not due to changes in the innate responsiveness of the pituitary-adrenal system to hypothalamic stimulation. Alternatively, selection for temperament could affect the neural circuits connecting the perception of the stressors to the activation of the HPA axis.
Introduction

The response of the hypothalamic-pituitary-adrenal (HPA) axis to stressors is protective, enabling the individual to overcome threats by temporarily maximising the availability of resources to essential processes, and diverting resources away from non-essential functions (Matteri et al., 2000; Charmandari et al., 2005). Therefore, changes in responsiveness to stressors are potentially problematic, because hyper-responsiveness may cause damage by altering resource partitioning more than necessary, whilst hypo-responsiveness will inhibit the ability to overcome or remove the cause of the stress (Matteri et al., 2000; Charmandari et al., 2005). Temperament may influence the ability of an individual to appropriately respond to and cope with threats because firstly, it is associated with variation in the responsiveness of the HPA axis to stressors, and secondly, it is associated with differences in metabolic factors that may influence activity of the HPA axis.

Temperament is associated with variation in the responsiveness of the HPA axis to stressors. For example, ‘nervous' individuals are more behaviourally responsive to stressors and generally have larger responses within the HPA axis to stressors, than their less responsive, or ‘calm', counterparts (Gunnar et al., 1989; Sternberg et al., 1992; Windle et al., 1998a; Curley et al., 2006; Marquez et al., 2006; Curley et al., 2008; Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013). The mechanism through which temperament affects the responsiveness of the HPA axis to a stressor is not clear, but in rats and cattle, the differences appear to be due to altered responsiveness of the pituitary-adrenal axis to hypothalamic signals, the peptides arginine vasopressin (AVP) and corticotrophin releasing hormone (CRH; Windle et al., 1998a; Capitanio et al., 2004; Curley et al., 2008). However, these findings have not been verified in a model with a known genetically-based temperament.
In addition to variation in the activity of the HPA axis, temperament is also associated with differences in a range of metabolic factors, including growth rate (Burrow and Dillon, 1997; Gerra et al., 1999; Gerra et al., 2000; Cafe et al., 2011a; Lennartsson and Jonsdottir, 2011), thermogenesis (Henry et al., 2010), circulating metabolite concentrations (Gerra et al., 2000), and feed intake and efficiency (Amdi et al., 2010). These effects may, at least to some extent, be mediated through insulin, a key metabolic hormone, because calm female sheep and male cattle have greater circulating concentrations of insulin than nervous conspecifics (Burdick Sanchez et al., 2014; Chapter 3). Variations in metabolism may affect the activity of the HPA axis because the metabolic system and the HPA axis act in concert to control energy balance (Pierluissi et al., 1986; Dallman et al., 1995). For example, in sheep, the magnitude of the cortisol response of the adrenal gland to adrenocorticotrophin (ACTH) is associated with reduced feed efficiency (Viengchareun et al., 2004; Knott et al., 2008), greater adiposity (Knott et al., 2010; Lee et al., 2014a), and greater thermogenesis in muscle tissue, both post-prandially (Mastronardi et al., 2000) and in response to an immune challenge (Lee et al., 2014b). Therefore, the relationship between temperament and the responsiveness of the HPA axis to stimulation may be driven by, or at least associated with differences in the metabolism of calm and nervous animals.

To investigate the effects of temperament on the responsiveness of the pituitary-adrenal axis to hypothalamic releasing hormones, AVP and CRH, we used Merino sheep that have been selectively bred for two decades for a nervous or calm temperament (Blache and Bickell, 2011b). The two temperament groups differ in their cortisol responses to isolation (one of the selection stressors) that could be due to innate differences in the responsiveness of the pituitary-adrenal axis to hypothalamic stimulation by AVP and/or CRH (Blache and Bickell, 2011b; Hawken et al., 2012a; Hawken et al., 2013). The calm and nervous animals also differ in
metabolic factors, including circulating concentrations of the key metabolic hormone insulin (Chapter 3), so we investigated whether the responsiveness of the pituitary-adrenal axis to stimulation was associated with changes in insulin concentrations. Therefore, we tested the following 3 hypotheses: that 1) treatment with a fixed dose of exogenous AVP and/or CRH will induce a greater increase in the secretion of adrenocorticotrophin (ACTH) and cortisol in nervous sheep than in calm sheep; 2) calm sheep will have greater circulating concentrations of insulin than nervous sheep; and 3) greater circulating concentrations of insulin will be associated with lower secretion of adrenocorticotrophin (ACTH) and cortisol in response to treatment with AVP and/or CRH.

Materials and methods

Animals

This experiment was carried out in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and was approved by the Animal Ethics Committee of The University of Western Australia under RA 33/100/1036.

The ACTH and cortisol responses to treatment with AVP and CRH were measured in calm (n = 8) and nervous (n = 8) 14 month-old, castrated male Merino sheep from the ‘UWA Temperament Flock’.

The ‘UWA Temperament Flock’ flock has been selectively bred for divergent behavioural reactions to the stressors of isolation and human presence (Murphy et al., 1994). Animals are tested at 3-4 months of age in two behavioural tests, the isolation box and the arena, as described previously (Murphy, 1999; Beausoleil et al., 2000).
al., 2008). The results of these tests are combined to produce a temperament score and males with the most extreme scores are kept for breeding (Blache and Bickell, 2011b). The calm animals are behaviourally hypo-reactive to stressors, a reaction interpreted as reflecting a lower level of fearfulness than their nervous counterparts. These measures of temperament therefore reflect a genetically determined ability to cope with stress, and the trait is moderately heritable (0.2 - 0.6; Murphy et al., 1994; Blache and Ferguson, 2005; Beausoleil et al., 2008; Blache and Bickell, 2011b).

**Treatments and experimental design**

The animals were housed indoors in individual pens and habituated to housing and human handling for 3 weeks prior to the experiment. Animals were subjected to natural lighting during this time (approximately 13L:11D), and housing was not climate controlled, but followed external temperature patterns. Temperament groups were balanced for weight (nervous: 45.6 ± 1.54 kg and calm: 46.9 ± 1.24 kg). Each animal received 4 treatments over 4 sessions, in a Latin square design. Animals were split into 4 groups, and all animals within a group received the same type of treatment during each session. Each group received the treatments in a different order, to avoid any effect of day or order in receiving treatment types. Over the 4 sessions, each animal received an intravenous bolus of saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW, i.v.), corticotrophin releasing hormone (CRH, 0.5 µg/kg BW, i.v.), and a combination of AVP (0.1 µg/kg BW, i.v.) and CRH (0.5 µg/kg BW, i.v.), via jugular cannula. Doses were based on previous studies (Copinschi et al., 1975; Gardner et al., 2006; Sloboda et al., 2007).

During each session blood was sampled via jugular cannula every 10 min for 4 h before and 4 h after the treatment was administered. Sampling began at 0900 h on each day. Blood samples were centrifuged immediately after collection for 10 min
at 3000 rpm, and the plasma was decanted and stored in plastic tubes at −20°C until radioimmunoassay.

Sampling and treatment sessions were held on Days 1, 3, 8, and 10 of the experiment and jugular cannulas were fitted on Days 0 and 7, so that each cannula was used for 2 sessions. The cannulae were flushed twice daily and after every sample during sampling with normal saline containing 5000 i.u. per litre heparin sodium BP (Pfizer Australia, Sydney, NSW).

One nervous animal had to be replaced during experimentation, due to a suspected infection prior to the commencement of sampling on day 10. This animal was replaced with a peer of similar weight, who had been kept with the other sheep for the duration of the experiment.

**Isolation box test**

To determine whether habituation to housing and human interaction altered the behavioural responses of the calm and nervous sheep to the stressor, the animals were subjected to the isolation box test 2 d before commencing habituation to the animal house and 2 d after the end of the experiment. This test is a valid test for the emotional state of the animals because it forms part of the selection criteria for the flock, and the behavioural response is robust and repeatable over the life of the animal (Blache and Ferguson, 2005). During the test, each animal was placed in a completely enclosed plywood box (1.5 m³) for 5 minutes, out of visual contact with peers (Murphy et al., 1994; Blache and Ferguson, 2005). The box is fitted with a digital meter that measures the amount of vibration caused by the animal's movement and vocalisations. Prior to testing, the meter was calibrated using a mechanical device that was placed inside the box and programmed to move 4 piston-driven ‘legs’ for 1 min, mimicking activity within the box at 3 levels of activity (Murphy et al., 1994; Blache and Ferguson, 2005). The sensitivity of the
agitation meter was adjusted to score 60 (low activity), 90 (medium), and 120 (high) arbitrary units. The calibration process was repeated at the end of testing, to ensure the meter had not drifted during the testing process. The agitation score was read at 1 and 5 min after the sheep entered the box.

**Hormone measurements**

*Cortisol*

Plasma concentrations of cortisol were analysed in samples collected between 30 min before treatment to 4 h after treatment (28 samples per animal) using commercial radioimmunoassay kits (Diasorin Australia Ltd. NSW), with a modified method validated for sheep, as described by Fulkerson and Tang (1979). The sensitivity of the assay was 1.25 ng/mL. Quality control samples (13.9 ng/mL and 30.5 ng/mL) were used to calculate inter-assay (14.0 % and 14.3 %) and intra-assay (8.4 % and 7.9 %) variation.

*ACTH*

Plasma sampled 10 min after treatment (one sample per animal) was also assayed for ACTH using in duplicate 100 µl aliquots and a human ACTH immunoradiometric kit (DiaSorin, Stillwater, OK, USA). Extra standards were made by diluting standards with the zero standard to extend the limit of detection of the assay to 3.12 pg/mL. All samples were processed in the same assay, and 2 quality control samples (33.48 pg/mL and 106.19 pg/mL) were included to assess intra-assay variation (2.0 % and 0.6 %).

*Insulin*

Insulin concentrations were measured in plasma sampled at -2, -1, 0, 1, 2, 3, and 4 h relative to treatment (seven samples per animal). Insulin was assayed in duplicate with a double-antibody radioimmunoassay (Tindal et al., 1978). All
samples were processed in a single assay and the limit of detection was 0.78 \( \mu U/mL \). Six replicates of three control samples containing 3.2, 5.2 and 11.9 \( \mu U/mL \) were included in the assay and were used to estimate the intra-assay coefficients of variation (8.6 %, 5.1 % and 6.0 %).

**Statistical analysis**

**Data preparation**

A cortisol response to treatment with AVP/CRH or AVP+CRH was defined as a rise in plasma concentrations of cortisol of more than 3 standard errors above the pre-treatment concentrations, within 30 min of the treatment (Bloomfield et al., 2003; Gardner et al., 2006; Sloboda et al., 2007). Two animals (1 calm, 1 nervous) showed a rise in cortisol concentrations during their control treatment period that was not deemed to be a response to treatment by the above criteria. Cortisol concentrations were not expected to rise during the control treatment, and did not rise in the other 14 animals, as determined by the above criteria. Therefore, these 2 animals may have been responding to other stimuli within the animal house, and these data were excluded from analysis.

All statistical tests were conducted using Genstat (Twelfth Edition, VSN International Pty Ltd, UK). Differences were considered significant if \( P \leq 0.05 \), and a tendency if \( P \leq 0.10 \), and so \( P \leq 0.10 \) reported as exact values. Prior to analysis, all data were assessed for normality using the Shapiro-Wilk test and for homogeneity of variance using Bartlett’s test. Where data were not normally distributed, or where variance was not homogenous across treatments, the data were transformed as described below. All data are presented as untransformed values for ease of interpretation.
All statistical models included day and order of receiving treatment as random factors (block). The effect of day was particularly of interest, as during day 3 and 4 of testing the ambient temperatures (33.5 and 36˚C) were greater than day 1 and 2 (23.8 and 23.9˚C). Daily maximum temperature was obtained from climate data available online from the Australian Government Bureau of Meteorology for Swanbourne, Western Australia located 2.8 km from the experimental site (Australian, 2013).

ACTH response to AVP and CRH treatment

ACTH concentrations at 10 min after treatment administration were subjected to split-plot ANOVA, using hormone treatment and temperament as treatments, day of treatment as a blocking factor, animal as whole plots, and order of treatment as subplots. Where a significant effect of a treatment factor was found, data were compared between treatments using Student’s paired t-test, and between temperaments using Student’s t-test.

Characteristics of the cortisol response to AVP and CRH treatment

The following characteristics of the plasma concentrations of cortisol in response to treatment were analysed: pre-treatment baseline concentrations, maximum concentration, amplitude of the response (maximum minus baseline concentrations), area under the curve, rate of response, time to maximum, duration of response, time from maximum to baseline, and area under the curve to maximum. Baseline was calculated as the average of the hormone concentrations prior to treatment (4 samples). Area under the curve was calculated as area under the curve relative to ground (AUCG), as total area under the curve, and as area under the curve relative to increase (AUCI), that is, exclusive of the area below the pre-treatment baseline, as described by Pruessner et al. (2003). Area under the curve to maximum was calculated only relative to increase above the pre-treatment
baseline. Return to baseline was determined by the time when concentrations fell to the pre-treatment baseline mean ± 1 standard deviation (Curley et al., 2008). The baseline concentrations, the rate of increase, and duration of the response required logarithmic (base 10) transformation before analysis. The area under the curve relative to ground, the amplitude of response, the maximum concentrations, and the area under the curve relative to increase required square-root transformation prior to analysis.

*Statistical analysis of the cortisol response to AVP and CRH treatment*

Data describing the cortisol response to treatment were subjected to a split-plot ANOVA. To determine differences between treatments, and demonstrate a difference between the AVP/CRH treatments and the control, the maximum concentration of cortisol, AUCG, and baseline concentrations of cortisol were analysed using a 3-way split plot ANOVA, with temperament, AVP, and CRH as treatment factors. Day was used as a blocking factor, and animal used as the whole-plot factor.

Measures of the cortisol response that were not meaningful for the control treatment were analysed using a 2 factor split-plot ANOVA, with temperament and treatment (AVP, CRH, and AVP+CRH) as treatment factors. Day was used as a blocking factor, animal used as the whole-plot factor, and order of treatment as the sub-plot factor. Day and order of treatment was not found to have any effect on any aspect of the cortisol response to treatment. The amplitude of the cortisol response, rate of the cortisol response, AUCI, and duration of response were analysed under this model.

The time from treatment to maximum concentration and the area under the curve to maximum (relative to increase) could not be transformed to establish normality and homogenous variance, and so were analysed using non-parametric tests. In
this case, variables were compared between treatments using Friedman’s non-parametric ANOVA, and where significant differences found, Wilcoxon matched-pairs test was used for post-hoc analyses. Temperament groups were compared within treatments with the Mann-Whitney U test.

*Insulin concentrations*

Insulin concentrations were compared over time between temperament and hormone treatments using a doubly repeated measures ANOVA, with animal and day of administration as blocking factors. Where a significant effect of time or treatment factor was found, data were compared between time points or treatments using Student’s paired t-test, and between temperaments using Student’s t-test. Prior to analyses, insulin data were subjected to logarithmic transformation (base 10). Day had a significant effect on insulin concentrations, and this effect was associated with variability in the ambient temperature. Therefore, we separated the data into the lower temperature days (Days 1 and 2, < 25°C) and greater temperature days (Days 3 and 4, > 33°C) and conducted the repeated measures ANOVA on each group separately, in addition to the analysis described above.

*Relationship between insulin and the pituitary-adrenal response*

Regression analyses were performed to determine whether insulin concentration affected the response of the pituitary-adrenal axis to AVP and CRH treatment. ACTH concentrations at 10 min post-treatment were compared with insulin concentrations at 0 h relative to treatment. Pre-treatment insulin concentrations (mean of -1 and 0 h) were compared with pre-treatment cortisol concentrations, the amplitude of the cortisol response was compared with the mean of insulin concentrations at 0 – 1 h, and the AUC was compared with the mean of insulin
concentrations between 0 – 4 h. All comparisons were done for each treatment separately, and temperament was included in the model as a grouping factor.

*Isolation box agitation scores*

The agitation scores from the isolation box tests, before and after the experiment, were subjected to repeated measures ANOVA, with temperament as treatment factor. Where a significant effect of time was found, data were compared using paired Student’s t-test, and where an effect of temperament or interaction were found, data were compared using unpaired t-tests. Due to the abnormal distribution of the data, the agitation scores were subjected to square-root transformation prior to analysis.

**Results**

**Pituitary-adrenal axis response to AVP and CRH**

Treatment affected plasma concentrations of ACTH (P < 0.001), but there was no effect of temperament (P > 0.10), or an interaction between temperament and treatment (P > 0.10). Specifically, administration of CRH and the combination of AVP + CRH increased the plasma concentrations of ACTH (CRH: P = 0.040; AVP + CRH: P < 0.001; Table 6.1) compared to the control (i.e. administration of saline) at 10 min post-treatment. Treatment with AVP alone did not increase ACTH concentrations at 10 min post-treatment compared to control in either calm or nervous animals (P > 0.10). Treatment with AVP + CRH yielded the greatest ACTH response (all P < 0.001; Table 6.1).

Treatment with CRH, AVP and AVP + CRH increased the plasma concentrations of cortisol compared to the control, as determined by maximum concentration achieved (all P < 0.001) and AUCG (all P < 0.001; Figures 6.1 and 6.2), irrespective
Table 6.1. Plasma ACTH concentrations (pg/mL) of calm or nervous sheep (n = 16) 10 min after intravenous treatment with saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW), corticotrophin releasing hormone (CRH, 0.5 µg/kg bodyweight), or a combination of AVP and CRH. All values are mean ± S.E.

<table>
<thead>
<tr>
<th>Temperament</th>
<th>Control</th>
<th>AVP</th>
<th>CRH</th>
<th>AVP + CRH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calm</td>
<td>2.00 ± 1.01a</td>
<td>4.51 ± 1.15ab</td>
<td>5.33 ± 2.01b</td>
<td>22.14 ± 5.24c</td>
</tr>
<tr>
<td>Nervous</td>
<td>4.29 ± 0.99a</td>
<td>3.10 ± 0.97ab</td>
<td>7.19 ± 1.54b</td>
<td>16.89 ± 3.30c</td>
</tr>
</tbody>
</table>

Different superscripts denote significant differences between treatments (P < 0.05)

Figure 6.1. Cortisol response (ng/mL) of calm (n = 8; solid lines) or nervous (n = 8; dashed lines) sheep to treatment with either a) saline (control), b) arginine vasopressin (AVP, 0.1 µg/kg BW), c) corticotrophin releasing hormone (CRH, 0.5 µg/kg BW), or d) the combination of AVP and CRH. Arrows indicate time of treatment administration. Mean standard error bars shown for each treatment.
of temperament. The combination of AVP+CRH yielded the largest and longest cortisol response compared to treatment with AVP or CRH (mean ± S.E. shown throughout; amplitude: 51.9 ± 3.80 ng/mL, all P < 0.001; duration of response: 132 ± 12.2 min, all P < 0.01). Treatment with AVP alone (amplitude: 35.2 ± 2.84 ng/mL; duration: 69 ± 5.3 min) resulted in a larger, but not longer, cortisol response than the treatment with CRH (amplitude: 26.5 ± 3.39 ng/mL, P = 0.007; duration: 79 ± 8.9 min, P > 0.10; Figures 6.1 and 6.2). There was no effect of day on any measure of ACTH or cortisol.

There was no effect of temperament or interactions between temperament and treatment on the magnitude of cortisol responses (all P > 0.10; Table 6.1). Although visually there appeared to be a difference between temperament lines in the magnitude of the response to the AVP+CRH treatment, temperament had no effect on any measure of the cortisol response to any treatment (i.e. baseline concentrations, maximum concentration of cortisol, AUCG, AUCI, the amplitude of the cortisol response, rate of the cortisol response, time to maximum, duration of response, or AUCI to maximum; all P > 0.10; Figures 6.1 and 6.2).

**Insulin**

Overall, nervous animals had greater insulin concentrations than calm animals (P = 0.007; Figure 6.3). There was no effect of treatment (i.e. AVP/CRH or AVP + CRH) or interactions between temperament and treatment on the plasma concentrations of insulin (P > 0.10).

There was an effect of day (P < 0.001), but not the order of receiving treatment (P > 0.10) on the plasma concentrations of insulin. Specifically, the concentrations of insulin were lower on Days 1 and 2 of sampling than on Day 3 and 4 (6.2 ± 0.15 μU/ml vs. 10.5 ± 0.27 μU/ml; P < 0.001 Figure 6.3), regardless of temperament or treatment. Interestingly, the ambient temperature was greater on day 3 and 4 of
Figure 6.2. Measures of the cortisol response (ng/mL) of calm (n = 8; white bars) or nervous (n = 8; black bars) sheep to treatment with either saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW), corticotrophin releasing hormone (CRH, 0.5 µg/kg BW), or the combination of AVP and CRH. Mean ± standard error shown.

testing (33.5 and 36°C) than Day 1 and 2 (23.8 and 23.9°C). To determine if the differences in temperature were masking any effects of treatment or temperament on plasma concentrations of insulin, we re-categorised the days by temperature and repeated the analysis. Insulin concentrations measured only on Days 1 and 2 were not affected by temperament (P > 0.10). However, analysis of insulin concentrations measured only on Days 3 and 4, the warmer days, revealed an effect of temperament (P = 0.042), with nervous animals having overall greater concentrations of insulin than the calm animals (11.6 ± 0.44 µU/mL vs. 9.4 ± 0.29 µU/mL).
Figure 6.3. Insulin concentrations (µU/mL) of calm (n = 8; solid lines) or nervous (n = 8; dashed lines) sheep on days where the ambient temperature was a) below 24˚C or b) above 33˚C. Arrows indicate timing of an intravenous injection with either saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW), corticotrophin releasing hormone (CRH, 0.5 µg/kg BW), or the combination of AVP and CRH. Mean standard error bar shown for each pair of days.

Relationship between pituitary-adrenal axis responsiveness and insulin

There was no significant correlation between plasma concentrations of insulin and any indicators of pituitary-adrenal responsiveness (i.e. ACTH concentration, peak cortisol concentration, AUC of the cortisol response, and pre-injection cortisol during any treatment; P > 0.10; Table 6.2), regardless of temperament. There was also no interaction of insulin concentration and temperament on any of these measurements (P > 0.10).

Table 6.2. Relationship between insulin concentrations and the pituitary-adrenal response to treatment with saline (control), arginine vasopressin (AVP, 0.1 µg/kg BW), corticotrophin releasing hormone (CRH, 0.5 µg/kg bodyweight), or a combination of AVP and CRH in calm or nervous sheep (n = 16). R² value shown, all P > 0.05. AUCG – area under the curve relative to the ground.

<table>
<thead>
<tr>
<th></th>
<th>ACTH</th>
<th>Amplitude</th>
<th>AUCG</th>
<th>Baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.5%</td>
<td>n/a</td>
<td>-6.9%</td>
<td>-6.5%</td>
</tr>
<tr>
<td>AVP</td>
<td>17.0%</td>
<td>-4.5%</td>
<td>-7.0%</td>
<td>-5.0%</td>
</tr>
<tr>
<td>CRH</td>
<td>3.8%</td>
<td>-7.4%</td>
<td>-1.3%</td>
<td>-1.9%</td>
</tr>
<tr>
<td>AVP + CRH</td>
<td>-6.2%</td>
<td>-5.5%</td>
<td>1.3%</td>
<td>-6.5%</td>
</tr>
</tbody>
</table>
Isolation box tests

The agitation scores for nervous animals were significantly greater than calm animals both before and after the experiment (P < 0.001; Table 6.3). There was no difference in the scores between tests for either temperament (P > 0.10; Table 6.3). During both tests, the agitation scores of nervous animals increased more between 1 and 5 minutes than the scores in calm animals (P < 0.001).

Table 6.3. Level of agitation in calm and nervous sheep (n = 16) exposed to a 5 min isolation stressor, before (test 1) and after experimentation (test 2) involving frequent human handling. All values are mean ± S.E.

<table>
<thead>
<tr>
<th>Temperament</th>
<th>Time (min)</th>
<th>Test 1</th>
<th>Test 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calm</td>
<td>1</td>
<td>49 ± 10</td>
<td>76 ± 19</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>118 ± 22 *</td>
<td>167 ± 36 *</td>
</tr>
<tr>
<td>Nervous</td>
<td>1</td>
<td>183 ± 17 a</td>
<td>177 ± 28 a</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>603 ± 63 a*</td>
<td>660 ± 141 a*</td>
</tr>
</tbody>
</table>

* Different from same measure in calm (P < 0.05)
  a Different from 1 min score, within temperament and test (P < 0.05)

Discussion

There was no difference between calm and nervous sheep in either the ACTH or cortisol response to treatment with exogenous AVP and/or CRH, and therefore we reject our first hypothesis. Furthermore, contrary to our second hypothesis, nervous animals had greater concentrations of insulin overall than calm animals. Finally, insulin concentrations were not associated with the magnitude of the pituitary-adrenal axis response to treatment with exogenous AVP and/or CRH, thus rejecting our third hypothesis. Thus it appears that that the disparity in the cortisol response of calm and nervous sheep to social stressors is not due to differences in the responsiveness of the pituitary gland to AVP/CRH or the adrenal gland to ACTH, but is likely to be mediated by supra-pituitary mechanisms.
We hypothesised that the calm and nervous sheep would have differential responses to fixed doses of AVP/CRH or a combination of the two neuropeptides because nervous sheep have a larger and longer cortisol, response to social stressors than calm sheep (Blache and Bickell, 2011a; Hawken et al., 2012a; Hawken et al., 2013). However, we found that they exhibited a similar ACTH and cortisol response to AVP and CRH treatment, both separately and in combination. The sheep were still deemed ‘calm’ or ‘nervous’ at the end of the experiment, as indicated by their scores in response to the isolation box test. The dose rates used in this study reflect physiological concentrations of AVP and CRH release, as they yielded cortisol responses comparable to those seen previously in this temperament model in response to stressors, using the same hormone assay methods (Hawken et al., 2012a; Chapter 4). Therefore, we can conclude that the divergent physiological responses of calm and nervous sheep to social stressors are not due to differences in their pituitary-adrenal responsiveness to hypothalamic stimulation. This observation is supported by previous work in cattle where the magnitude of the pituitary-adrenal axis response of female calves to exogenous CRH (0.03 μg/kg BW, i.v.) and ACTH (0.016 i.u./kg BW, i.v.) was not affected by their behavioural or endocrine response to novelty (Van Reenen et al., 2005). In contrast, in rats, the relatively hyper-reactive Fischer rat has a larger ACTH response to 0.5 μg CRH, and a more rapid, but not larger overall, response to 2 μg CRH than the hypo-reactive Lewis rats (Spinedi et al., 1994; Windle et al., 1998a). However, the literature is far from clear cut and even within a species, the relationship between HPA activity and temperament is very variable. For example, in a cattle model where temperament was determined by the speed that they left a squeeze chute, more ‘excitable’ castrated males had greater ACTH, but not cortisol, responses to AVP (1.0 μg/kg BW, i.v.) than in ‘calm’ animals (Curley et al., 2010). In contrast, within the same model, the calm young females had larger pituitary-adrenal axis
responses to CRH (0.1 μg/kg BW, i.v.) and ACTH (0.1 i.u./kg BW, i.v.) treatment than the excitable females (Curley et al., 2008), possibly indicating a gender-dependent relationship. The variation in the results from the current study and previous work show that the link between temperament and pituitary-adrenal responsiveness to AVP and CRH is highly complex and dependent on various aspects of the animal model studied, including the species, gender, temperament trait, and genetic basis of the model. The sheep in the current study have been selectively bred for their behavioural reactivity to social stressors for over 20 generations, and their behavioural and neuroendocrine reactivity to stress is both sustained and heritable, with a known genetic basis (Bickell et al., 2009). In contrast, the model used by Curley Jr (2008; 2010), used unselected cattle that were deemed to be ‘excitable’ or ‘less excitable’ based on the results of a single test, within 1 generation. Using unselected animals potentially increases the influence of other factors on the stress response, such as maternal influence, early life history, recent experience, social rank, or, in females, stage of oestrous cycle (Birke and Archer, 1975; Grandin, 1997; Meaney, 2001). Furthermore, comparisons with the Lewis and Fischer model must be interpreted with care, as the rats are not selected on temperament, but on inflammatory responses, which directly involves the secretion of glucocorticoids, possibly confounding the temperament – HPA axis relationship (Anderle et al., 1985; Sternberg et al., 1989a; Sternberg et al., 1989b; Spinedi et al., 1994; Windle et al., 1998a). Therefore, we conclude that not all studies of temperament or reactivity to stress are likely to have the same physiological basis so care must be taken in making generalised conclusions from the literature. However, at least within the calm and nervous sheep, pituitary-adrenal responsiveness does not appear to drive their divergent cortisol responses to social stress. 

123
Pituitary-adrenal responsiveness does not appear to be responsible for the differences in the cortisol response of calm and nervous sheep to stressors, and so the cause likely lies in supra-pituitary mechanisms, such as within the hypothalamus itself or in the brain regions that process emotion. For instance, within the hypothalamus, there may be temperamental divergence in expression of CRH and/or AVP. This concept is supported by observations from rodent models of anxiety, such as the high (HAB)/low (LAB) anxiety model, and the Lewis and Fischer rat model. Lewis rats have relatively low behavioural and cortisol responses to open field exposure, and have decreased CRH mRNA expression after restraint compared to the relatively hyper-reactive Fischer rats (Sternberg et al., 1992). HAB/LAB rats are selected for their divergence in anxiety-related behaviours during an elevated plus maze, and the more anxious HAB rats have higher rates of mRNA expression and release of AVP in the hypothalamus paraventricular nucleus (PVN) after exposure to the open arms of this maze than the LAB rats (Wigger et al., 2004). HAB mice and rats also showed greater neuronal activation in the anxiety and fear circuitry in the PVN in response to open exposure than LAB rats (Salome et al., 2004; Muigg et al., 2009). In addition to the hypothalamus, differences in the HPA axis response may be due to variation within the amygdala, a brain region controlling emotional responses that facilitates stimulation of the HPA axis. Emotional processing is chiefly controlled by γ-aminobutyric acid (GABA)-ergic control, and in HAB mice, basal levels of mRNA and protein for GABA synthesising enzymes in the amygdala were increased compared to mice displaying normal anxiety-related behaviours (Tasan et al., 2011). In summary, in rodents, variation in responsiveness to stressors is associated with differences in activity in key regions of the brain related to anxiety, fear and stress which control AVP and CRH production and release. No studies to date have quantified the expression of AVP, CRH, or GABA in any large animal
models of anxiety or stress, but the observations in the current study would indicate that the divergent cortisol responses of calm and nervous sheep to social stressors may be mediated at the level of the hypothalamus or the amygdala.

Interestingly, overall the plasma concentrations of insulin were greater in nervous sheep than in calm sheep, although insulin concentrations were not related to the pituitary-adrenal response to treatment. Insulin is an important hormone in the regulation of metabolism, and this observation suggests that insulin may mediate the impact of temperament on metabolic factors previously observed in both the temperament flock (Amid et al., 2010; Henry et al., 2010; Hawken et al., 2012b) and in other models (cattle (Burrow and Dillon, 1997; Cafe et al., 2011a; Cafe et al., 2011b); sheep (Knott et al., 2008; Lee et al., 2014a; Lee et al., 2014b)). However, our results suggest this relationship does not appear to be due to direct interaction between insulin and the pituitary-adrenal axis. Instead, the nexus between metabolism and temperament is likely to be mediated at the level of the brain, as previous studies have shown that both cortisol and insulin affect the neural network regulating energy balance, and this neural network appears to regulate the responsiveness of the HPA axis (Dallman et al., 1995; Lennartsson and Jonsdottir, 2011). However, the relationship between temperament and insulin does not appear to be straightforward, as previously we found that over a 24 h period insulin concentrations in calm female sheep were similar to or greater than in nervous female sheep, in non-stressed animals (Chapter 3). This divergence in findings may reflect a gender difference in the relationship between temperament and insulin activity, as gender appears to affect insulin activity in a range of species, including sheep (Gatford et al., 2004); humans (Clausen et al., 1996); and rats (Gerra et al., 1999; Yayou et al., 2010). In conclusion, the relationship between temperament and metabolism is likely to be mediated at the level of the brain, and seems to be context-dependent.
The day of the experiment appeared to have an effect on insulin concentrations, apparently due to high ambient temperature (maximum > 33°C) on 2 d of testing. This relationship, though unexpected, was not surprising because insulin concentrations are sensitive to high ambient temperature (Achmadi et al., 1993; Itoh et al., 1997; Itoh et al., 1998a; Itoh et al., 1998b; Itoh et al., 2001). However, it is interesting to note that, when the hot and cool days are considered separately, there is a difference between temperament groups in insulin concentrations on the hot days suggesting that nervous animals might be more responsive to warm ambient temperatures than calm animals. Studies in cattle have shown that a more ‘excitable’ temperament was associated with lower resistance to heat stress than calmer animals (Prayaga and Henshall, 2005; Brown-Brandl et al., 2006). A similar relationship might exist in sheep, and so the greater insulin concentrations found in the nervous animals compared to the calm animals may indicate a divergence in responsiveness to warmth, which may be investigated by testing the impact of the response to a heat load on metabolism in the two lines.

In summary, the responsiveness of their pituitary-adrenal axis to hypothalamic peptides does not differ between calm and nervous sheep. Therefore, the origin of the disparate responses of the HPA axis to stress lies elsewhere in the hypothalamic-pituitary-adrenal axis. We propose that it may originate from differences in neural expression of CRH and/or AVP in the hypothalamus, similar to those reported in rats selected for high or low anxiety. Nervous sheep appeared to have greater plasma concentrations of insulin than calm sheep, but a link with ambient temperature makes interpretation of the data difficult.
Chapter 7.

General discussion

The general aim of this thesis was to investigate the effect that selection for fear-related temperament in Merino sheep has upon the activity of the hypothalamic-pituitary-adrenal (HPA) axis. A secondary aim was to test whether the relationship between fear-related temperament and the HPA axis affects activity of other hormonal systems, in particular insulin, as well as leptin and prolactin. We found that selection for temperament has not affected the resting activity of the HPA axis (Chapters 3 and 4) or the responsiveness of the pituitary-adrenal axis to stimulation (Chapters 5 and 6). As in previous work, we have found that nervous animals show greater responses of the HPA axis than calm animals to isolation, one of the stressors on which the UWA temperament flock are selected (Blache and Bickell, 2011b; Hawken et al., 2012a; Chapter 4). We found that temperament was associated with differences in insulin secretion. Therefore, we conclude that selection for temperament seems to affect HPA axis activity during exposure to stressors only, and that the mechanism for this divergence does not affect either the resting activity of the HPA axis, or the responsiveness of the pituitary-adrenal axis to stimulation with exogenous hormones. The divergence in the responsiveness of the HPA axis to stressors between the temperament groups is likely to be mediated at the level of the brain and/or of the negative feedback of glucocorticoids of the HPA axis. Furthermore, temperament seems to affect the activity of insulin independent of changes within the HPA axis.
Although selection for temperament in Merino sheep affects the response of the HPA axis to stressors, it does not affect the resting activity of the HPA axis, or the responsiveness of the pituitary-adrenal axis to stimulation. Findings on the activity of the HPA axis within the temperament flock are both supported and contradicted by findings in other animal models based upon selection for fear-related temperament. For example, our findings are similar to those from a rat and mouse model of high (HAB) and low (LAB) anxiety-related behaviour. HAB animals engage in more anxious behaviours than LAB animals when exposed to a variety of stressors, including an elevated plus maze (EPM; the selection test for the model), an open field test, and a light-dark box test (Liebsch et al., 1998b; Henniger et al., 2000; Salome et al., 2002; Kromer et al., 2005; Muigg et al., 2009). Adult male rats from the HAB line have greater ACTH and glucocorticoid responses to the open arm of the EPM than LAB animals, although both lines have similar concentrations of both hormones in the absence of stressors (Landgraf et al., 1999), as seen in our model (Chapters 3 and 4). Furthermore, HAB and LAB rats have similar ACTH and glucocorticoid responses to CRH treatment, indicating that pituitary-adrenal responsiveness is not responsible for the divergence in HPA axis responses to stressors (Liebsch et al., 1998a; Neumann et al., 1998). Therefore, selection for fear-related temperament based upon the EPM in rodents has a similar impact upon HPA axis activity as selection for fear-related temperament in our sheep model. Both models are based on selection for behaviour in response to fear-eliciting situations, and so it is likely that similar brain pathways are involved in mediating the divergence in responsiveness to stressors.

However, our findings contrast with other studies in genetically-based animal models of temperament. The Maudsley reactive (MR) rats freeze and defaecate more frequently, interpreted as being more fearful, during an open field test than non-reactive (MNR) rats (Denenberg, 1969; Broadhurst, 1975). As in our model, the
two lines of rats have similar glucocorticoid concentrations in the absence of stressors (Abel, 1991). However, MR and MNR rats show similar glucocorticoid responses to stressors such as the open field test, forced swimming and foot shock (Abel, 1991; Blizard and Adams, 2002). In contrast, in our model the calm and nervous animals show a divergence in the response of the HPA axis to isolation (Blache and Bickell, 2011b; Hawken et al., 2012a; Chapter 4), although there was no difference between the lines in the cortisol response to 10 min exposure to the arena test (Beausoleil et al., 2008). Furthermore, MNR, MR rats and unselected Wistar rats have similar glucocorticoid responses to restraint stress, although MNR rats had a smaller ACTH response to restraint stress than MR rats (Kosti et al., 2006). This finding was confirmed by in vitro tests, which showed that MR rats have lower adrenal responsiveness to ACTH stimulation than MNR (Kosti et al., 2006). Therefore, although there is some impact on adrenal sensitivity to stimulation, it appears that selection for fear-related temperament in rats based upon defaecation frequency during an open field test does not strongly impact glucocorticoid activity either at rest or in response to stressors.

Our findings also contrast with those from a model in Japanese quails (Coturnix coturnix japonica) bred for the duration of tonic immobility (TI) after an immobilisation stressor (Mills and Faure, 1991). Birds that remain immobile longer (LTI) are deemed to be more fearful than those that begin to move more quickly (STI), and STI birds seem generally less fearful than LTI birds (Jones et al., 1991; Mills and Faure, 1991; Faure et al., 2006) (Mills and Faure, 2000). Glucocorticoid concentrations are similar between the STI and LTI animals in the absence of stressors, similar to our findings in the UWA temperament flock (Hazard et al., 2005; Hazard et al., 2008a; Hazard et al., 2008b). However, STI birds have larger glucocorticoid responses to a restraint stress over 2 min or restraint in a crush cage than LTI birds, although the groups have similar responses to repeated induction
of TI and 1 min restraint stress (Hazard et al., 2005; Hazard et al., 2008a; Hazard et al., 2008b). This contrasts with our model, where the more fearful line of animals show larger HPA axis responses to stressors than the less fearful line (Blache and Bickell, 2011b; Hawken et al., 2012a; Chapter 4). The divergence in glucocorticoid responsiveness to stressors may be mediated by adrenal responsiveness, as STI birds have larger glucocorticoid responses to treatment with exogenous ACTH than LTI birds, although the relationship varied with gender and age, particularly in LTI birds (Hazard et al., 2005; Hazard et al., 2008b). Therefore, in quails, a less fearful temperament, rather than a more fearful temperament, as determined by immobility after restraint, is associated with greater responsiveness of the HPA axis to stressors.

Species difference is likely to contribute to the differences in HPA axis activity between genetically based models of fear-related temperament. However, for example, as shown above findings from the HAB/LAB rat model differ from the MNR/MR model in the same species, but are similar to those from our sheep model, indicating that the basis for selection is likely to play a stronger role in the relationship between temperament and the HPA axis. Although, the physiological response to stressors was once thought to be a uniform response to all stressors, it has become clear that the response to stressors varies as much as the stressors themselves (Selye, 1950; Moberg, 2000). Therefore, how HPA axis activity is affected by selection for temperament is likely to be determined by to what degree the HPA axis is involved in the response to the stressor(s) chosen as the basis for selection, and whether the mechanism for the divergence in the behavioural response to the stressor has a strong influence on the activity of the HPA axis. For example, in the Maudsley rats, the lack of a relationship between defecation frequency in response to a stressor and HPA axis activity in this model may be because defaecation in responsive to fear-eliciting situations is reflexive and results
from the lifting of inhibition via the parasympathetic nervous system (Maggi et al., 1988). In contrast, the UWA temperament flock and HAB/LAB models measure complex behavioural responses to stressors that are controlled centrally, and so it is likely that similar pathways control the divergence in responses between the temperament groups in these models. Therefore, the relationship between HPA axis activity and fear-related temperament depends on the behaviour chosen as the basis for selection. In our model of fear-related temperament in sheep, it seems that selection based upon the behavioural response to the stressors of isolation and human presence affects the response of the HPA axis to stress, but not activity in the absence of stressors.

Our findings may have been influenced by the gender of sheep used in our studies, because sex steroids affect HPA axis activity and responsiveness in sheep (Tilbrook et al., 2000). For example, intact rams have higher concentrations of AVP in the median eminence than intact ewes, and gonadectomy increased concentrations of AVP in both sexes (Canny et al., 1999). There are differences between genders at the level of the adrenal gland, as intact ewes show greater glucocorticoid responses to ACTH than gonadectomised ewes and intact and gonadectomised rams (van Lier et al., 2003). Interestingly, the response of rams to the ACTH treatment was greater in the non-breeding season than in the breeding season (van Lier et al., 2003). However, under basal conditions, intact rams have greater glucocorticoid production in vitro than gonadectomised rams and intact ewes (Canny et al., 1999). Regardless of gonadal status, rams show greater cortisol responses to insulin-induced hypoglycaemia than ewes, whilst ewes have a greater cortisol response to isolation plus restraint stress than rams (Turner et al., 2002). Gender affects HPA axis activity both at rest and during stimulation, and gender may interact with temperament to affect HPA axis activity. For example, Fischer rats are hyper-reactive to stressors compared to the Lewis rats (Windle et al., 1998a). Female
Lewis rats show a typical circadian rhythm of plasma concentrations of glucocorticoids, whereas female Fischer rats lack the trough period of the rhythm, resulting in elevated concentrations of glucocorticoids throughout the day (Windle et al., 1998a). In comparison, male Fischer rats show a typical circadian rhythm in glucocorticoid concentrations, but in comparison, male Lewis rats seem to have a blunted rhythm, having a smaller peak of the rhythm (Griffin and Whitacre, 1991). Therefore, although we did not find any effect of temperament on HPA axis activity at rest in ewes or wethers (male sheep gonadectomised before puberty; Chapters 3 and 4) or pituitary-adrenal responsiveness in wethers (Chapters 5 and 6), it is possible that different results would be found if these studies were repeated in rams.

Our study showed no evidence that selection for temperament affected resting activity of the HPA axis or the responsiveness of the pituitary-adrenal axis to stimulation. Furthermore, although we did not directly test it, we found no evidence that temperament affects the negative feedback processes of the HPA axis in the suppression of cortisol production with dexamethasone, or in the length of the cortisol responses and return to baseline after treatment with AVP, CRH, and ACTH (Chapters 5 and 6). Together, this evidence indicates that the divergence in the response of the HPA axis to stressor exposure between the calm and nervous animals is not because of changes in the basal activity of the axis or of differences in the responsiveness of the pituitary and/or adrenal glands to stimulation or possibly negative feedback. Instead, the source of divergence in stress responsiveness is likely to be in the brain, as the site of the perception of stressors and the initiation, modulation and termination of the stress response (Matteri et al., 2000; Moberg, 2000). This hypothesis is supported by previous work in the temperament flock which suggests that there are differences in how the calm and nervous animals perceive and assess stressors. Calm and nervous animals did not
show differences in their glucocorticoid and behavioural responses to an initial exposure to isolation plus a novel object (flapping white plastic), a non-selection stressor (Bickell, 2005; Blache and Bickell, 2011b). However, upon subsequent exposures, the calm animals maintained a similar glucocorticoid and behavioural response to the stressor, whilst the nervous animals had increased responses. Furthermore, nervous animals appeared to anticipate the novel object stressor, showing heightened stress responses even when the novel object was not present, whilst the calm animals did not appear to anticipate the novel object, suggesting differences between the temperament groups in cognitive processes. Therefore, it seems likely that the divergence in cortisol and behavioural responses to stressors between the lines of the UWA temperament flock are mediated within the brain.

There are several neurological pathways that affect behaviour and HPA axis activity which may be responsible for modulating emotional reactivity in the UWA temperament flock, including AVP, CRH, γ-aminobutyric acid (GABA), serotonin, and dopamine. Directly in control of glucocorticoid release, AVP and CRH are obvious candidates for controlling the magnitude of the HPA axis response to stressors. In addition, both peptides can affect fear- and stress-related behaviours in rodents (Stenzel-Poore et al., 1994; Liebsch et al., 1996; Davis, 1999; Ebner et al., 1999; Karolyi et al., 1999; Koob and Heinrichs, 1999; van Gaalen et al., 2002; Wigger et al., 2004), and, as discussed in Chapter 6, rats and mice that engage in more fear- and anxiety-related behaviours when exposed to a stressor show higher activity in the CRH and AVP secretion pathways when exposed to a stressor compared to their relatively hypo-reactive counterparts (Sternberg et al., 1992; Keck et al., 2002; Salome et al., 2004; Wigger et al., 2004; Muigg et al., 2009). A functional polymorphism in the AVP-locus is associated with over-expression of AVP in HAB rats (Murgatroyd et al., 2004), and functional polymorphisms in genes of the CRH-locus are associated with HPA axis reactivity and behavioural...
inhibition in humans and emotional reactivity in cattle and Rhesus macaques (Smoller et al., 2003; Smoller et al., 2005; Barr et al., 2008; Pugh et al., 2011; Sheikh et al., 2013). Therefore, both AVP and CRH can affect stress responsiveness in a genetic manner, and are likely candidates for mediating the divergence in responsiveness of the HPA axis to stressors between temperament groups in the UWA temperament flock.

Serotonin and dopamine are neurotransmitters that can affect the expression of fear and anxiety-related behaviours, and so may play a role in the divergence in stress-responsiveness in our model. Low concentrations of serotonin are associated with increased expression of anxiety and fear behaviour (Lesch et al., 1996; Parks et al., 1998; Ramboz et al., 1998; Li et al., 1999; Holmes et al., 2003; Hariri and Holmes, 2006). Serotonin is involved in genetically-based stress resilience, with functional polymorphisms in genes associated with serotonin transporters shown to be related to larger cortisol responses to challenge (Jabbi et al., 2007; Gotlib et al., 2008; Mueller et al., 2010), and higher susceptibility to depression after stressful life events in humans (Caspi et al., 2003; Zalsman et al., 2006; Kim et al., 2007). Dopamine plays important roles in higher cognitive function and in seeking behaviours (Arnsten et al., 2012), and dopaminergic pathways are associated with stress-related behaviours, particularly during chronic stress (Muscat et al., 1990; Chaudhury et al., 2013; Tye et al., 2013). Polymorphisms in the dopaminergic pathways are associated with behavioural traits in boys (Noble et al., 1998) and differences in the susceptibility to depression after stressful life events (Elovainio et al., 2007). Therefore, serotonin and dopamine pathways may contribute to divergence in responsiveness of the HPA axis to stressors between temperament groups in the UWA temperament flock.

Finally, GABA, a neurotransmitter controlling emotional processing in the amygdala, may play a role in the divergence in stress responsiveness between the
calm and nervous sheep. Activity of GABA within the amygdala has previously been related to temperament in mice and humans, with more anxious individuals displaying higher activity in the GABA-ergic systems (Stein et al., 2007; Tasan et al., 2011). Furthermore, GABA appears to influence the behavioural response of sheep to isolation, as administration of agonistic benzodiazepines reduces the level of agitation shown by isolated sheep (Drake et al., 2004). Interestingly, lavender oil has been shown to have divergent effects on the stress response of calm and nervous sheep (Hawken et al., 2012a). As the reported anxiolytic properties of lavender oil appear to be mediated by the GABA-ergic system, in a similar manner to benzodiazepines, this finding may indicate that there are differences within the GABA pathways between the two temperament groups (Aoshima and Hamamoto, 1999; Bradley et al., 2007; Shaw et al., 2007; Woelk and Schläfke, 2010). In summary, there are several neurological pathways that, either individually or interactively, may mediate the divergence in the responsiveness of the HPA axis to stressors between the temperament groups.

Selection for temperament is seen as a tool that can improve animal health, welfare, and productivity in livestock production systems (Pottinger, 2000). A more fearful temperament is seen as disadvantage, as these individuals seem more disturbed by stressors and probably expend more resources on the stress response. Furthermore, a more responsive temperament has been associated with an elevated or even lack of a trough in the circadian rhythm, resulting in higher mean concentrations of glucocorticoids in the absence of stressors (Windle et al., 1998a; Capitanio et al., 2004). High concentrations of glucocorticoids suppress other functions such as growth, immunity, and reproduction (Elsasser et al., 2000; Moberg, 2000; Tilbrook et al., 2002), and so nervous animals may have reduced productivity and health due to relative hyperactivity of the HPA axis. However, our study showed no evidence that selection for fear-related temperament in Merino
sheep affects the activity of the HPA axis outside of the response to stressors, indicating that, in the absence of stressors, nervous animals are probably not disadvantaged by impaired diurnal rhythms or exposure to excess glucocorticoids compared to calm animals. Because the HPA axis strongly influences other systems within the body and prolonged changes in the activity of the HPA axis can result in dysfunctioning within those systems, it seems beneficial for health and welfare that the resting activity of the HPA axis should be highly robust, and therefore minimally affected by factors such as temperament.

Temperament seems to affect insulin activity; however, our findings are not consistent across experiments. In Chapter 3 we found that calm ewes had higher insulin concentrations than nervous animals between 1300 – 1900 h, and a greater amplitude of the diurnal pattern of insulin secretion. In chapter 4, we found no relationship between insulin activity, temperament and repeated exposure to stressors in wethers (males castrated before puberty). In this experiment, insulin was measured between 0900 – 1500 h, and animal numbers were restricted to 4 per group. However, the relationship becomes more complex in Chapter 6, where we measured insulin whilst stimulating cortisol production with AVP and/or CRH in wethers, between 1000 – 1600 h. There was no relationship between insulin and the cortisol response to stimulation, but nervous animals had higher insulin concentrations than calm animals. These were confounded by warmer ambient temperatures experienced on some days of experimentation. A possible explanation of the findings may be that at rest, calm animals have higher insulin (chapter 3), which may indicate that they are directing more energy into storage rather than immediate use, possibly making them more efficient than nervous animals. The absence of a relationship between temperament and plasma concentrations of insulin in chapter 4 may be due to gender differences (Clausen et al., 1996; Gerra et al., 1999; Gatford et al., 2004; Yayou et al., 2010) and/or the low number of
animals. However, the findings of Chapter 6 may indicate that nervous animals are more responsive than calm animals to the stimulus of warmer ambient temperatures, reversing the relationship found in Chapter 3, although the disparity between chapters 3 and 6 may also reflect a gender difference. Our experiments were not primary designed to investigate the relationship between temperament and insulin secretion, so further work is needed to investigate the complex and context-dependent relationship between temperament and insulin.

Throughout this thesis, we have found links between fear-related temperament and insulin activity. Insulin is a major hormone in the control of metabolism, and our findings may indicate that insulin mediates the relationship between temperament and aspects of metabolism seen in previous studies (Amdi et al., 2010; Henry et al., 2010). For example, previous work in this model has found that nervous animals have lower core body temperatures when fed below maintenance requirements than their ‘calm’ counterparts (Henry et al., 2010), and that nervous animals seem to be more feed efficient than calm animals in a housed situation (Amdi et al., 2010). Furthermore, temperament is associated with metabolism in cattle, as ‘calm’ animals have higher growth rates and carcass weights than ‘temperamental’ animals (Burrow and Dillon, 1997; Cafe et al., 2011a). There seems to be a link between stress, insulin activity and other aspects of metabolism, as stress in humans is associated with higher insulin activity as well as increased abdominal fat, and high concentrations of glucose and lipids in the blood (Raikkonen et al., 1996). However, in our model, the relationship between fear-related temperament and insulin activity seems to be independent of the HPA axis. Instead, a possible candidate is the sympatho-adrenomedullary axis, which is responsive to stress, and affects insulin concentrations (Ahrén et al., 1981; Richter et al., 1981). Selection for temperament can affect the responsiveness of the sympatho-adrenomedullary axis, as HAB rats display hypo-responsiveness of the
sympatho-adrenomedullary axis to exposure to the open arm of the EPM compared
to rats from the LAB line (Salome et al., 2006). There seems to be a relationship
between fear-related temperament and insulin that may be mediated by the
sympatho-adrenomedullary axis and may explain other differences in metabolism
found between the temperament groups. However, further work is necessary to
determine the exact nature of how temperament affects metabolism.

The gut-brain relationship may also play a role in mediating the divergences in
both metabolism and stress-responsiveness in the calm and nervous sheep. Whilst
the gut is obviously involved in metabolism, recently much attention has been paid
to its potential role in affecting behaviour, emotionality and stress responsiveness
(Clarke et al., 2014; Keightley et al., 2015; Luna and Foster, 2015). For example,
manipulation of gut biota has been shown to affect behavioural and HPA axis
responses to stressors in mice (Keightley et al., 2015). In children, temperament
measures have been associated with the composition of the gut microbiome
(Christian et al., 2015) Therefore, gut flora may play a role in mediating the
difference between the lines in the UWA temperament flock, but further work is
needed.

In the absence of stressors, the calm and nervous animals are indistinguishable in
behaviour and HPA axis activity, but when presented with isolation, one of the
stressors this flock is selected upon, nervous animals are hyper-reactive compared
to the calm animals (Chapters 3, 4, and 6; (Blache and Bickell, 2011b; Hawken et
al., 2012a; Hawken et al., 2013)). Divergence between the temperament groups
only in the presence of challenge is seen within other systems in the UWA
temperament flock. For example, in Chapter 6, we found differences in insulin
concentrations between the temperament groups only existed on warm days (> 33°C),
but not on the cooler days (< 24°C). The increased heat load on the animals
on the warmer days presents a physiological challenge, to which the nervous
138
animals seem to be more responsive. Another example of divergence between the temperament groups is seen in lambing. Murphy (1994; 1999) found that calm ewes displayed better maternal behaviour post-parturition and had lower lamb mortality than nervous ewes. In contrast, Bickell (2009; 2011) found fewer effects of temperament on maternal behaviour and no effect on lamb survival. In Murphy’s study, humans were present during lambing and lambs were tagged immediately after birth, whereas during Bickell’s study, human presence was minimal, suggesting that the stressor of human presence interacts with temperament to affect ewe behaviour and lamb behaviour. Therefore, behavioural and physiological differences between the temperament groups may only be present when faced with external stimuli, whether a psychosocial stressor like human presence or isolation, or a physiological stressor such as a heat or nutritional challenge.

In summary, this thesis has demonstrated that selection for temperament has not resulted in changes in the resting activity of the HPA axis or in the responsiveness of the pituitary-adrenal axis to stimulation. Therefore, the mechanism for divergence in the responsiveness of the HPA axis to isolation between the calm and nervous animals is most likely due to changes within the brain systems which control activation of the HPA axis. In addition, we hypothesised that temperament would be related to insulin activity, through activity of the HPA axis. Although we found that selection for temperament is related to insulin activity, this relationship appears to be independent of the HPA axis. Furthermore, this relationship appears to be context dependent, as the nature of the relationship differs between experiments.


corticotropin-releasing hormone, hypothalamic-pituitary-adrenal axis activity, temperament, and alcohol consumption in Rhesus macaques. *Arch Gen Psychiatry* **65**, 934-44.


Bickell, S. L. (2009). Does the selection on temperament in Merino sheep significantly affect the establishment of the ewe-lamb bond or lamb survival?, The University of Western Australia, Perth.


selectively bred for high- and low-anxiety-related behavior.

_Neuropsychopharmacol_ 19, 381-96.


Masuzaki, H., Ogawa, Y., Hosoda, K., Miyawaki, T., Hanaoka, I., Hiraoka, J.,
Glucocorticoid regulation of leptin synthesis and secretion in humans:
elevated plasma leptin levels in Cushing’s syndrome. *J Clin Endocrinol Met*
*82*, 2542-7.

Matsumoto, H., Maruyama, M., Noguchi, J., Horikoshi, Y., Fujiwara, K., Kitada,
Stimulation of corticotropin-releasing hormone-mediated
adrenocorticotropic secretion by central administration of prolactin-

Matsumoto, H., Noguchi, J., Horikoshi, Y., Kawamata, Y., Kitada, C., Hinuma, S.,
release by prolactin-releasing peptide in rats. *Biochem Biophys Res Comm*
259, 321-4.

animal welfare"* (G. Moberg and J. A. Mench, eds.). CABI Publishing,
Wallingford.

Mattson, C., Lai, M., Noble, J., McKinney, E., Yau, J. L., Seckl, J. R., and Walker,
B. R. (2003). Obese Zucker rats have reduced mineralocorticoid receptor and
11beta-hydroxysteroid dehydrogenase type 1 expression in hippocampus-
implications for dysregulation of the hypothalamic-pituitary-adrenal axis in
obesity. *Endocrinology* 144, 2997-3003.

releasing factor alone, but not arginine vasopressin alone, stimulates the
release of adrenocorticotropic in the conscious intact sheep. *Endocrinology*
136, 1821-1827.


Putu, G. (1988). Maternal behaviour in Merino ewes during the first two days after parturition and survival of the lamb, The University of Western Australia, Perth.


vasopressin-, and neurotensin-immunoreactive neurons in the hypothalamus of the male rat. *J Neurosci* 4, 1118-29.


185


Twenty four-hour profiles of metabolic and stress hormones in sheep selected for a calm or nervous temperament

Authors: S.E. Rietema\textsuperscript{1}, M.A. Blackberry\textsuperscript{1}, S.K. Maloney\textsuperscript{2}, G.B. Martin\textsuperscript{1}, P.A.R. Hawken\textsuperscript{2}, D. Blache\textsuperscript{1}

Addresses: \textsuperscript{1}The UWA Institute of Agriculture and School of Animal Biology, and \textsuperscript{2}The School of Anatomy, Physiology and Human Biology, The University of Western Australia, Crawley, Western Australia, 6009, Australia

Corresponding author: Dominique Blache, The School of Animal Biology M085, The University of Western Australia, Crawley, Western Australia, 6009, Australia
dominique.blache@uwa.edu.au

+61 8 6488 6763
Abstract

Even in the absence of stressors, temperament is associated with changes in the concentration of stress-responsive hormones and, possibly because of such changes, temperament can affect metabolism. We tested whether, in sheep bred for temperament for 14 generations, ‘nervous’ females have greater concentrations of stress-responsive hormones in the absence of stressors than ‘calm’ females, and whether these differences are associated with changes in the concentrations of metabolic hormones. In resting ‘calm’ (n = 8) and ‘nervous’ (n = 8) sheep, concentrations of cortisol, prolactin, leptin and insulin were measured in blood plasma sampled via jugular catheter every 20 min for 24 h. The animals were individually penned, habituated to their housing and human handling over 7 wk, and fed before sampling began. Diurnal variation was evident for all hormones, but a 24-h cortisol pattern was detected in only 7 individuals. There was no effect of temperament on any aspect of concentrations of cortisol or prolactin, but ‘calm’ animals had greater concentrations of insulin in the early afternoon than ‘nervous’ animals (14.5 ± 1.1 vs. 10.0 ± 1.6 μU/mL; P = 0.038), and a similar tendency was seen for leptin (P = 0.092). We conclude that selection for temperament affects the concentration of metabolic hormones in the absence of stressors, but this effect is independent of stress-responsive hormones.

Keywords: Temperament, cortisol, prolactin, insulin, leptin
1. Introduction

Temperament determines, at least in part, how an individual responds to stressful situations and can vary widely even within species and genders [1]. A highly responsive temperament can change the pattern of secretion of the stress-responsive hormones such as cortisol and prolactin in the presence of stressors [1-3] but also in the absence of stressors [1-3]. Furthermore, temperament can also affect metabolism [4-7], however, the mechanisms behind these relationships are not well understood.

Temperament is reported to be affected by the diurnal pattern of the pulsatile secretion of cortisol in rats and macaques [8, 9]. Prolactin secretion is also affected by temperament, but most studies have investigated the prolactin response to stimuli, rather than in the absence of stressors [10-13]. The magnitude of the prolactin response to stimuli is correlated with temperament in humans and cattle [10-13], and, in humans, prolactin responses to stimuli are positively correlated with the cortisol response [13-15]. However, to our knowledge, the impact of temperament on the diurnal pattern of prolactin secretion in the absence of stressors, in relationship with the activity of the HPA axis, has not been investigated.

Differences in the diurnal pattern of stress-responsive hormones could help to explain the apparent effects of temperament on metabolism because cortisol directly affects the circulating concentrations of the two primary metabolic hormones, insulin and leptin. Glucocorticoids stimulate leptin secretion [16, 17] and acute increases in cortisol concentrations suppress insulin secretion in response to nutrient intake [18, 19]. The role of prolactin as a regulator of metabolism is still being elucidated but, in rodents and humans, prolactin is reported to stimulate and potentiate leptin secretion [20-25], although a suppressive effect has also been found [25-27]. Therefore, cortisol and prolactin can affect the secretion of metabolic hormones, but it is not known whether an increased responsiveness to stressors can increase the concentrations of the stress-
responsive hormones in a non-stressed state, and thereby lead to changes in metabolic
hormone balance.

To further understand the relationship between temperament, the resting state of the
hypothalamic-pituitary-adrenal (HPA) axis and metabolic hormones, we measured the
concentrations of cortisol, prolactin, insulin and leptin in sheep that had been selectively
bred to be of ‘calm’ or ‘nervous’ temperament on the basis of hypo- or hyper-reactivity to
isolation and human presence. The diurnal patterns of secretion were described in the
absence of any additional stressors (i.e. at rest). We hypothesised that, compared to
‘calm’ animals, ‘nervous’ animals will have: 1) greater plasma cortisol concentrations,
with less pronounced variation over 24 h; 2) greater plasma concentrations of prolactin;
and 3) greater plasma concentrations of leptin, with leptin values greatest after the
peak in the patterns of cortisol and prolactin concentrations; and 4) decreased plasma
concentrations of insulin.

2. Methods

2.1 Experimental design

This experiment was carried out in accordance with the Australian Code of Practice for
the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and was
approved by the Animal Ethics Committee of The University of Western Australia
(RA/13/1003/333).

2.2 Animals

The UWA temperament flock comprises two lines of Merino sheep that have been bi-
directionally selected for 14 generations for extreme behavioural reactions to the
stressors of social isolation and human presence. This phenotypic trait is heritable [28].
Locomotor activity and vocalisations are measured in two tests, the isolation box and
novel arena tests, as described elsewhere [29,30]. Mobility and vocalisation measures
are used to calculate a temperament score, and hypo-reactive sheep are termed ‘calm’
whilst hyper-reactive sheep are termed ‘nervous’ [28,29]. For the present experiment, 2-
year-old ewes were selected from the calm (n = 8) and nervous (n = 8) lines of the
population. These animals had divergent scores in the arena test (CROSS: calm 6.3 ±
2.16 v nervous 18.4 ± 4.58 crosses; BLEAT: calm 5.3 ± 3.36 v nervous 39.4 ± 13.70) and
isolation box test (BOX: calm 24.5 ± 10.13 v nervous 71.8 ± 7.70) conducted at weaning.
Furthermore these animals were subjected to the isolation box test for 1 min, 2 d before
commencing habituation to the animal house.

For the duration of the experiment, animals were individually penned indoors, in 1
room, for 7 wk, beginning on November 16 (mid-non-breeding season), at the University
of Western Australia (31° 58′ S). Photoperiod was 12L:12D with lights on at 0600 h. The
animals were subjected to minimal human contact and were under constant video
surveillance. Pen-cleaning and feeding followed a regular schedule, with the animals fed
before 0700 h daily. Animals were fed a diet of hammer-milled oaten chaff with 20%
lupin seed and 2.5% mineral mixture (Siroman, Narrogin Mineral Stockmix, Narrogin,
WA, Australia) providing 9.2 MJ of metabolisable energy and 13% crude protein per kg
DM. Each animal was fed enough to maintain bodyweight. Feed intake was monitored,
and all animals finished their daily ration within 90 min.

2.3 Sampling

After 38 d of habituation, the animals were fitted with an in-dwelling jugular cannula
with an extension to the shoulder. The following morning, the animals were fed at 0600
h. We decided to feed the animals prior to the commencement of sampling, because
although feeding stimulates secretion of leptin and insulin, missing a feed is a stressor,
and not feeding the animals can potentially lead to hypoglycaemia, which can trigger
cortisol and prolactin responses, and disrupt the normal circadian rhythm of these
hormones [31,32].

Blood sampling commenced at 0700 h and continued every 20 min for 24 h. Lights were kept on for sampling at night. Samples were centrifuged at 2000 g for 10 min so that plasma could be separated, and stored in plastic tubes at −20°C until assay.

2.4 Hormone analysis

2.4.1 Cortisol

Plasma cortisol concentrations were measured in duplicate 100 µL aliquots [33]. The limit of detection was 0.20 ng/mL. Six replicates of two control samples were included in the assay to estimate the intra-assay coefficients of variation of 7.9 % at 3.8 ng/mL and 18 % at 2.5 ng/mL.

2.4.2 Prolactin

Plasma prolactin was measured with a homologous double antibody RIA [34]. The samples were assayed in duplicate 10 µL aliquots and the limit of detection was 0.26 ng/mL. The assay included six replicates of three control samples which were used to estimate the intra-assay coefficients of variation of 6.7 % at 0.5 ng/mL, 8.1 % at 1.0 ng/mL and 3.8 % at 2.0 ng/mL.

2.4.3 Insulin

Plasma insulin was assayed in duplicate by a double-antibody radioimmunoassay [35]. All samples were processed in a single assay and the limit of detection was 0.78 µU/mL. The assay included six replicates of three control samples which were used to estimate the intra-assay coefficients of variation of 6.7 % at 2.5 µU/mL, 5.2 % at 3.7 µU/mL and 5.6 % at 11.1 µU/mL.

2.4.4 Leptin

6
Plasma leptin was measured in duplicate by a double-antibody radioimmunoassay
developed for sheep in our laboratory [36]. All samples were processed in a single assay
and the limit of detection was 0.10 ng/mL. The assay included six replicates of three
control samples which were used to estimate the intra-assay coefficients of variation of
1.9 % at 0.42 ng/mL, 6.8 % at 0.91 ng/mL and 4.9 % at 1.4 ng/mL.

2.5 Data analysis

Pulses of cortisol and prolactin secretion were detected with MUNRO (Zaristow
Software, West Morham, Haddington, East Lothian, UK) [37]. For the present study,
the G parameters were 3.98, 2.4, 1.68, 1.25, and 0.93 for G1-G5, respectively. For cortisol,
the Baxter parameters were -0.751 (B1, the y-intercept), 0.3404 (B2, the x coefficient),
and -0.00008 (B3, the x^2 coefficient); for prolactin, they were 0.6872 (B1), 0.0156 (B2), and
0.0003 (B3).

The hormone data were arbitrarily divided into four equal time periods of 6 h: (0700–
1300 h; 1300–1900 h; 1900–0100 h; 0100–0700 h) to allow analysis of variation in pulse
characteristics over the 24-h day [8]. This approach was based on a previous method for
description of diurnal variation in sheep [38]. The time periods encompass and separate
phases in the cycle (peak, trough), and are long enough to include sufficient pulses
(about 3) to enable statistical analysis.

All statistical tests were conducted using Genstat (Genstat 5. Second Edition, Lawes
Agricultural Trust, Rothamsted Experimental Station, Hertfordshire UK). Prior to
statistical analysis, all data were assessed for normality using the Shapiro-Wilk test and
for homogeneity of variance using Bartlett’s test. Where data were not normally
distributed, or where variance was not homogenous across treatments, the data were
transformed as described below. All data are presented as untransformed values for
case of interpretation.
For cortisol and prolactin, we compared the following pulse characteristics between time periods and between temperaments: amplitude, area under the pulse curve, nadir, baseline, mean concentrations, and pulse frequency. Pulse frequency was calculated as the number of pulses per h. Baseline concentrations for cortisol and prolactin were determined for each individual animal by selecting the 7 lowest values in each 6 h time period, and averaging these [39]. Over a 24-h period, the lowest 28 points were used.

Insulin and leptin concentrations were not pulsatile and so we analysed only the mean concentrations.

Pulse amplitude, area under the pulse curve, nadir, baseline, and mean concentrations for cortisol and prolactin, and mean concentrations of insulin and leptin were initially compared over time and between temperaments using repeated measures ANOVA. Differences were considered significant if $P \leq 0.05$, and a tendency if $P \leq 0.10$. Where significant differences were found, paired 2-tail t-tests were used to compare pulse characteristics among time periods, and Student’s t-tests were used to compare characteristics between temperaments in the same time period. For both cortisol and prolactin, values for the pulse amplitude, area under the pulse curve, nadir, baseline and mean concentration were transformed logarithmically (base 10) before analysis to correct for variance being proportional to the mean. Total number of pulses over 24 h was compared between temperament groups using Student’s t-test.

Pulse frequency data were not normally distributed and so were compared among time periods using Friedman’s test and between temperaments using the Mann-Whitney U test. Where Friedman’s test showed significance, time period pairs were compared using Wilcoxon Matched-Pairs tests.

To analyse the diurnal variation in hormone patterns, all hormone profiles were fitted with a cosine curve using cosinor analysis, restricted to a 24 h period [40]. Where the fit of the curve was significant, the mesor, amplitude, and timing of the acrophase (in...
degrees) of the curve were returned. Not all hormone profiles returned a significant

cosine curve, restricting the sample size to 7 animals for cortisol (calm: n = 3, nervous: n
= 4), 12 for prolactin (calm: n = 6, nervous: n = 6), 15 for insulin (calm: n = 8, nervous: n
= 7), and 13 for leptin (calm: n = 6, nervous: n = 7). The acrophase, mesor, and
amplitude of the curves were compared between temperaments using 2-tailed t-tests.

Because only 7 out of 16 cortisol profiles returned a significant cosinor pattern, and this
outcome was not associated with temperament, it appeared that there were 2 groups of
animals, those with and those without diurnal variation. Therefore, the cortisol dataset
was re-analysed with the relevant statistical tests, as described above, but including the
presence or absence of a rhythm as a factor.

Scores from the isolation box test were compared between the temperaments using
Student’s t-test.

3. Results

3.1 Isolation box test

Nervous animals had significantly greater agitation scores from the isolation box test
than calm animals (nervous 54 ± 9.1 vs. calm 21 ± 5.2 (arbitrary units – see methods); P
= 0.007).

3.2 Cortisol

All of the animals showed discrete pulses of cortisol secretion over 24 h, with pulses
peaking up to 35 ng/mL (Figure 1). The total number of pulses over 24 h (mean ± S.E.
shown throughout; calm 13.4 ± 0.5 vs. nervous 12.8 ± 0.6; P = 0.418) was similar
between temperaments.
There was a significant effect of time on mean concentrations ($P = 0.043$), and baseline ($P = 0.033$), but not on area under the pulse curve ($P = 0.316$), or nadir ($P = 0.279$; Figure 2). Pulse frequency varied with time in both temperaments (calm: $P = 0.016$, nervous: $P = 0.043$; Figure 2). Pulse amplitude varied greatly, both within (c.v. 69 ± 4.5 %) and between (c.v. 39 %) individuals and, although each animal appeared to have a pattern of larger and smaller peaks, the patterns were not consistent among individuals (Figure 1) and so there was no difference in pulse amplitude among time periods ($P = 0.112$; Figure 2). There was no effect of temperament or an interaction between time and temperament on any characteristic of cortisol secretion.

Specifically, pulse frequency was decreased between 0700 – 1300 h compared to 1300 – 1900 h ($P < 0.001$) and 1900 – 0100 h ($P = 0.029$), and decreased between 0100 – 0700 h compared to 1300 – 1900 h ($P = 0.003$; Figure 2), regardless of temperament. Baseline concentrations of cortisol were greater between 0100 – 0700 h than between 0700 – 1300 h ($P = 0.0013$), 1300 – 1900 h ($P = 0.017$), and 1900 – 0100 h ($P = 0.025$). Mean concentrations of cortisol were greater between 0100 – 0700 h than between 0700 – 1300 h ($P = 0.045$) and 1300 – 1900 h ($P = 0.024$).

Cosinor analysis of cortisol profiles returned a significant curve for 7 out of 16 animals (Table 1). Within these animals, calm animals had a decreased minimum compared to nervous animals ($P = 0.034$), but there was no difference between temperaments in mesor, amplitude, acrophase, or maximum.

The cortisol dataset was re-analysed including the presence or absence of a diurnal rhythm as a factor in the ANOVA model. There was an effect of an interaction between time, temperament and presence of a diurnal rhythm on mean cortisol concentrations ($P = 0.028$) and a tendency for an effect on area under the pulse curve ($P = 0.051$; Figure 2).

There was an effect of an interaction between temperament and presence of a diurnal rhythm on mean cortisol concentrations ($P = 0.006$), pulse amplitude ($P = 0.048$), and
pulse nadir ($P = 0.003$). There was an effect of time on mean cortisol concentrations ($P = 0.023$) and baseline concentrations ($P = 0.009$). There was a tendency for an effect of temperament on pulse nadir, with nervous animals having slightly greater concentrations than calm animals ($P = 0.075$).

Specifically, between 0700 – 1300 h calm animals with a diurnal rhythm (DR) had decreased mean cortisol concentrations compared to calm animals with no diurnal rhythm (NDR; $P = 0.005$), or any nervous animals (DR: $P = 0.044$, NDR: $P = 0.043$; Figure 2). Calm DR animals also had decreased mean cortisol concentrations compared to calm NDR ($P = 0.010$) and nervous DR animals ($P = 0.029$) between 1300 – 1900 h.

Regardless of time period, calm DR animals had decreased mean cortisol concentrations compared to calm NDR ($P = 0.002$) and nervous DR animals ($P < 0.001$); nervous NDR animals had decreased mean cortisol concentrations compared to calm NDR ($P = 0.024$) and nervous DR ($P = 0.0021$) animals; and calm NDR tended to have decreased mean concentrations compared to nervous NR ($P = 0.062$). Regardless of temperament and presence of a diurnal rhythm, mean cortisol concentrations were greater between 0100 – 0700 h than 0700 – 1300 h ($P = 0.043$) and 1300 – 1900 h ($P = 0.024$).

Regardless of time, nervous DR had greater pulse amplitudes than calm DR ($P = 0.037$) and nervous NDR ($P = 0.021$), and tended to be greater than calm NDR ($P = 0.063$).

Furthermore, calm DR had decreased pulse nadirs compared to calm NDR ($P < 0.001$), nervous DR ($P < 0.001$) and NDR ($P = 0.018$), regardless of time period. Nervous DR had greater pulse nadirs than calm NDR ($P = 0.029$) and nervous NDR ($P = 0.004$). Presence of a rhythm did not affect pulse frequency.

3.3 Prolactin
All of the animals showed discrete pulses of prolactin secretion over 24 h (Figure 1), and there was no difference between the temperament lines in the number of pulses detected over 24 h (calm $12.9 \pm 0.5$ vs. nervous $12.3 \pm 0.9$, $P = 0.543$).

There was a significant effect of time on the baseline ($P = 0.021$), mean ($P = 0.006$) and nadir ($P = 0.023$) concentrations of prolactin and a tendency for an effect on area under the pulse curve ($P = 0.053$). As for the cortisol profiles, the amplitude of prolactin pulses appeared to vary throughout the day for individual animals but was highly variable both within (c.v. $77 \pm 6.5\%$) and between (c.v. $63\%$) animals (Figure 1 and 3), and no effect of time was found on pulse amplitude ($P = 0.326$). There was no effect of temperament on the baseline ($P = 0.709$), mean concentration ($P = 0.592$), nadir ($P = 0.796$), or pulse amplitude ($P = 0.747$), or area under the pulse curve concentrations of prolactin ($P = 0.286$). There was no effect of an interaction between temperament and time on the baseline ($P = 0.293$), mean concentration ($P = 0.358$), nadir ($P = 0.310$), or pulse amplitude ($P = 0.295$), or area under the pulse curve concentrations of prolactin ($P = 0.162$). Nervous animals had a decreased pulse frequency between 1900 – 0100 h compared to calm animals ($P = 0.011$; Figure 3), although neither temperament showed diurnal variation in pulse frequency (calm: $P = 0.576$, nervous: $P = 0.241$; Figure 3).

Mean concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h ($P = 0.013$), 1900 – 0100 h ($P = 0.0078$), and 0100 – 0700 h ($P = 0.016$; Figure 3). Similarly, baseline concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h ($P = 0.0042$), 1900 – 0100 h ($P = 0.024$), and 0100 – 0700 h ($P = 0.019$; Figure 3). Also, nadir concentrations of prolactin were decreased between 0700 – 1300 h compared to between 1300 – 1900 h ($P = 0.028$), 1900 – 0100 h ($P = 0.0623$), and 0100 – 0700 h ($P = 0.033$; Figure 3).

Cosinor analysis of prolactin concentrations yielded significant curves for 12 out of 16 animals (Table 1). The acrophase was advanced in the nervous animals compared to the
calm animals by around 2 h ($P = 0.047$). However, there was no difference in the mesor ($P = 0.632$), amplitude ($P = 0.607$), minimum ($P = 0.673$), or maximum ($P = 0.604$) between temperaments (Table 1).

### 3.4 Leptin

There was an overall effect of time on plasma concentrations of leptin ($P < 0.001$; Figure 1) but no effect of temperament ($P = 0.191$) and a tendency for an interaction between temperament and time ($P = 0.092$). Specifically, plasma concentrations of leptin were decreased between 0100 – 0700 h compared to 0700 – 1300 h ($P < 0.001$), 1300 – 1900 h ($P < 0.001$), and 1900 – 0100 h ($P < 0.001$; Figure 4). Concentrations of leptin were greater between 1300 – 1900 h than 1900 – 0100 h ($P = 0.001$), and tended to be greater than between 0700 – 1300 h ($P = 0.077$; Figure 4).

Cosinor analysis showed that 13 animals (calm: n = 6; nervous n = 7, Table 1) had a significant diurnal rhythm in leptin secretion. Within these animals, there was a tendency for nervous animals to have an advanced acrophase compared to calm animals ($P = 0.052$; Table 1). Temperament did not affect the mesor ($P = 0.286$), amplitude ($P = 0.833$), minimum ($P = 0.280$), or maximum ($P = 0.315$; Table 1) of the fitted cosine curvus.

### 3.5 Insulin

There was an effect of time ($P < 0.001$) but not temperament ($P = 0.110$) on plasma concentrations of insulin. However, there was an interaction between temperament and time ($P = 0.033$). Plasma concentrations of insulin were greater during 0700 – 1300 h than between 1900 – 0100 h ($P = 0.0019$) and 0100 – 0700 h ($P < 0.001$). Similarly, plasma concentrations of insulin were greater during 0700 – 1300 h than between 1900 – 0100 h ($P < 0.001$) and 0100 – 0700 h ($P < 0.001$). Mean plasma concentrations of
insulin were greater in calm than nervous animals between 1300 – 1900 h (P = 0.038) and tended to be greater between 0700 – 1300 h (P = 0.063; Figure 4).

Cosinor analysis of insulin concentrations yielded significant curves for 15 out of 16 animals (calm: n = 8; nervous n = 7, Table 1). Within these animals, cosinor curves fitted to calm animals had a greater amplitude than in the nervous animals (P = 0.019; Table 1), and tended to have a greater maximum (P = 0.063; Table 1). Temperament had no effect on the mesor (P = 0.184), acrophase (P = 0.471), or minimum (P = 0.813) of the cosinor curve of plasma concentrations of insulin (Table 1).

4. Discussion

Temperament did not affect the pulse characteristics, mean concentrations, or the expression of a diurnal variation of cortisol or prolactin, but nervous animals had decreased concentrations of insulin in the afternoon hours, resulting in a smaller diurnal variation, than in calm animals. There was a similar tendency for leptin concentrations. We therefore conclude that, in the absence of stressors, selection for temperament does not affect the activity of the HPA axis or prolactin secretion, but does lead to differences in the concentrations of insulin, indicating possible effects on the regulation of glucose homeostasis and perhaps adipose homeostasis.

The lack of a difference between calm and nervous sheep in the pattern or magnitude of cortisol secretion indicates that selection for behavioural reactivity to the stressors of isolation and human presence does not affect the resting activity of the HPA axis. The minimum of the cosinor curve describing the pattern of cortisol concentrations was decreased in calm animals compared to nervous animals; however, this result must be interpreted with caution, as it is based on a subset of animals (n = 7), and was not reflected in the baseline or pulse nadir concentrations of cortisol in the full set of animals. Our findings are consistent with a rodent model of anxiety, where high anxiety
(HAB) rats secrete more glucocorticoids and ACTH when faced with their selection
stressor than low anxiety (LAB) rats, but have similar concentrations of corticosterone
and ACTH at rest [41]. Our experiment was conducted in anoestrous ewes, and because
of the interactions between sex steroids and the HPA axis we acknowledge that we may
have had different findings if our experiment was conducted in oestrous-cycling ewes or
males [42]. It is possible that stage of the oestrous cycle can affect the diurnal activity of
the HPA axis, and for this reason, we chose to maximize our ability to detect a difference
in HPA axis activity by eliminating potentially confounding effects and conducting the
experiment in anoestrous ewes. We conclude that in anoestrous ewes selected for
temperament the resting activity of the HPA axis is not affected by the mechanisms that
drive the divergence in cortisol secretion in response to the stressors of isolation and
novelty [28,43,44].

A diurnal pattern of cortisol secretion was detected in less than half of the animals, and
was independent of temperament. Although it is widely accepted that cortisol is secreted
in a diurnal pattern in a number of species [40], it is not unusual for individuals within
a population to not display a diurnal rhythm in cortisol secretion [45,46]. Within sheep,
the existence of a diurnal rhythm itself is questionable because the studies that detected
a rhythm used only 3 animals. Because the animals were kept in a single room under
identical conditions, were of similar age and weight, and the experiment was conducted
in the middle of the non-breeding season, thus eliminating any effects of the oestrous
cycle [47], it is difficult to attribute our findings to any factor. Interestingly, in horses
the absence of routine and/or hyper-reactivity of an individual to the sampling protocol
was found to likely result in enhanced cortisol secretion that masks the daily rhythm
[48]. Calm and nervous sheep have similar cortisol responses to some novel stressors
[44], and so the reactivity of the individual animals to the change of routine on the day
of sampling could have affected their diurnal pattern of cortisol secretion. The incidence
and biological significance of a diurnal rhythm in cortisol secretion in sheep requires further investigation.

Interestingly, analysis of the cortisol data, including the presence of a diurnal rhythm as a factor into the statistical model, suggests that temperament can affect the HPA axis activity but only in animals that present a diurnal rhythm. This observation reflects data from South African Merino sheep that had been selected for high multiple-rearing ability and that are less behaviourally responsive during an arena test, indicating a ‘calm’ temperament [49]. In these sheep, the magnitude of the cortisol response to an insulin challenge is affected by an interaction between phenotype and a polymorphism in a gene coding for a steriodogenic enzyme, CYP17, that controls cortisol production [50]. Thus, the subtle differences between subsets of temperament groups in the current study could be due to polymorphisms affecting adrenal steroidogenesis. The small sample size restricts interpretation, but these observations suggest a need for further investigation into the relationship between temperament and the HPA axis.

Plasma concentrations of insulin were decreased in nervous animals compared to calm animals only in the afternoon, at the peak of the pattern, indicating an interaction between temperament and the diurnal variation in the secretion of insulin. The variation in insulin concentrations over the day may reflect a true circadian rhythm as, in monogastric animals, there appears to be a circadian rhythm in insulin secretion even when blood glucose concentrations are constant [51,52]; however, the decline in insulin concentrations over the day may also simply reflect that the animals were fed 1 hour prior to sampling [53]. Differences in insulin concentrations between temperaments could be related to the apparent difference between temperaments in the minimum of the cortisol rhythm, but, as discussed above, the finding for cortisol is based on a subset of animals, and so must be interpreted with caution. Nevertheless, our findings are supported by other studies of temperament in ruminants. For instance, calm bulls have
greater concentrations of insulin than 'temperamental' bulls, both before and in 
response to an endotoxin challenge [54], whilst 'temperamental' heifers presented a 
greater insulin response to cannulation than calm heifers [55]. Our findings suggest 
that a more behaviourally-responsive temperament is associated with a greater energy 
requirement that, in turn, possibly leads to greater circulating concentrations of blood 
glucose and therefore decreasing concentrations of insulin. This concept is supported by 
studies in cattle, where calm animals have greater growth rates and carcass weights 
than 'temperamental' animals [6,7]. Interestingly, temperament differences in plasma 
concentrations of metabolites in response to handling were not correlated with HPA axis 
activity [7]. Overall, it appears that divergence between temperament groups in 
metabolic factors is not mediated solely by the HPA axis, but more likely to involve 
other systems, such as the sympatho-adrenal-medullary system.

However, the relationship between temperament and metabolism in the calm and 
nervous sheep is far from simple and appears to be dependent on environmental factors 
such as nutrition. For example, nervous sheep fed at maintenance produce less 
colostrum than calm sheep; however, when animals were supplemented with barley, 
this difference disappeared, indicating that there may be a nutrition x genotype 
interaction in the control of the metabolism of calm and nervous sheep [56]. Similarly, 
diet determined whether there were differences in core body temperature or adipose 
tissue temperatures in response to feeding in calm and nervous sheep [5]. In summary, 
the relationship between temperament and metabolism is highly complex, but the 
observation that nervous female sheep fed at maintenance have decreased 
concentrations of insulin in the afternoon compared to calm sheep offers an insight into 
the mechanism behind the relationship between temperament and metabolism.

Contrary to our hypothesis, plasma concentrations of prolactin were not related to 
temperament, an observation that is coherent with the lack of difference between the
temperament groups in cortisol secretion because the two hormones are regulated by some of the same mechanisms [57-60]. Therefore, it is possible that, as with cortisol, prolactin secretion will differ between the temperament groups only during exposure to a stressor; however, no study to date has looked at prolactin concentrations in these sheep. This concept is supported by a study in heifers where behavioural reactivity to stressors was correlated with the prolactin response to those stressors, but not with concentrations of prolactin at rest [61]. In contrast to our findings, in both the HABLAB and Maudsley reactive and non-reactive models, the line of rats that appear more disturbed by stressors have greater plasma concentrations of prolactin at rest than rats that are less disturbed, in the absence of any differences in corticosterone or ACTH [62-64]. These discrepancies may reflect differences between species and in protocols for selection and assessment of temperament. We therefore need to test whether prolactin concentrations change in parallel with cortisol concentrations in the calm and nervous sheep subjected to their selection stressors.

In summary, selection for fear-related temperament affects the magnitude of the response of HPA axis to stressors of sheep, but does not alter HPA activity in the absence of stressors in anoestrous ewes. However, selection for temperament does affect insulin secretion, through an unknown process possibly independent of the HPA axis. This effect could explain the differences in metabolism reported for calm and nervous animals.

5. References


[9] Capitanio JP, Mendoza SP, Bentzon KL. Personality characteristics and basal cortisol concentrations in adult male rhesus macaques (Macaca mulatta).


209


211

[30] Murphy PM. Maternal behaviour and rearing ability of Merino ewes can be improved by strategic feed supplementation during late pregnancy and selection for calm temperament. The University of Western Australia, 1999.


Tables.

Table 1. Coefficients of a cosine model fitted to 24 h plasma hormone profiles of calm and nervous ewes. Values shown are the mean ± S.E for only animals that showed a significant rhythm in plasma hormone concentration, as determined by cosine analysis.

<table>
<thead>
<tr>
<th>Hormone</th>
<th>Moser ng/mL</th>
<th>Amplitude ng/mL</th>
<th>Acrophase Degrees (h ± min)</th>
<th>Maximum ng/mL</th>
<th>Minimum ng/mL</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>Calm 6.1 ± 0.32</td>
<td>2.6 ± 0.43</td>
<td>227 ± 12 (2206 h ± 48 min)</td>
<td>8.7 ± 0.75</td>
<td>3.5 ± 0.12</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>Nervous 7.7 ± 1.49</td>
<td>3.0 ± 0.71</td>
<td>173 ± 53 (1833 h ± 213 min)</td>
<td>10.6 ± 2.19</td>
<td>4.7 ± 0.81*</td>
<td>4</td>
</tr>
<tr>
<td>Prolactin</td>
<td>Calm 58 ± 3.4</td>
<td>27 ± 5.2</td>
<td>229 ± 19 (2215 h ± 78 min)</td>
<td>85 ± 7.2</td>
<td>31 ± 4.9</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Nervous 69 ± 7.6</td>
<td>31 ± 14.3</td>
<td>199 ± 18* (2014 h ± 73 min)</td>
<td>91 ± 21.5</td>
<td>29 ± 7.7</td>
<td>6</td>
</tr>
<tr>
<td>Insulin</td>
<td>Calm 10.7 ± 0.96</td>
<td>3.3 ± 0.35</td>
<td>111 ± 7 (1424 h ± 27 min)</td>
<td>14.0 ± 1.14</td>
<td>7.40 ± 0.89</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>Nervous 9.8 ± 0.57</td>
<td>2.5 ± 0.44*</td>
<td>128 ± 30 (1534 h ± 120 min)</td>
<td>12.3 ± 1.14*</td>
<td>7.27 ± 1.00</td>
<td>7</td>
</tr>
<tr>
<td>Leptin</td>
<td>Calm 1.9 ± 0.06</td>
<td>0.2 ± 0.02</td>
<td>125 ± 7 (1520 h ± 23 min)</td>
<td>2.0 ± 0.07</td>
<td>1.7 ± 0.04</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>Nervous 1.8 ± 0.14</td>
<td>0.2 ± 0.03</td>
<td>111 ± 11* (1425 h ± 44 min)</td>
<td>1.9 ± 0.13</td>
<td>1.6 ± 0.14</td>
<td>7</td>
</tr>
</tbody>
</table>

* Different from calm, \( P < 0.05 \)

* Tendency to be different from calm, \( P < 0.10 \)
Figure captions.

Figure 1. Examples of 24-h profiles in the plasma concentrations of stress and metabolic hormones in 4 ewes that had been genetically selected for either a calm or nervous temperament. Cortisol and prolactin pulses, as determined by the analysis program Munro, are marked by an asterisk. Profiles were chosen randomly.

Figure 2. Characteristics of cortisol secretion over 24 h in anoestrous Merino ewes bred for calm or nervous temperament that lack (black bars) or display a diurnal rhythm (white bars) in cortisol secretion as determined by cosinor analysis. Time had an effect on pulse frequency, mean concentrations, pulse amplitude and nadir. There was an effect of an interaction between temperament and the presence of a diurnal rhythm on mean concentrations and baseline, and an effect of an interaction between time, temperament and the presence of a diurnal rhythm on mean concentrations. Bars indicate significant difference between groups within temperament and time period. For other significant differences, please refer to text (all values are mean ± S.E; n = 16).

Figure 3. Characteristics of plasma prolactin pulses over 24 hours in anoestrous Merino ewes bred for calm (white bars) or nervous (black bars) temperament. There was an effect of time on mean concentrations, baseline, and pulse nadir. Bars indicate significant difference between temperaments within time period. For other significant differences, please refer to text (all values are mean ± S.E; n = 16).

Figure 4. Plasma leptin and insulin concentrations over 24 h in anoestrous Merino ewes bred for a calm (white bars) or nervous (black bars) temperament. There was an effect of time on mean leptin and insulin concentrations and an effect of an interaction between time and temperament on insulin concentrations. Bars indicate significant difference between temperaments within time period. For other significant differences, please refer to text (all values are mean ± S.E; n = 16).
Figure 1.

Figure 2.
Figure 3.

- a) Pulse frequency
- b) Pulse amplitude
- c) Mean concentrations
- d) Area under the pulse curve
- d) Baseline
- c) Pulse nadir

Figure 4.

- a) Leptin
- b) Insulin