Gene polymorphisms associated with temperament in
Merino sheep

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Declaration

The work presented in this thesis is original work of the author, and none of the material in this thesis has been submitted either in full, or part, for a degree at this or any other university or institution. The experiments and manuscript preparation were carried out by myself after discussion with my supervisors, Dr. Dominique Blache, Professor Graeme B. Martin and Dr. Shimin Liu.

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Summary

When individuals are exposed to stressful environmental challenges, the response varies widely in one or more of three components of the stress response: psychological (emotional), behavioural and physiological. This variability among individuals can be defined as temperament. Temperament affects not only the perception and responses to the stressor, but also can affect cognition. The genetic basis of temperament is not clearly understood, but it could be explained by polymorphisms in genes that regulate brain activity and glucocorticoid synthesis. In this thesis, I tested the general hypothesis that polymorphisms of specific genes will be associated with the difference in behavioural reactivity and physiological response in sheep of known temperament (nervous or calm). I mainly used sheep from the UWA Merino flock that had been selected over 20 generations for two temperaments: a nervous line and a calm line, based on high or low reactivity to humans and social isolation.

Firstly, using nervous (n = 58) or calm (n = 59) ewes from lines that had been selected for temperament for 20 generations, we identified polymorphisms in a specific gene responsible for cortisol production (CYP17), and in three specific genes associated with personality and behavioural traits: dopamine receptors 2 and 4 (DRD2, DRD4), and monoamine oxidase A (MAOA). The frequencies of CYP17 SNP628 and DRD2 SNP939 differed significantly between the two temperament lines, but those for DRD2 SNP483 and the 2 MAOA SNP genotypes did not.

To validate the correctness of the two SNPs associated with temperament (CYP17 SNP628 and DRD2 SNP939) as genetic markers for prediction of sheep temperament, we genotyped 278 non-selected sheep for the DRD2 SNP939 and CYP17 SNP628 polymorphisms. We then tested their behavioural response to the challenges used to assess temperament) and their cortisol response to exogenous ACTH. The results showed that responses to the behavioural tests (‘arena test’ and ‘isolation box’ test) were affected by the DRD2 SNP939 genotype and the cortisol response to ACTH challenge was affected by the CYP17 SNP628 genotype. We concluded that, for sheep, a combination of the DRD2 SNP939 C allele and the CYP17 SNP628 A/A genotype could be a genetic marker for prediction of nervous temperament, and that a combination of DRD2 SNP939 T/T and CYP17 SNP628 G/G could be a genetic marker.
for prediction of calm temperament.

We then calculated the heritability of behavioural reactivity to isolation (one measurement of temperament) based on the 2 SNPS genotypes associated with temperament phenotype. The outcome suggested that the phenotypic heritability of reactivity to social isolation, as measured by the agitation score, can be improved by genetic selection based on these 2 SNPs genotypes, and that the combination of the 2 SNPs genotypes was associated with the highest heritability. This result confirmed the above conclusions that DRD2 SNP939 and CYP17 SNP628 genotypes are associated with the two temperaments.

In addition to the association of emotional reactivity (or ‘temperament’) with behavioural and physiological responses, previous studies have suggested that temperament is associated with cognition. Moreover, polymorphism in DRD2 has been implicated in variability in cognitive functions in humans. However, the effect of the interaction between emotional reactivity and DRD2 polymorphism on cognitive function is poorly understood. Therefore, we investigated the association of our temperament-associated DRD2 SNP939 genotypes with cognitive learning abilities in sheep and found that the ‘calm’ temperament-associated DRD2 SNP939 T/T genotype was linked to a greater ability in reversal learning of spatial discrimination than the ‘nervous’ temperament-associated C/C +C/T genotype.

In conclusion, this thesis is a first step in the identification of potential genetic markers for prediction of calm or nervous temperament in sheep. The DRD2 SNP939 is specifically associated with behavioural reactivity and cognitive learning ability, and the CYP17 SNP628 is specifically associated with the physiological cortisol response. The combination of these 2 SNPs could be used as a genetic marker for the temperament prediction. However, many other genes involved in brain neurotransmitter systems and in hypothalamic-pituitary-adrenal axis could also be associated with sheep temperament, so, perhaps using genome-wide association study (GWAS), we need to investigate the association of other genes with sheep temperament.
Acknowledgements

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Publications

Peer-reviewed papers


Conference Contributions


List of abbreviations

AADC aromatic amino-acid decarboxylase
ACTH adrenocorticotrophic hormone
ADHD attention deficit hyperactivity disorders
ADR2A adrenergic receptor 2A
AUCG area under the curve calculated to ground
AUCB area under the curve calculated to baseline
AVP arginine vasopressin
BDNF brain-derived neurotrophic factor
COMT catechol-O-methyltransferase
CRH corticotrophin-releasing hormone
CRH-R1 CRH receptor 1
CYP17 cytochrome P450 17
DAT dopamine transporter
DBH dopamineβ-hydroxylase
DNA deoxyribonucleic acid
DOPA dihydroxyphenylalanine
DRD1 dopamine receptor 1
DRD2 dopamine receptors 2
DRD4 dopamine receptors 4
EDS extradimensional shifts
GWAS genome-wide association study
HPA hypothalamo-pituitary adrenal
5-HT 5-serotonin
5-HTP 5-hydroxy tryptophan
5-HTT 5-serotonin transporter
IBT isolation box test
IDS intradimensional shifts
MAO monoamine oxidases
MAOA monoamine oxidase A
NET norepinephrine transporter
NO nitric oxide
17-OHPREG 17-hydroxypregnenolone
17-OHPROG  17-hydroxyprogesterone
PCR       polymerase chain reaction
PREG      pregnenolone
PROG      progesterone
RFLP      restriction fragment length polymorphism
PVN       paraventricular nucleus
RIA       radioimmunoassay
SNPs      single nucleotide polymorphisms
TCI       temperament character inventory
TH        dihydroxyphenylalanine
TPH       tryptophan hydroxylase
VMAT      vesicular monoamine transporter
VNTRs     variable number of tandem repeats
General Introduction

When individual humans or animals are exposed to a stimulus, they first evaluate the situation on the basis of parameters such as suddenness, familiarity and pleasantness through brain processing (Boissy 1995). After this evaluation, they build and express psychological, behavioural and physiological responses (Corr 2009). In animals, only the behavioural and physiological responses can be directly measured and assessed. The usual indicator of the physiological response is cortisol secretion (Corr 2009). The behavioural response varies among individual animals exposed to the same stimulus, and this variability is known as temperament (Strelau 1987; Rothbart et al., 2000; Mervielde et al., 2005; Shiner and DeYoung 2011; Blache and Bickell 2010, 2011).

Variation among individuals in temperament is the result of variation in genetics (Strelau 1987; Rothbart et al., 2000; Mervielde et al., 2005; Saudino 2005; Krueger and Johnson 2008) and in life experience (Rothbart et al., 2000; Krueger and Johnson 2008). Sheep of calm or nervous temperament show different physiological responses (cortisol secretion) and behavioural responses (motor activity) following exposure to stressors such as novelty and isolation. The genetic basis of temperament has been confirmed in these animals, but the physiological mechanisms that implement the effects of temperament on physiological and behavioural responses to a stimulus are not understood. Hypothetically, the genes associated with temperament could be involved in i) the perception or the process of evaluation at brain level, before responses are elicited; or ii) the modulation of the physiological response (cortisol production as a result of activation of the HPA axis) or the behavioural response.

At the brain level, human personality traits (such as temperament) have been linked to neurotransmitter systems (Cloninger 1987) and genetic differences in these systems could be the neurological basis of temperament. For example, in humans, polymorphisms of genes encoding for dopamine receptors (DRD4, DRD2) and monoamine oxidase A (MAOA), an enzyme responsible for the metabolism of 5-serotonin, dopamine as well as norepinephrine, have all been studied and found to be
associated with psychological disorders and behavioural traits. Similarly, genetic
differences in enzymes involved in the HPA axis could explain the effect of
temperament on glucocorticoid production. Indeed, it has been shown that the amplitude
of the cortisol response is affected by temperament (behaviour) in humans, monkeys,
dogs, cattle and sheep. Moreover, in the South African Angora goat, polymorphisms of
CYP17, an enzyme involved in the synthesis of adrenal cortisol (Nakajin et al., 1981;
Vander et al., 2001), affect its activity and, consequently, cortisol production in response
to a metabolic stress (Storbeck et al., 2007). However, the existence of this
polymorphism and its potential role as a mediator of the effect of temperament on
cortisol secretion have never been investigated.

In addition to the association of emotional reactivity (‘temperament’) with the
behavioural and physiological responses, previous studies have suggested that
temperament is also associated with cognition. Lower levels of emotional activity were
found to be linked with better performance in cognitive tasks, including spatial learning,
discrimination and recognition (Mendl 1999; Erhard et al., 2004; Lansade and Simon
2010; Bickell et al., 2011; Butts et al., 2013). In addition, polymorphism in the DRD2
has been implicated in variability in cognitive function in humans and animals (Smith et
al., 1999; Kruzich and Grandy 2004; Lee et al., 2007; Jocham et al., 2009). However,
the effects of interactions between emotional reactivity and DRD2 polymorphism on
cognitive function are poorly understood.

The general hypothesis of this thesis is that polymorphisms of specific genes will be
associated with differences in behavioural reactivity and in physiological response to
stressors in sheep of known temperament (calm or nervous). The general aim of this
thesis is to identify a genetic marker for temperament (calm and nervous) that can be
used for selection in sheep. A step towards the identification of this genetic marker will
provide useful information for sheep reproduction, evolution, welfare, conservation in the
wild, and future breeding programs. To achieve this aim, four experiments were
conducted:

1) Investigation of the association of polymorphisms of four candidate genes (CYP17,
   DRD4, DRD2, MAOA) with temperament (calm and nervous) in Merino sheep
   (Chapter 4);
2) Verification that the polymorphisms (identified in Chapter 4) affect the behavioural
responses and physiological responses of sheep of unknown temperament (Chapter 5);

3) Calculation of the phenotypic heritability of temperament measured by the reactivity to social isolation (IBT) based on the polymorphisms (identified in Chapters 4 and 5) associated with temperament (Chapter 6);

4) Investigation of the association of temperament-associated polymorphisms (identified in Chapters 4 and 5) with temperament-associated variation in cognitive learning ability (Chapter 7).
## Chapter 2

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Animals of different temperament show different physiological responses (e.g., cortisol secretion) and behavioural responses (e.g., motor activity) to exposure to stressors such as novelty and isolation. The genetic basis of these differences is not understood and is therefore the focus of the work described in this thesis. After discussing the nature of temperament and the associated physiological and behavioural traits, this review of literature focuses on the neuroendocrine and genetic basis of temperament, considers candidate genes that could be used as genetic markers for temperament, and outlines phenotype tests for the validation of genetic results.

2.1 Introduction of temperament

2.1.1 Definition of temperament

The very existence of emotion in animals has long been debated until, in 1872, Darwin recognized the existence of emotion in animals and suggested that that animals can experience different responses, including both positive and negative reactivity, displayed in terms of pleasure or fearful behaviours, when exposed to triggering psychological, physical or physiological situations. Boissy (1995) drew on the previous centuries of research and concluded that animals had a strong fearful response when exposed to unfamiliar situation or threatening circumstances, in similar ways to humans. A recent study showed that when sheep are exposed to unfamiliar and threatening circumstances, they display fear or anger responses dependent on whether or not the situation was under their control (Greiveldinger 2007). These responses in sheep are somewhat similar to those observed in humans in both their nature and their individual variability (Boissy 1995). When individual humans are provoked by a stimulus or a response-eliciting situation, they evaluate the situation on the basis of seven different parameters: suddenness, familiarity, pleasantness, controllability, predictability, expectations, and social norms. For animals, all of the the first six parameters are demonstrable (Boissy 1995). After evaluation of the situation, the response has three aspects (Corr 2009): 1) The psychological response or feelings, including positive and negative feelings; 2) The behavioural response; and 3) The physiological response that involves changes in, for example, adrenaline, noradrenaline, serotonin, dopamine and cortisol (Deckersbach 2006; Haas 2007; Corr 2009). Cortisol is usually used as the indicator of physiological response (Corr 2009). A combination of all of these responses constitutes the emotional reactivity to stressors or eliciting situations. Differences among individuals in the overall evaluation are the basis of variability in individual
emotional reactivity, and this variability among individuals is called temperament (Strelau 1987; Rothbart et al., 2000; Mervielde et al., 2005; Blache and Bickell 2010, 2011; Shiner and DeYoung 2011). During the past 20 years at UWA, a flock of Merino sheep has been selected to generate two lines according to their different responses to stimuli: i) The "calm" line is less fearful of humans and less reactive to isolation; ii) The "nervous" line displays more fearful responses. These two lines have proven to be a most valuable model for experimentation on productivity and on the behaviour and physiology of stress.

2.1.2 The dimensions of temperament
In humans, it is thought that the temperament system is an important sub-system of personality (Corr 2009). Thomas and Chess (1977) conceptualized temperament in nine broad dimensions: level of activity, approach/withdrawal, intensity, threshold, adaptability, rhythmicity, mood, persistence of attention span, and distractibility. This array is similar to the classic ‘big five’ personality factors (N, E, O, A, C) defined by McCrae and colleagues (McCrae and Costa 1986, 1987; McCrae and John 1992): Neuroticism (N), the tendency to be anxious, nervous, self-pity, hostile, impulsive, self-conscious, irrational, depressed, and low self-esteem; Extraversion (E), the tendency to be positive, assertive, energetic, social, talkative, and warm; Openness (O), the tendency to be curious, artistic, insightful, flexible, intellectual and original; Agreeableness (A), the tendency to be forgiving, kind, generous, trusting, sympathetic, compliant, altruistic, and trustworthy; and Conscientiousness (C), the tendency to be organized, efficient, reliable, self-disciplined, achievement-oriented, rational, and deliberate. Similar arrays can be used for animals (Rothbart 2007). These dimensions of temperament can be linked to processes and reactions, related to stress and emotion, that are induced by exposure to stressors and displayed in various psychological disorders or behavioural traits, including depression, anxiety, fear, panic, neuroticism, schizophrenia, obesity, substance dependence and abuse, pathologic gambling, impulsivity, aggression, violence, suicidal behavior, child abuse behavior, youth progression, novelty seeking and attention deficit hyperactivity disorder (McCrae and Costa 1986; Hooker, Frazier and Monahan 1994).

2.1.3 The importance of temperament
There is a large body of evidence for a strong relationship between animal temperament
and reproductive performance, production traits and animal welfare.

In sheep, studies have shown that temperament affects reproductive performance (Price 2002; Gelez et al., 2003; Hart et al., 2006, 2008; Bickell et al., 2009a). In the UWA flock, calm ewes show an increased proceptivity during the mating period, a higher spontaneous ovulation rate, a higher ovulation rate in response to oestrus synchronization, better embryo survival and organ development, a higher twin-bearing percentage and a higher survival rate of lambs compared to nervous ewes (Gelez et al., 2003; Hart et al., 2006; Hart et al., 2008; Hart et al., 2008; Bickell et al., 2009a; Blache and Bickell 2010). It has been also suggested that calm animals had higher daily weight gains, improved ease of milking, increased quantity of milk production, higher concentration of protein in milk and also lower production costs (less feed required and less incidence of illness) compared to nervous animals (Burrow 1997; Murray et al., 2009; Pajor et al., 2010; Horton and Miller 2011).

In addition to reproductivity and production traits, selection for temperament could improve the welfare of sheep. Animals in a state of good welfare can show better adaptation to their environment and this is beneficial to their reproductive performance and production traits under intensive farm conditions, whereas animals in a state of poor welfare usually suffer and do not produce as well (Veissier and Boissy 2007). In addition, animals that are more fearful of humans and more reactive to a stimulus (nervous) show poor ability to adapt to their captive environment and therefore have a more stressful experience, reducing their level of welfare (Bickell et al., 2009). Reducing stress in farm animals can be achieved by improving farm management or implementing pharmacological treatment. Selection for temperament offers an alternative by modifying behavioural reactivity.

### 2.2 The impact of temperament on behavioural response to stressors

Stress is defined as a challenge to, or disruption of, homeostasis. Tilbrook and Clarke (2006) added more detail by defining stress as “a complex physiological state that embodies a range of integrative physiological and behavioral processes that occur when there is a real or perceived threat to homeostasis”. There are two types of stressor (acute or chronic) depending on intensity and timing. Acute stressors induce the release of catecholamines in both the sympathetic and central nervous systems. By contrast, responses to chronic or intermittent stressors involve the activation of the hypothalamic-pituitary-adrenal axis (Greenberg, Carr and Summers 2002). The
responses to stressors are not only physiological but also include both psychological and behavioural components.

### 2.2.1 Individual variability of behavioural response

It is clear that there is variability in the behavioural response to the same stimulus or in the same eliciting situation between individuals, which is the basis that the temperament is defined. For example, in humans, this variance between individuals were indicated in many emotional reactivity and behavioural traits including depression, anxiety, fear, panic, substance dependence and abuse, impulsivity, aggression, violence, suicidal behaviour, child abuse behaviour, youth progression, novelty seeking and attention deficit hyperactivity (Rothbart et al., 1994; Corr 2009). Similar results were also observed in animal species (Table 2.1).

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2.2.2 Neurophysiological basis

It has been suggested that the individual variance in behavioural response depends on the variance in the perception or evaluation of the situation before the response happened (Boissy 1995). Lazarus and his colleagues (Lazarus and Folkman 1984) have argued that the major determinant of coping responses is the perception of the situation by the individual. Therefore, the perception or evaluation process in the brain is mainly responsible for the variance of behavioural response to stimulus. It has been long acknowledged that the dopaminergic and serotonergic systems are the two main central nervous systems that are involved in the perception or evaluation process in brain and are most widely implicated in the regulation of various psychological disorders or behavioural traits (Reif and Lesch 2003; Noblett and Coccaro 2005; Tilbrook and Clarke 2006; Turner et al., 2012).

The dopaminergic pathway

Dopamine

Since the first study suggesting that human personality traits are linked to neurotransmitter systems (Cloninger 1987), the dopaminergic system has become the subject of intensifying focus. Dopamine is one of the catecholamine neurotransmitters and is found in humans and animals, including invertebrates. Dopaminergic neurons project to many regions of brain, including prefrontal cortex, striatum, nucleus accumbens and olfactory tubercle and pre-synaptic membrane in limbic area (Moore and Bloom 1978; Swanson 1982; Sobel and Corbett 1984; Goldman-Rakic 1987; Tzschentke 2001; Gong et al., 2012). Dopamine exerts its function by binding to its specific membrane receptors. So far, five dopamine receptors named as DRD1-DRD5 have been isolated. DRD1 and DRD5 are mainly distributed at cerebral cortex, amygdala, hippocampus and in the parafascicular nucleus of the thalamus; DRD2 is mainly located in the striatum, olfactory tubercle and nucleus accubens; DRD3 is mainly distributed at pre-synaptic membrane in limbic area; and DRD4 is mainly distributed at the prefrontal cortex, midbrain and the medulla (Vallone et al., 2000).
Among these regions, the prefrontal cortex and the striatum have been widely acknowledged to be involved in the mediation of various psychological disorders or behavioural traits including depression, anxiety, fear, panic, ADHD (attention deficit hyperactivity disorders), impulsive and aggression related behaviours (Goldman-Rakic 1987, 1990; Wise and Rompre 1989; Tzschentke 2001). Therefore, DRD2 (main distribution in the striatum) and DRD4 (main distribution in the prefrontal cortex) have been investigated in depth to be involved in the mediation of various psychological disorders or behavioural traits in humans and other species (Table 2.2).

Synthesis and catabolism of dopamine

The synthesis and catabolism of dopamine is illustrated in Figure 2.1. Tyrosine, the precursor, enters the brain from the blood with the aid of a specific amino acid transporter. In dedicated neurons, tyrosine is converted into dihydroxyphenylalanine (DOPA) by tyrosine hydroxylase (TH), a step that is believed to be rate-limiting in dopamine synthesis. Dopamine is synthesized from DOPA by aromatic amino-acid decarboxylase (AADC) and then transported into vesicles via vesicular monoamine transporter (VMAT) and stored in the terminals before being released into the synaptic cleft during activation of the neuron by electrical impulses. A dopamine transporter (DAT) can re-uptake dopamine from the synaptic cleft and return it into the nerve terminals. Dopamine is degraded by the monoamine oxidases (MAO) for which there are two isoforms, MAOA and MAOB (Lan et al., 1989), with MAOA being most important and playing a role regulation of psychological disorders and behaviours as depression, alcoholism and impulsive behaviours (Sullivan et al., 1990; Brunner et al., 1993; Devor et al., 1993; Faraj et al., 1994; Verkes et al., 1998; Eensoo et al., 2005; Nilsson et al., 2006).
Table 2.2 The investigations of DRD2 and DRD4 involved in the variance of behaviours in human and animal species

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Behaviours</th>
<th>Species</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD2</td>
<td>schizoid/avoidant behaviour</td>
<td>human</td>
<td>Blum et al., 1997</td>
</tr>
<tr>
<td></td>
<td>pathological gambling</td>
<td>human</td>
<td>Comings et al., 1996a</td>
</tr>
<tr>
<td></td>
<td>ADHD</td>
<td>human</td>
<td>Comings et al., 1996b</td>
</tr>
<tr>
<td></td>
<td>extraversion/agreeableness</td>
<td>human</td>
<td>Jonsson et al., 2003</td>
</tr>
<tr>
<td></td>
<td>impulsivity/sensation-seeking</td>
<td>human</td>
<td>Hamidovic et al., 2009;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Esposito-Smythers et al., 2009;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Kawamura et al., 2013</td>
</tr>
<tr>
<td></td>
<td>childhood aggression</td>
<td>human</td>
<td>Zai et al., 2012</td>
</tr>
<tr>
<td></td>
<td>substance addiction</td>
<td>human</td>
<td>Noble et al., 1991, 1994a, b;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Lawford et al., 1997;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Sullivan et al., 2013;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Wang et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Han et al., 2007; Blum et al., 2011</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Johnson and Kenny 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>rats</td>
<td></td>
</tr>
<tr>
<td>DRD4</td>
<td>impulsive behaviour</td>
<td>human</td>
<td>Kirley et al., 2004; Congdon et al., 2008;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Reiner and Spangler 2011</td>
</tr>
<tr>
<td></td>
<td>novelty seeking</td>
<td>human</td>
<td>Ono et al., 1997; Noble et al., 1998; Strobel et al., 1999; Keltikangas-Jarvinen et al., 2003</td>
</tr>
<tr>
<td>ADHD</td>
<td></td>
<td>human</td>
<td>LaHoste et al., 1996; Rowe et al., 1998;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Smalley et al., 1998; Swanson et al., 1998;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Faraone et al., 1999; Miglia et al., 2000;</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Holmes et al., 2000; Sunohara et al., 2000;</td>
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<td></td>
<td></td>
<td></td>
<td>Tahir et al., 2000; Roman et al., 2001;</td>
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<td>Curran et al., 2001; Mill et al., 2001;</td>
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<td></td>
<td></td>
<td></td>
<td>Kramer et al., 2009</td>
</tr>
<tr>
<td></td>
<td>novelty-seeking</td>
<td>monkey</td>
<td>Bailey and Julia 2007</td>
</tr>
<tr>
<td></td>
<td>curiosity and vigilance</td>
<td>horse</td>
<td>Morozawa et al., 2005</td>
</tr>
<tr>
<td></td>
<td>aggressiveness</td>
<td>dog</td>
<td>Ito et al., 2004</td>
</tr>
<tr>
<td></td>
<td>early exploratory behaviour</td>
<td>bird</td>
<td>Fidler et al., 2007</td>
</tr>
</tbody>
</table>
Figure 2.1 Overview of the dopaminergic synthesis and catabolism (adapted from Bah Rosman 2008). TH= Tyrosine hydroxylase; AADC= Aromatic amino acid decarboxylase; DOPA= Dihydroxyphenylalanine; DA=Dopamine; VMAT= Vesicular monoamine transporter; DAT=Dopamine transporter; MAO=Monoamine oxidase

The serotonergic pathway

Serotonin

Since the initial time of the identification of serotonin by Rapport et al. (1948), there has been a major increase in investigations of its anatomic location and functions in brain.
Some studies suggested that there is projection of 5-serotonin (5-HT) to the prefrontal cortex (Lewis et al., 1986) where there is an overlap of dopamine and serotonin receptors (Goldman-Rakic et al., 1990), the main innervation targets for 5-HT neuron seem to be the brain stem and spinal cord (Jacobs and Klemfuss 1975; Goldman-Rakic 1987; Tork 1990; Mann 1999; Muller 2009). Over the last 30 years, a variety of studies with 5-HT receptor agonists has established the role of serotonin in the psychiatric related disorders and behaviours, including major depression, anxiety, schizophrenia, substance dependence and abuse, eating disorders in humans and rats (Tork 1990; Jacobs and Azmitia 1992; Schreiber and De Vry 1993; Baldwin and Rudge 1995; Mann 1999; Muller 2009). The effect of serotonin on the cell or neurons targets is mediated by serotonin receptors. So far, 14 sub-types (labeled 5-HT1-14) have been identified (Hoyer and Martin 1996). Of these, 5-HT1, 5-HT2 and 5-HT7 have been investigated in depth to be involved in psychiatric disorders related behaviours including anxiety, depression, schizophrenia and substance dependence in humans, mice and rats (Table 2.3).

**Synthesis and Metabolism of 5-serotonin**

As shown in Figure 2.2, tryptophan is the precursor for serotonin synthesis and is also transported from the blood into the brain via a specific amino acid transporter. In dedicated neurons, tryptophan is converted into 5-hydroxy tryptophan (5-HP) by tryptophan hydroxylase (TPH), a step that is believed to be rate-limiting in serotonin synthesis. Aromatic amino-acid decarboxylase (AADC) also converts 5-HTP into 5-serotonin (5-HT) that is then transported into vesicles via vesicular monoamine transporter (VMAT) and stored in the terminals before being released into the synaptic cleft during activation of the neuron by electrical impulses. A 5-serotonin transporter (5-HTT) can re-uptake 5-HT from the synaptic cleft, thus returning it into the nerve terminal. As with dopamine, 5-HT is degraded by MAOA and MAOB (Lan et al., 1989) and, again, MAOA is most important role and thought to play an important role regulation of psychological disorders and behaviours (Sullivan et al., 1990; Brunner et al., 1993; Devor et al., 1993; Faraj et al., 1994; Verkes et al., 1998; Eensoo et al., 2005).
Table 2.3 The investigations of 5-HT1, 5-HT2 and 5-HT7 involved in psychiatric disorder related behaviours in human and animals

<table>
<thead>
<tr>
<th>Serotonin receptor</th>
<th><em>behaviours</em></th>
<th>Species</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-HT1</td>
<td>anxiety</td>
<td>human</td>
<td>Jin et al., 1992; Huang et al., 2004</td>
</tr>
<tr>
<td></td>
<td>depression</td>
<td>human</td>
<td>Blier and Ward 2003; Przegalinski et al., 1995; Overstreet et al., 2003</td>
</tr>
<tr>
<td></td>
<td>depression</td>
<td>rat</td>
<td>Przegalinski et al., 1995; Overstreet et al., 2003</td>
</tr>
<tr>
<td></td>
<td>substance dependence</td>
<td>human</td>
<td>Lappalainen et al., 1998</td>
</tr>
<tr>
<td></td>
<td>substance dependence</td>
<td>mouse</td>
<td>Brunner and Hen 1997; Rocha et al., 1998</td>
</tr>
<tr>
<td>5-HT2</td>
<td>schizophrenia</td>
<td>human</td>
<td>Williams et al., 1996, 1997; Tsuang et al., 2013</td>
</tr>
<tr>
<td></td>
<td>food intake</td>
<td>human</td>
<td>Lappalainen et al., 1995</td>
</tr>
<tr>
<td></td>
<td>food intake</td>
<td>mouse</td>
<td>Tecott et al., 1995</td>
</tr>
<tr>
<td></td>
<td>substance dependence</td>
<td>human</td>
<td>Holmes et al., 1998; Virkkunen et al., 2008</td>
</tr>
<tr>
<td>5-HT7</td>
<td>depression</td>
<td>mouse</td>
<td>Guscott et al., 2005</td>
</tr>
<tr>
<td></td>
<td>anxiety</td>
<td>rat</td>
<td>Wesolowska et al., 2006</td>
</tr>
<tr>
<td></td>
<td>anxiety</td>
<td>mouse</td>
<td>Wesolowska et al., 2006</td>
</tr>
</tbody>
</table>

In summary, the two nervous systems (dopaminergic and serotonergic systems) including dopamine and serotonin synthesis and metabolism process and their receptors are involved in above mentioned various psychological disorders and behaviours. Serotonergic system is more linked to psychological disorders related behaviours like depression, anxiety, schizophrenia, food intake and substance abuse, whereas dopaminergic system is more linked to the behavioural reactivity including fear, ADHD, impulsivity and aggression related behaviours.
Figure 2.2 Overview of the serotonergic synthesis and catabolism (Bah Rosman 2008).
TPH= Tryptophan hydroxylase; AADC= Aromatic amino acid decarboxylase; 5-HP= 5-hydroxytryptophan; 5-HT=5-serotonin; VMAT= Vesicular monoamine transporter; 5-HTT=5-serotonin transporter; MAO=Monoamine oxidase

2.3 The impact of temperament on physiological response to stressors

2.3.1 The physiological mechanism in stress response
The hypothalamo-pituitary adrenal (HPA) axis, a classic neuroendocrine system, is the primary mediator of physiological responses to stressors so it is often considered to be
the main ‘stress system’. The HPA axis controls the production of glucocorticoids (Figure 2.3).

![Diagram of the HPA axis](image.png)

**Figure 2.3** Schematic diagram of the HPA axis (Tilbrook and Clarke 2006).

Its essential role was understood early in the development of modern endocrinology after it was realized that the adrenal gland was important for dealing with stress (Selye 1946). The adrenal activity is controlled and regulated by signals from the hypothalamus that are translated and magnified by the anterior pituitary gland. Exposure to a stressor leads to the secretion of corticotrophin-releasing hormone (CRH) and arginine vasopressin (AVP) by neurons located paraventricular nucleus (PVN) that project their terminals in the median eminence. CRH and AVP are released into the hypophyseal portal system, a specialized circulatory system connecting the hypothalamus to anterior pituitary gland, and then reach the anterior pituitary. In the anterior pituitary gland, CRH and AVP stimulate the responsive and specialised cells, the corticotrophs, to produce the adrenocorticotropic hormone (ACTH). ACTH acts on the adrenal cortex to stimulate the synthesis of glucocorticoids. Glucocorticoids regulate
the secretion of CRH, AVP and ACTH through negative feedback actions in the brain, to ultimately inhibit the synthesis and secretion of CRH and AVP, and anterior pituitary gland, where corticotrophs become less responsive to CRH and AVP. The nature of the glucocorticoids varies between species but cortisol and corticosterone are the main active glucocorticoids (Tilbrook and Clarke 2006). In rodents and avian species, the predominant glucocorticoid is corticosterone, whereas in humans and many mammalian species, the predominant glucocorticoid is cortisol.

Therefore, the cortisol or corticosterone, as the final effector of HPA axis, is known to play an important role in stress responses, hence its frequent use as an indicator of the physiological response to stressors (Corr 2009).

2.3.2 Individual variance in cortisol or corticosterone response
A large body of studies has reported that different temperaments can result in the differences in the amplitude of the increase in plasma concentrations of cortisol in humans, monkeys, dogs and cattle and also differences in plasma concentrations of corticosterone in avian species (Table 2.4). In sheep, the cortisol response to stressors has been studied to be different in two lines selected for divergence in temperament (calm and nervous; Hawken et al., 2013) and in two lines selected for or against ability to rear multiple offspring (Vander Walt et al., 2009; Hough et al., 2013).

Therefore, temperament can affect the physiological response to stressors and the variation in the physiological response could be linked to the variations in temperament.
Table 2.4 The investigations of variance in cortisol/corticosterone response to stressors between individuals with different temperament in human and animals

<table>
<thead>
<tr>
<th>Glucocorticoid</th>
<th>Species</th>
<th>Stressor (Challenge)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortisol</td>
<td>human</td>
<td>completion</td>
<td>Donzella et al., 2000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>social competence</td>
<td>Gunnar et al., 1997, 2003; Tout et al., 1998; Dettling et al., 1999</td>
</tr>
<tr>
<td></td>
<td>dog</td>
<td>restraint, noise, shock</td>
<td>Beerda et al., 1998</td>
</tr>
<tr>
<td></td>
<td></td>
<td>social and spatial restriction</td>
<td>Beerda et al., 1999; Hennessy et al., 2001; De Palma et al., 2005</td>
</tr>
<tr>
<td></td>
<td>monkey</td>
<td>novelty</td>
<td>King et al., 2003</td>
</tr>
<tr>
<td></td>
<td>cattle</td>
<td>fearful eliciting situation</td>
<td>Kalin et al., 1998</td>
</tr>
<tr>
<td></td>
<td>sheep</td>
<td>ACTH</td>
<td>Curley et al., 2008</td>
</tr>
<tr>
<td></td>
<td></td>
<td>transportation</td>
<td>Burdick et al., 2010</td>
</tr>
<tr>
<td></td>
<td></td>
<td>restraint, human presence, dog barking</td>
<td>Hawken et al., 2013</td>
</tr>
<tr>
<td></td>
<td></td>
<td>insulin challenge</td>
<td>Vander Walt et al., 2009; Hough et al., 2013</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>bird</td>
<td>handling</td>
<td>Cockrem and Silverin 2002; Fraisse and Cockrem 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>restraint</td>
<td>Satterlee and Johnson 1988; Jones et al., 1992; 1994a, 1994b</td>
</tr>
<tr>
<td></td>
<td>chicken</td>
<td>handling</td>
<td>Fraisse and Cockrem 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>restraint</td>
<td>Beuving et al., 1989; Jones et al., 1995; Korte et al., 1997</td>
</tr>
</tbody>
</table>
2.4 The impact of temperament on cognition

2.4.1 Individual variance in cognition
In a challenging or continuously changing environment, the ability of individuals to learn and memorise associations between stimuli and actions, make the appropriate decision and adapt with the appropriate behaviours, is a vital determinant of survival (Provenza 1995; Shettleworth 2001). Previous studies have suggested that there is variability in this ability between individuals with different emotional reactivity (or called temperament) in humans and animal species (Table 2.5). Lower levels of emotional reactivity are associated with better performance in various cognitive tasks, including spatial learning, discrimination and recognition tasks (Mendl 1999; Erhard et al., 2004; Lansade and Simon, 2010; Bickell et al., 2011; Butts et al., 2013).

Table 2.5 The investigations of variance in cognitive function between individuals with different emotional reactivity in animal species

<table>
<thead>
<tr>
<th>Species</th>
<th>Cognitive function</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Horse</td>
<td>instrumental learning</td>
<td>Lindberg et al., 1999; Visser et al., 2003; Lansade and Simon, 2010</td>
</tr>
<tr>
<td></td>
<td>spatial learning</td>
<td>Heird et al., 1986</td>
</tr>
<tr>
<td></td>
<td>visual discrimination</td>
<td>Fiske and Potter 1979; Mader and Price 1980</td>
</tr>
<tr>
<td>Bird</td>
<td>avoidance learning</td>
<td>Richard et al., 2000</td>
</tr>
<tr>
<td>Cattle</td>
<td>decision-making</td>
<td>Grandin et al., 1994</td>
</tr>
<tr>
<td>Rat</td>
<td>spatial learning</td>
<td>Brush et al., 1985; Teskey et al., 1998; Herrero et al., 2006</td>
</tr>
<tr>
<td></td>
<td>cognitive flexibility</td>
<td>Butts et al., 2013</td>
</tr>
<tr>
<td>Dog</td>
<td>spatial learning</td>
<td>Svartberg 2002</td>
</tr>
<tr>
<td>Sheep</td>
<td>decision-making</td>
<td>Rushen, 1986b</td>
</tr>
<tr>
<td></td>
<td>cognitive flexibility</td>
<td>Erhard et al., 2004</td>
</tr>
<tr>
<td></td>
<td>face discrimination</td>
<td>Bickell et al., 2011</td>
</tr>
<tr>
<td>Deer</td>
<td>decision-making</td>
<td>Pollard et al., 1994</td>
</tr>
<tr>
<td>Pig</td>
<td>decision-making</td>
<td>Van Rooijen and Metz 1987</td>
</tr>
<tr>
<td></td>
<td>spacial learning</td>
<td>Mendl et al., 1997</td>
</tr>
<tr>
<td>Chicken</td>
<td>memory</td>
<td>Regolin et al., 1995</td>
</tr>
</tbody>
</table>
2.4.2 The mechanism involved in the cognition

The neurobiology of the cognitive functions is complex, but the location of the brain regions and neuronal pathways involved starting to be understood. The prefrontal loop and striatum are mainly responsible for attention and directing the behaviour toward a given goal in a given situation (Posner and Petersen 1990). Studies suggest that the dysregulation of the brain prefrontal cortex and striatum activity may underlie many of the behavioural disorders of executive attention (Sagvolden and Sergeant 1998; Rubia et al., 1999; Castellanos and Tannock 2002; Johansen et al., 2002). It has been shown that dopamine exerts a strong regulatory effect on prefrontal cortex and striatum neuronal activity (Missale et al., 1998; Haber et al., 2000; Schultz 2002), with many studies in humans, monkeys and rats implicating the activity of midbrain dopaminergic neurons and their projections to the prefrontal cortex and striatum has been implicated in a variety of cognitive and executive functions in human, monkeys, and rats, including working memory (Brozoski et al., 1979; Sahakian et al., 1985; Cai and Arnsten 1997; Floresco and Phillips 2001; Cools et al., 2002), behavioural flexibility (reversal learning and shifting of attentional set including extradimensionalshifts (EDS) and intradimensional shifts (IDS) of attention) (Owen et al., 1991, 1993; Roberts et al., 1994; Birrell and Brown 2000; McAlonan and Brown 2003; Yang et al., 2004) and decision-making (Bechara et al., 2001; Ersche et al., 2005; Shurman et al., 2005; Denk et al., 2005; Winstanley et al., 2006). Dopamine exerts its effects on prefrontal cortex and striatum neural activity via multiple receptor subtypes, with dopamine receptor 1 (DRD1) and receptor 2 (DRD2) being suggested as mediating the cognitive learning abilities in human and mice (Smith et al., 1999; Kruzich and Grandy 2004; Floresco and Magyar 2006; Lee et al., 2007; Jocham et al., 2009). For example, DRD2 knockout mice had impaired ability to acquire odor-driven discrimination (Kruzich and Grandy 2004).

In summary, there is variance in behaviours, physiological cortisol response, and cognition between individuals with different emotional reactivity or temperament. The HPA axis is mainly responsible for the physiological response; the serotonergic system is more linked to the variance in psychological disorders related behaviours like depression, anxiety, schizophrenia and substance abuse, whereas dopaminergic system is more linked to the variance in behavioural reactivities including fear, ADHD, impulsivity and aggression related behaviours and cognition. The question is: does such
2.5 The genetic basis of temperament

Variation in temperament could be caused by differences in genetics (Strelau 1987; Rothbart et al., 2000; Mervielde et al., 2005; Saudino; Krueger and Johnson 2008; Bickell et al., 2009b) or environment (Rothbart et al., 2000; Krueger and Johnson 2008). However, the shaping of individual temperament by the complex interactions between genetic and environmental factors is not well understood and little studied. One study indicated that early experience and 5HTT gene variation interact to have a mutual influence on the aggressive behaviors in rhesus macaques (Bennett et al., 2002). It was suggested that variation in 5HTT was associated with aggressive behaviours, but the behaviours of offspring were influenced by genotype in paternal-reared individuals but not for maternal-reared subjects. By contrast, in sheep, a study using a cross-fostering procedure has suggested that sheep temperament is caused by genetic inheritance rather than learned behaviours (Bickell et al., 2009b). Newborn lambs born to calm ewes were fostered to either other calm ewes or to nervous ewes and, lambs from nervous ewes were fostered to other nervous ewes or to calm ewes, and their emotional reactivity was measured. Lambs born from calm ewes, even if fostered by nervous ewes, were still calm and showed little fear response, whereas lambs born from nervous ewes, even if raised by calm ewes, still displayed fearful responses similar to those of nervous lambs. The clear conclusion was that temperament in Merino sheep is mainly determined by genetic transmission of the trait rather than from behaviour learned from the mother. Therefore, although there are complex cross-connections between nature and nurture, in general, it seems likely that sheep temperament is at least partially determined by genetics.

2.5.1 Molecular genetics

Gene structure
The genome comprises a very large number of genes, each of which is a segment of deoxyribonucleic acid (DNA) composed of 4 different nucleotides (or bases): adenine (A), cytosine (C), guanine (G) and thymine (T) assembling in a double helix (Watson and Crick, 1953). The 4 bases are matched in a specific way – A with T and C with G
allowing the DNA replication occurs in a unique way using one strand as template of the other one. Three bases constitute a codon. A specific amino acid is usually encoded by one codon or different codons in some instance due to total 64 possible codons but only 20 amino acids. A specific gene is composed of coding region called exons and non-coding sequence called introns. ATG is the start codon and TGA is the stop codon which are responsible for the initiation and termination of the translation of protein respectively. The promoter region is a special non-coding sequence located before coding region which is responsible for the start of DNA transcription.

**Gene polymorphisms**

Gene polymorphisms (or mutations) refer to the base changes in the DNA sequence, of which there are several different types:

1) Single nucleotide polymorphisms (SNPs), in which one base is mutated into another base (Brookes 1999), is the most common form with, to date, over 11 million available in two databases including dbSNP (http://www.ncbi.nlm.nih.gov/SNP/) and UCSC (http://genome.ucsc.edu/);
2) Insertions and deletions (I/D) in which base-pairs (from 1 to several thousand) are added or removed from the DNA sequence;
3) Restriction fragment length polymorphism (RFLP), a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA with specific restriction endonucleases ( Tanksley et al., 1989);
4) Repeat polymorphisms in which there is a variable number of repeats of a certain fragment, usually in one of two main patterns: microsatellites (small di- or tri-nucleotide repeats; Jeffreys et al., 1985), or a variable number of tandem repeats (VNTRs) of up to 100 nucleotides (Nakamura et al., 1987);
5) Copy number polymorphisms, comprising larger deletions and duplications (>1 kbp in size).

Whether the polymorphism can affect the phenotype depends on its location in the DNA sequence. A polymorphism located in coding regions can cause amino acid substitutions and will thus be functional. However, even if it is located in non-coding regions, a polymorphism can affect transcription rates by, for example, being located within the promoter region, or at the binding sites of transcription factors.
Methods for genetic studies
Psychological characteristics can vary between individuals because of genotype, as illustrated by genetic variation in personalities and temperaments and differences in the response of individuals to the same stimulus. An increasing body of work is focused on the association of genetics with personality, psychological disorders and behavioural traits. Some diseases, such as Huntington’s disease, are ‘monogenic’, having been found to be the result of a single mutation in a single gene (Gusella et al., 1983). However, complex behavioural disorders such as attention deficit hyperactivity disorder (ADHD), Tourette’s syndrome, depression, and substance addiction, and some behavioural traits such as aggression, impulsiveness, novelty seeking, harm avoidance, are believed to be determined by multiple genes rather than a single gene, so are ‘polygenic’ (Bah Rosman 2008; Corr 2009). In polygenic disorders, it is a difficult to identify and associations with specific gene(s).

One method for identifying specific genes associated with personality, psychological disorders and behavioural traits is ‘association studies’ (Glazier et al., 2002; Bah Rosman 2008) which assume that the underlying pathological and physiological mechanisms of your target phenotype disorder or behaviour is well understood. Candidate genes can thus be investigated for mutation, usually with a classical Case-Control experimental design, where individuals with the target disorder or behaviour (Case) and individuals without the disorder or behaviour (Control) are compared. Allele and genotype frequencies of polymorphisms in the candidate genes are detected, measured and compared between the case and control groups, as in the present study.

2.5.2 Candidate genes involved in temperament

Candidate genes involved in the variability in behaviours
As mentioned above, the dopaminergic and serotonergic systems, in particular the receptors and the catabolic enzymes (MOA) are involved in expression and variability of behaviour linked to personality disorders and other phenotypic traits related to temperament. In the following section, I will review the literature concerning polymorphisms in the genes encoding expression of monoamines receptors and monoamine oxidase and their relationships with expression of phenotypic traits related
to temperament and their variability among individuals.

**Gene polymorphisms in the dopaminergic pathway**

*TH and DAT genes*

In humans, to date, there have been few studies of the TH gene in the context of psychological disorders and behavioural traits, the most relevant being the discovery of a functional tetranucleotide VNTR (variable number of tandem repeats) polymorphism (TCATn) that is related to neuroticism (Persson et al., 2000). Similarly, for the gene controlling DAT, and thus the re-uptake of dopamine, there is also little information linking polymorphisms to personality traits, although a VNTR polymorphism with 40-bp repeat unit has been identified and proposed to play a role in substance addiction and attention-deficit/hyperactivity disorder in humans (Parsian and Zhang 1997; Sabol et al., 1999; Ueno et al., 1999; Daly et al., 1999; Barr et al., 2001).

*DRD4 and DRD2 genes*

In contrast with the TH and DAT genes, the genes for DRD4 and DRD2 have been investigated in depth. Three polymorphisms of the DRD4 gene has been identified in the human: i) a VNTR polymorphism with 48-bp repeat unit in the third intracellular loop of the receptor molecule; ii) 2 SNPs (-616 C/G and -521 C/T) in the promoter region; and iii) a VNTR polymorphism with repeats of 120-bp in the 5’ un-translated region (Cichon et al., 1995; Liu et al., 1996). Among them, the VNTR polymorphism with 48-bp repeats has been the subject of many studies and the consensus is that it is associated with impulsive behaviour, novelty-seeking and attention deficit/hyperactivity disorder (Lahoste et al., 1996; Ono et al., 1997; Noble et al., 1998; Rowe et al., 1998; Smalley et al., 1998; Swanson et al., 1998; Faraone et al., 1999; Holmes et al., 2000; Sunohara et al., 2000; Miglia et al., 2000; Tahir et al., 2000; Roman et al., 2001; Curran et al., 2001; Mill et al., 2001; Kirley et al., 2004; Keltikangas-Jarvinen et al., 2004; Congdon et al., 2008; Kramer et al., 2009; Reiner and Spangler 2011). In addition, the VNTR polymorphism has been detected in the same region in other species, although the repeat base pair unit and the number of repetitions differes with the species. In monkeys, the VNTR polymorphism (48-bp repeat unit) was found to be associated with novelty-seeking behaviour (Bailey and Julia 2007). In dogs, on the other hand, two VNTR polymorphisms (with 12-bp or 39-bp repeat unit) were detected and found to be associated with aggressiveness (Ito et al., 2004).
For DRD2, several polymorphisms have been studied, the most common being two forms of a TaqI RFLP, TaqI A and TaqI B (Reif and Lesch 2003). In addition, a SNP in intron 6 with a Ser-Cys mutation and a -141C ins/del variant in the promoter region have also been detected (Jonsson et al., 2003; Reif and Lesch 2003). The DRD2 TaqI A RFLP was found to be linked to dopaminergic activity and to the quantity of D2 dopamine receptors in brain tissue, as well as to responses to stress, dopamine-related disorders and variation in personality traits and behaviours including behavioural inhibition and impulsivity, novelty-seeking and attention deficit/hyperactivity disorder (Noble et al., 1991; Noble et al., 1994 a,b,c,d; Comings et al., 1996a,b; Noble et al., 1997; Lawford et al., 1997; Blum et al., 1997; Berman and Noble, 1997; Noble et al., 1998; Lee et al., 2007). The DRD2 polymorphism in the promoter, -141C ins/del, was suggested to be related to personality detachment in terms of extraversion and agreeableness (Jonsson et al., 2003). Two SNPs of DRD2 (rs4648317 and rs12364283) are associated with behavioural inhibition and impulsivity/sensation-seeking (Hamidovic et al., 2009) and three DRD2 polymorphisms (2 SNPs, -241A/G and rs1079598, and a TaqIA RFLP) are associated with childhood aggression (Zai et al., 2012).

In summary, polymorphisms of the TH and DAT genes are linked to substance addiction, attention-deficit/hyperactivity disorder and neuroticism rather than personality or temperament traits. By contrast, polymorphisms of DRD4 and DRD2 genes are found in different regions for different species yet seem to be associated with the level of behavioural reactivity, including impulsive behaviour and aggression, a trait that is closely related to the phenotype of temperament and personality.

**Gene polymorphisms in the serotonergic pathway**

*TPH and 5-HTT genes*

For the human TPH gene, polymorphisms have been detected in various different regions. A SNP (labeled alleles U and L) has been identified in Intrion 7 at Position 218 (-218A/C). The L allele has been linked with suicidality, alcoholism and aggression (Nielsen et al., 1994, 1997, 1998; New et al., 1998; Nolan et al., 2000). In addition, SNPs in the promoter region of TPH gene were found to be associated with suicidal behaviour in a Chinese population (Liu et al., 2006). However, other studies failed to detect an association between TPH polymorphism and suicidality (Bennett et al., 2000) or with scores in temperament character inventory (TCI), a general assessment of personality traits (Ham et al., 2004). It is feasible that TPH polymorphisms play a role
in predisposition to some temperament characteristics and behavioural traits, but further studies are needed for clarification.

With respect to serotonin re-uptake, three polymorphisms have been identified for the gene encoding for 5-serotonin transporter (5-HTT) (De Mel et al., 2012). One polymorphism is a VNTR polymorphism within intron 2 and the other two are both located in the promoter area, the most extensively studied one being a VNTR polymorphism (named 5-HTTLPR) with a long variant (L: 16 copies of 22 bp repeat unit) and a short variant (S: 14 copies of 22 bp repeat unit. Within this VNTR polymorphism in promoter area, a SNP (A/G) has also been detected. The 5-HTTLPR L/S polymorphism has been the main target main of investigation and appears, in humans, to be strongly associated with behaviours related to psychiatric disorders, including schizophrenia, substance dependence and abuse, eating disorders, anxiety and depression, fear and panic, comorbid dysthymia and suicidal behaviour (Lesch et al., 1996; Rotondo et al., 2002; Golimbet et al., 2004; Retz et al., 2004; Baca-Garcia et al., 2005; Kilpatrick et al., 2007; Lonsdorf et al., 2009; Verhagen et al., 2009; Klumpers et al., 2012).

5-HT receptor genes

Fourteen sub-types (labeled 5-HT1-14) have been identified for the 5HT receptor (Hoyer and Martin 1996). Both 5-HT1 and 5-HT2 have been reported to be involved in behaviours related to personality and physiological disorders in the human (Roth 1994). A SNP of the 5HT1B gene (-861G/C) is associated with alcoholism (Lappalainen et al., 1998) and 2 SNPs (-102T/C and -14238A/G) in the 5HT2A gene have been linked to schizophrenia and post-traumatic stress disorder (Williams et al., 1996, 1997; Lee et al., 2007). A SNP of the 5HT2C gene (Cys23Ser) appears to be related to Alzheimer’s disease and alcoholism (Holmes et al., 1998).

In summary, the gene polymorphisms involved in serotonergic system seemed to be more linked to psychiatric disorders, including schizophrenia, substance dependence and abuse (alcoholism), eating disorders, anxiety and depression rather than personality or temperament traits.

Gene polymorphisms in MAO

Of the two isoforms, MAO-A and MAO-B, MAOA is thought to be the prevailing and essential isoform in the brain (Weyler 1990; Shih 1999) where it plays an important role
in the genetic regulation of psychological disorders and behaviours. Low levels or abnormalities in MAOA activity are implicated in psychiatric disorders such as depression, alcoholism and impulsive behaviours (Sullivan et al., 1990; Brunner et al., 1993; Devor et al., 1993; Faraj et al., 1994; Verkes et al., 1998; Eensoo et al., 2005). Abnormalities in MAOA activity that are apparently associated with mutations in MAOA gene (Sabol et al., 1998; Deckert et al., 1999) seem to cause various psychiatric disorders, such as impulsive or aggressive behaviour and attempted suicide (Brunner et al., 1993a, b). In humans, the most common polymorphism, a 30-bp VNTR, is in the promoter region and is associated with panic and fear behaviours, depression, aggressiveness and impulsiveness (Deckert et al., 1999; Schulze et al., 2000; Manuck et al., 2000, 2002; Du et al., 2002; Parsian et al., 2003; Ito et al., 2003). In rhesus monkeys and in Macaques (Newman et al., 2005; Wendland et al., 2006), the repeat polymorphism in MAOA promoter region has been found to be associated with aggression-related behaviour. In chickens, there is breed difference in two microsatellites polymorphisms for MAOA in Intron 4 (a thymine repeat, named CMin4T) and Intron 9 (a adenine repeat, named CMin9A), and a 128-bp allele in Intron 4 was observed, only in the Nagoya breed that shows very cowardly behaviour (Hong et al., 2008b).

Therefore, although the polymorphisms differed in different regions and also differed among the species, the polymorphism of MAOA gene seemed to be associated with the level of behavioural reactivity including fear, impulsive and aggressive behaviours that are closely related to the phenotype of temperament and personality.

In summary, polymorphisms of the TH and DAT genes involved in dopaminergic system and the gene polymorphisms involved in serotonergic system seemed to be more linked to psychiatric disorders, which appear to differ fundamentally from the phenotype of our temperament. By contrast, although their polymorphisms differed in different regions and also differed among the species, the polymorphism of DRD2, DRD4 and MAOA genes seemed to be associated with the level of behavioural reactivity including fear, impulsive and aggression that are closely related to our temperament phenotype.

**Candidate genes involved in the variability along the HPA axis**
As outlined above, variation in the genes involved in the regulation of the HPA axis, including the production of CRH in hypothalamus and the production of cortisol in adrenal cortex, can probably explain variations in temperament. Here we will explore this hypothesis.

**Gene polymorphisms related to hypothalamus CRH production**

The studies have suggested that gene polymorphisms related to CRH production is linked to some psychological disorders related behaviours in humans and monkeys. In humans, a SNP (-201 C/T) in the promoter region of CRH gene appears to increase the alcohol consumption in humans (Baerwald et al., 1999). The same SNP (-248C/T) was also detected in the corresponding region of the monkey CRH gene, also apparently related to increased risk for disorders of alcohol-use in stress-exposed individuals (Barr et al., 2009). In addition, the SNP in the gene of CRH receptor-1 (CRH-R1) has been found to be associated with psychological disorders related behaviours in humans, including depression, alcohol consumption and suicidal behaviour (Licinio et al., 2004; Keck 2006; Liu et al., 2006, 2007; Keck et al., 2008; Bradley et al., 2008; Blomeyer et al., 2008; Wasserman et al., 2008, 2009; Schmid et al., 2010; Binder and Nemeroff 2010).

Therefore, the gene polymorphisms related to CRH production seemed to be more linked to psychiatric disorders, including schizophrenia, substance dependence and abuse (alcoholism), anxiety and depression, which not directly relevant to the phenotype expression of temperament. So far, few studies investigate the ACTH related gene polymorphisms and behaviours.

**Gene polymorphisms involved in adrenal cortisol production**

As mentioned above, differences in the amplitude of the cortisol response have been reported to be associated to temperament in humans, monkeys, dogs and cattle. The gene CYP17 plays a central role in the regulation of cortisol production in the mammalian adrenal cortex (Nakajin et al., 1981) in both of the two principal pathways –Δ5 and Δ4 (Figure 2.4). Pregnenolone (PREG) is the specific substrate for Δ5 pathway while progesterone (PROG) is the specific substrate for the Δ4 pathway. The hydroxylation activity of CYP17 causes 17-hydroxylation of PREG (Δ5) to 17-hydroxypregnenolone (17-OHPRG) and PROG (Δ4) to 17-hydroxyprogesterone
The 17, 20-lyase activity of CYP17 converts 17-OHPREG (Δ5) to dehydroepiandrosterone (DHEA) and 17-OHPROG (Δ4) to androstenedione (A4), two androgen precursors (Figure 2.4).

![Figure 2.4](image-url)  
**Figure 2.4** The Δ5 and Δ4 pathways catalysed by CYP17 responsible for the production of androgens or glucocorticoids by the adrenal gland.

Differences in the hydroxylase and the lyase activities give CYP17 its crucial role in the determination of the direction of steroidogenesis towards the production of mineralocorticoids, glucocorticoids or androgens by the adrenal gland, and these differences seem to depend on the CYP17 genes. In African Angora goats, Boer goats and Merino sheep, certain CYP17 genotypes are strongly associated with the cortisol response to stressors (Storbeck et al., 2007, 2008, 2009). The Angora and Boer goats have different physiological characteristics that are linked to the high mortality rate in Angoras when they are exposed to cold stress (Wentzel et al., 1979). In addition, the plasma cortisol concentrations are lower and the cortisol response to CRF is smaller in Angora than in Boer goats or Merino sheep (Herselman and Vanloggerenberg 1995). It was therefore suggested that, in Angora goats, but not Boer goats or Merino sheep, CYP17 was primarily involved the Δ5 pathway and conversion of PREG to DHEA rather than the Δ4 pathway leading to the production of cortisol (Engelbrecht and Swart 2000). Two CYP17 isoforms, CYP17 ACS− and CYP17 ACS+ with four single nucleotide differences have been identified in goats (Slabbert 2003). Interestingly, of the 83 Angora goats that were genotyped, 24 were ACS−/ACS−, the remaining 59 were ACS−/ACS+, and no goats with ACS+/ACS+ were detected, whereas all Boer goats were ACS−/ACS+ (Slabbert 2003). The two alleles (ACS− and ACS+) were found to be located on different genes: in Angora goats, the ACS−/ACS− genotype is located on one
CYP17 gene (ACS−) whereas, in Boer goats, the ACS+/ACS− genotype is located on two different CYP17 genes (ACS+ and ACS−; Storbeck et al., 2007; Vander Walt et al., 2009). Therefore, ACS+ was always present with ACS−, and ACS+/ACS+ could not be found. When the ACS− and ACS+ genes were cloned, they displayed similar 17-hydroxylase activity but the ACS− isoform had a greater 17, 20-lyase activity, leading to greater androgen production and smaller glucocorticoid production (Storbeck et al., 2007), probably explaining the hypocortisolism observed in Angora goats with their extra ACS−. These observations agree with those of Engelbrecht and Swart (2000) who showed that the Angora produced significantly less glucocorticoid precursors, but more androgens. In summary, the CYP17 isoform has a strong association with cortisol production in South African Angora goats, with the ACS− isoform leading to lower cortisol outputs.

In addition, two CYP17 isoforms (WT1 and WT2) that differ by two single nucleotides were detected in South African Merino sheep by Storbeck et al. (2007). The sheep were from two divergent lines selected for Low (L-line) or high (H-line) ability to rearing single or multiple litters (Vander Walt et al., 2009). The H and L lines differ in their cortisol response to insulin injection, with H-line having an earlier cortisol peak and quicker glucose recovery (Vander Walt et al., 2009). However, the frequency distributions of WT1 and WT2 did not differ significantly between the two lines, and homozygous WT2 was not detected (Table 2.6). The difference between L-line and the H-line in the cortisol response to insulin challenge suggested that, in addition to CYP17, other genes are associated with the cortisol response in Merino sheep, so we have also explored other candidate genes.

Table 2.6 Frequency of the 3 CYP17 genotypes in sheep from the H-line and L-line (from Vander Walt D et al., 2009)

<table>
<thead>
<tr>
<th>Line</th>
<th>Homozygous WT1</th>
<th>Heterozygous WT1/WT2</th>
<th>Homozygous WT2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Number of sheep</td>
<td>%</td>
<td>Number of sheep</td>
</tr>
<tr>
<td>H-line</td>
<td>15</td>
<td>14.3</td>
<td>90</td>
</tr>
<tr>
<td>L-line</td>
<td>5</td>
<td>16.1</td>
<td>26</td>
</tr>
</tbody>
</table>

In summary, polymorphisms in the gene for CRH are more likely linked to the behaviours related to psychiatric disorders including depression, alcohol addiction and
suicidal behaviours that are relevant to the phenotype expression of temperament. On the other hand, polymorphisms in CYP17 are linked to differences in the cortisol response to exposure to stressors, so CYP17 is a good candidate as a genetic marker for temperament, especially because secretion of cortisol differs with different phenotypic traits for temperament in sheep (Hawken et al., 2013).

Besides the gene polymorphisms, reviewed above, in the dopaminergic and serotonergic system, and in the HPA axis, several other genes have also been associated with behaviour. For example, in the noradrenergic pathways, genes encoding for dopamineβ-hydroxylase (DBH; responsible for norepinephrine synthesis), the adrenergic receptor (ADRA2A), and norepinephrine transporter (NET1), have been suggested to play a role in ‘fight or flight’ response (Major et al., 1980). Similarly, within the GABAergic pathway, the GABA-A receptor gene has been shown to be involved in anxiety disorders (Crestani et al., 1999). Another example is the gene encoding for Nitric oxide (NO) synthase which has been proposed to be associated with aggressive and sexual behaviour (Nelson et al., 1995). However, very few studies have investigated these relationships in detail.

**Gene polymorphism and cognition**

Dopamine in the prefrontal cortex and striatum influences neural activity via multiple receptor subtypes that have been suggested to mediate cognitive learning abilities, including working memory and behavioural flexibility. In both human and nonhuman primates, memory has been linked to the actions of DRD1 receptors, with the antagonism or inhibition of DRD1 can improve memory ability (Sawaguchi and Goldman-Rakic 1991, 1994; Williams and Goldman-Rakic 1995; Murphy et al., 1996; Muller et al., 1998). By contrast, the behavioural flexibility has been suggested to be associated with DRD2 receptors and their polymorphisms (Ridley et al., 1981; Taghzouti et al., 1985; Farde et al., 1987; Goldman-Rakic et al., 1990; Lidow et al., 1991; Smith et al., 1999; Kruzich and Grandy 2004; Lee et al., 2007; Cools et al., 2001, 2007; Jocham et al., 2009; Gong et al., 2012). Potentially, therefore, DRD2 polymorphisms could help explain the variability in learning performance associated with different temperaments.

In summary, the gene polymorphisms including TH, DAT, DRD2, DRD4, TPH, 5-HTT,
5-HT receptors, MAOA, CRH, CYP17, DBH, ADRA2A, NET1, GABA-A receptor and NO all could be linked to the various dimensions of temperament, such as psychiatric disorders, substance addiction, attention-deficit/hyperactivity disorder, neuroticism, and behavioural reactivity (including fearfulness, impulsiveness and aggression). Genes or gene polymorphisms in TH, DAT, TPH, 5-HTT, 5-HT receptors and CRH seem to be more linked to psychiatric disorders, which appear to differ fundamentally from the phenotype of our temperament flock. By contrast, although their polymorphisms differed in different regions and also differed among the species, the polymorphism of DRD2, DRD4, MAOA and CYP17 genes seemed to be more associated with the level of behavioural reactivity including fearfulness, impulsiveness and aggression that are closely related to our temperament phenotype, while the associations of genes DBH, ADRA2A, NET1, GABA-A receptor and NO are not strong because they have been rarely studied. Therefore, considering the characteristics of our two temperament phenotypes, 4 genes (DRD2, DRD4, MAOA and CYP17) were chosen as candidates most likely associated with our two sheep temperaments.

2.6 The model of temperament flock

During the past 20 years at UWA, a flock of Merino sheep has been selected to generate two lines according to their different behavioural responses to the presence of human and social isolation, using two behavioural phenotype tests, the Arena test and the Isolation Box test (Murphy et al., 1994; Murphy 1999; Blache and Ferguson 2005). The two lines are labeled "calm" for sheep that are less fearful of humans and less reactive to isolation and "nervous" for sheep that display more fearful and more reactive responses. In this section, I will describe the two behavioural tests used to assess the phenotype of temperament.

Arena test

The arena test measures the behavioural response of sheep exposed to the challenge of having to pass a human to gain access to a pen of flock mates. The surface of the pens is divided into 4 sections by lines drawn on the ground (Figure 2.5). Flockmates are kept in a pen at the end of the arena with a human in front of them. A single test sheep is gently pushed in the arena through the entry door (Figure 2.5), where it experiences a conflict avoidance situation because it has to pass the human to approach the flockmates. The behavioural response is measured by quantifying locomotor activity by counting
the number of lines crossed over a period of 3 min (TOTAL CROSS). A high level of locomotor activity indicates a high degree of fear (Blache and Ferguson 2005). However, in this arena test, there is some ambiguity in the interpretation of the behaviour. It has been argued that immobilization might reflect an absence of fear in one situation, such as the presence of a human, and but it can also reflect a high degree of disturbance and nervousness in another situation (Vandenheede et al., 1998), and the inhibition of locomotor and vocal behaviour can be interpreted as a reaction towards a predator and thus can represent a higher degree of fear (Romeyer et al., 1992). Therefore, second test, the isolation box test, is used to complete the assessment of sheep temperament.

Figure 2.5 Schematic diagram of the arena test (Blache and Bickell 2011).

Isolation box test

The test sheep is isolated in a 1.5 x 1.5 x 1.5 m solid box (Figure 2.6) without visual contact. A digital agitation meter, calibrated for reflection of low, medium and high agitation levels before the test (Blache and Ferguson 2005), is used to record the vocalisations and agitation of the test animal and a score (BOX) is read after 1 min of isolation. The agitation score is an indicator of the inherent fear and ability to adapt to isolation (Blache and Ferguson 2005), with a high score indicating a high degree of fear.
Selection index

The level of vocalisation and locomotor activity of sheep exposed to various fear eliciting situations can be used as an indicator of their levels of fear (Romeyer et al., 1992; Vandenheede et al., 1998). An overall selection index (i) can thus be calculated based on the TOTAL CROSS and BOX measured in the two tests, according to the following equation (Murphy et al., 1994; Murphy 1999):

\[
\text{Selection score} = 100 + \frac{[\text{BOX}_i - \text{BOX}_x] + [\text{TOTAL CROSS}_i - \text{TOTAL CROSS}_x]}{\text{BOX}_{sd} + \text{TOTAL CROSS}_{sd}}
\]

\(i\) = individual score, \(x\) = flock mean, \(sd\) = standard deviation of mean

High and low values for the selection index are used to classify behavioural reactivity, with the high-index sheep labeled ‘nervous’ and the low-index sheep labeled ‘calm’.

2.7 Summary

Individuals display different behavioural and physiological responses to the same stimulus and this variability is called temperament. Sheep of calm or nervous temperament show different physiological responses (cortisol secretion) and behavioural responses (motor activity) following exposure to stressors such as novelty and isolation (the method for temperament assessment). In our model, the genetic basis is not understood. This thesis focuses on two systems that could be involved in the expression of temperament and in the consequences of differences in temperament on adrenal activity and on cognitive capacity. The experimental work aimed to explore the genes involved in the regulation of the activity of the dopamine and serotonin neurotransmitter systems, as well as a gene involved in the regulation of the HPA axis.
The serotonergic system and its related gene polymorphisms is more linked to the psychological disorders related behaviours like depression, anxiety, schizophrenia, and substance abuse, which are not directly relevant to the phenotype expression of personality or temperament; whereas, dopaminergic system and its related gene polymorphisms, although differed in different regions and also differed among the species, is more linked to the behavioural reactivity including fear, ADHD, impulsivity and aggression related behaviours and cognitive capacity, which are closely related to our temperament phenotype. The polymorphism of CYP17 gene is linked to differences in cortisol response to stressors which was tested to be different in sheep with different phenotypic traits for temperament. Therefore, taking into consideration of the phenotype trait of the model of our temperament flock, the genes DRD2, DRD4, MAOA and CYP17 are good candidates to look at the association of their polymorphisms with temperament.

The aim of this thesis is to contribute to the understanding of the genetic basis of temperament using the UWA flock, by examining:

1) The association of temperament with the polymorphisms of genes potentially involved in the perception and responses to stressors (Chapter 4);
2) The response to stressor of animals carrying combination of the identified polymorphisms (Chapter 5);
3) The heritability of behavioural reactivity to isolation (one measurement of temperament) of animals carrying the identified polymorphisms (Chapter 6);
4) The differences in cognitive ability of animals carrying the identified polymorphisms (Chapter 7).
Chapter 3

General Materials and Methods

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The materials and methods described in this chapter were used in several different experimental chapters, although some are repeated in experimental chapters that were written as journal manuscripts. Treatments and analyses that are specific to particular experiments are described in detail in the relevant experiment chapter. All experiments were conducted in accordance with the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition, 2004) and were approved by the Animal Ethics Committee of UWA (Approval number RA/3/100/1252).

3.1 Experimental location
The laboratory experiments were conducted at the University of Western Australia (UWA), Perth, Western Australia. Animal experiments were conducted at UWA Farm Ridgefield, a 1588-hectares farm near Pingelly, Western Australia (32°30’23” S, 116°59’31’’E) where the UWA Future Farm 2050 project is located. The climate is Mediterranean-type with 425 mm annual average rainfall, with mild wet winters and hot dry summers. Daylength ranges from 10 h to 14 h.

3.2 Experimental materials and animals
In Chapter 4, we used whole blood samples collected into EDTA vacutainers (Greiner Bio-One, Australia) from sheep from the two lines in the UWA Temperament Flock. This flock of Merino has been selected for 20 generations on the basis of the behavioural reactivities of lambs after weaning (at approximately 16 weeks of age) to humans and social isolation using two behavioural tests (Murphy et al., 1994; Murphy 1999; Blache and Ferguson 2005) described in Section 3.7. In Chapter 5, we used ewes of unknown temperament from a commercial flock kept at UWA Farm Ridgefield. In Chapter 6, the blood samples were obtained for another project (Blache and Ferguson 2009) from ewes and lambs that had been selected on the basis of the accuracy of their parentage. In Chapter 7, we used the Merino ewes with known DRD2 SNP939 genotypes.

3.3 Molecular techniques

**Blood genomic DNA extraction protocol**

Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the instructions provided by the manufacturer.

1) 20 μL proteinase K pipetted into a 1.5 mL microcentrifuge tube; 100 μL blood sample added, followed by 100 μL PBS.
2) 200 μL Buffer AL added (without added ethanol); reagents mixed thoroughly by vortexing, and incubated at 56°C for 10 min. We ensured that ethanol had not been added to Buffer AL. It was essential that the sample and Buffer AL were mixed immediately and thoroughly by vortexing to yield a homogeneous solution.

3) 200 μL ethanol (96-100%) added to the tube, and mixed thoroughly by vortexing. It was important that the sample and the ethanol were mixed thoroughly to yield a homogeneous solution.

4) The mixture from previous step was pipetted into the DNeasy Mini spin column placed in a 2 mL collection tube; after centrifugation at 8000 rpm for 1 min, flow-through and collection tube were discarded.

5) the DNeasy Mini spin column was placed in a new 2 mL collection tube and 500 μL Buffer AW1 was added; after centrifugation for 1 min at 8000 rpm, the flow-through and collection tube were discarded.

6) The DNeasy Mini spin column was placed in a new 2 mL collection tube and 500 μL Buffer AW2 was added. The DNeasy membrane was dried by centrifugation for 3 min at 14,000 rpm, and the flow-through and collection tube were discarded. It was important to dry the membrane of the DNeasy Mini spin column, since residual ethanol may interfere with subsequent reactions. This centrifugation step ensured that no residual ethanol would be carried over during the following elution. The DNeasy Mini spin column was then removed carefully so that the column did not come into contact with the flow-through, since this would have resulted in carryover of ethanol.

7) The DNeasy Mini spin column was then placed in a clean 1.5 mL microcentrifuge tube, and 200 μL Buffer AE was pipetted directly onto the DNeasy membrane. After incubation at room temperature for 1 min, it was centrifuged for 1 min at 8000 rpm to elute.

**Polymerase Chain Reaction (PCR)**

Fragments of the CYP17 and DRD4 genes were amplified by standard PCR. Amplification reactions (10 μL) contained 2 μL of 5×PCR buffer including 0.2 mM dNTPs (Fisher Biotec, Australia), 2.5 mM MgCl₂ (Fisher Biotec, Australia), 0.3 μM each of forward and reverse primers (GeneWorks, Australia), 1.1 U Taq DNA polymerase (5.5 U/μL; Fisher Biotec, Australia) and 20 ng genomic DNA.
Fragments of the DRD2 and MAOA genes were amplified by Hotstar PCR. Amplification reactions (50 µL) contained 5 µL of 10×Faststart PCR buffer including 2 mM MgCl₂ (Roche, Germany), 0.2 mM dNTP mix (Roche, Germany), 0.3 µM each of forward and reverse primers (GeneWorks, Australia), 2 U Faststart Taq DNA polymerase (5 U/µL; Roche, Germany) and 80 ng genomic DNA. The protocols for each fragment are listed in Table 3.1.

<table>
<thead>
<tr>
<th>Fragment</th>
<th>Initial denaturation</th>
<th>35 cycles</th>
<th>Final extension</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>94°C 5min</td>
<td>94°C 30s, 58°C 30s</td>
<td>72°C 15s, 72°C 7min</td>
</tr>
<tr>
<td>CYP17 SNP628</td>
<td>94°C 5min</td>
<td>94°C 30s, 68°C 30s</td>
<td>72°C 15s, 72°C 7min</td>
</tr>
<tr>
<td></td>
<td>94°C 5min</td>
<td>60°C 30s</td>
<td>72°C 20s, 72°C 7min</td>
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<tr>
<td>DRD4 conserved</td>
<td>94°C 5min</td>
<td>94°C 30s, 68°C 30s</td>
<td>72°C 15s, 72°C 7min</td>
</tr>
<tr>
<td>Exon1</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 45s, 72°C 7min</td>
</tr>
<tr>
<td>Exon2</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 20s, 72°C 7min</td>
</tr>
<tr>
<td>Exon3</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 25s, 72°C 7min</td>
</tr>
<tr>
<td>DRD2</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 25s, 72°C 7min</td>
</tr>
<tr>
<td>Exon4</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 25s, 72°C 7min</td>
</tr>
<tr>
<td>Exon5</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 25s, 72°C 7min</td>
</tr>
<tr>
<td>Exon6</td>
<td>95°C 6min</td>
<td>95°C 30s, 68°C 30s</td>
<td>72°C 45s, 72°C 7min</td>
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<tr>
<td>Exon7</td>
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<td>72°C 45s, 72°C 7min</td>
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<td>Exon1</td>
<td>95°C 6min</td>
<td>95°C 30s, 68°C 30s</td>
<td>72°C 42s, 72°C 7min</td>
</tr>
<tr>
<td>MAOA</td>
<td>95°C 6min</td>
<td>95°C 30s, 60°C 30s</td>
<td>72°C 15s, 72°C 7min</td>
</tr>
<tr>
<td>Exon15</td>
<td>95°C 6min</td>
<td>95°C 30s, 68°C 30s</td>
<td>72°C 42s, 72°C 7min</td>
</tr>
</tbody>
</table>

**Gel electrophoresis of PCR products**

The electrophoresis of PCR products was performed on 1.5% agarose gel. The gel was prepared by melting 1.5g agarose with 100 mL 1×TAE solution in a microwave oven, cooling it to 60 °C, and then pouring it into the electrophoresis tank and inserting the comb. After 0.5 h, a mixture of 4 µl PCR product and 1 µl loading buffer was loaded into each pole. The gel was run at 120V for 30 min and then stained with ethidium bromide (0.5 µg mL⁻¹) for 20-30 min and then placed on a UV-light transilluminator to be photographed.

**Genotyping by real-time PCR method**

Real-time PCR-based genotyping was carried out using an Applied Biosystems (California, USA) Vii7™ instrument. Amplification reactions (10 µL) were performed using 5 µL TaqMan® Genotyping Master Mix (2×) (Applied Biosystems, California, USA), 0.5 µL of custom TaqMan® SNP genotyping Assay (20×) containing two primers...
and two labelled probes (Applied Biosystems, California, USA) and 20 ng genomic DNA. The thermal profile is presented in Table 3.2. There was an extra step in the 40 cycles for CYP17 SNP628 with cooling to 52°C with a 30s hold before going to 60°C due to the lower annealing temperature of its primer. The transition rate between all steps was 1.6°C/s. Data were collected by the Vii7™ software. The VIC®-labelled sensor probe and the FAM™-labelled anchor probe were designed to be a perfect match for two allele sequences for each SNP. A no-template control (negative control) was also included in each assay. Each sample was done in triplicate on the 96-well plate.

<table>
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<tr>
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<td>60°C 30s</td>
<td>95°C 10min</td>
<td>95°C 15s</td>
<td>60°C 30s</td>
</tr>
<tr>
<td>CYP17 SNP628</td>
<td>60°C 30s</td>
<td>95°C 10min</td>
<td>95°C 15s</td>
<td>52°C 30s</td>
</tr>
</tbody>
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3.4 Cannulation of the jugular vein for serial blood collection

**Animal Preparation**

a. Wool was clipped close to the skin over the jugular vein, providing an area big enough to allow easy visualisation of and access to the jugular vein;

b. Local anaesthetic cream was applied (EmLa®; lignocaine 25mg/g, prilocaine 25 mg/kg) to the area –the time of application was noted;

c. The animal was then injected with sedative (ilium xylazil; 0.1mg/kg i.m.);

d. Approximately 30 min after application of the local anaesthetic cream, the animal was restrained in a standing position.

**Site Preparation**

a. On cannulation trolley, all needles, tubing and equipment are kept in 70% ethanol.

b. The shaved window was scrubbed with betadine surgical scrub.

c. The vein was clearly located by occlusion plus visualization of rise and fall with pressure and release, before moving on to the next step.

d. A small incision was made in the skin above the vein using a scalpel.

e. A 13G luer lock needle was inserted into the jugular vein and the cannula tubing was quickly fed into the lumen of the vein.

f. The needle was slid off the tubing and a 3-way tap was attached to the end of the cannula.

g. Blood flow was verified using a syringe filled with sterile, heparinised (40 iu/ml)
0.9% saline; the lumen of the cannula was rinsed by injection of 2-3 mL heparinised saline.

h. A piece of fabric tape was wrapped around the tubing at the point where it enters the skin, leaving two ‘wings’ on either side that could be sutured to the skin to secure the cannula in place.

i. Another piece of fabric tape was wrapped around the junction between the tap and tubing to reduce the risk of kinking and perforation of the tubing.

j. The cannula and tap were laid on the neck of the sheep and 2-3 layers of packing tape were wrapped around the neck to ensure that the cannula and tap were covered, with care taken that breathing was not restricted.

k. At the end of the experiment, the cannula was removed and the cannulation site checked for bleeding.

3.5 ACTH Test
Hormone injection and blood sampling were all done via indwelling jugular catheter that had been inserted one day before the test. The ACTH stimulation test was used to obtain a cortisol profile. The endogenously driven secretion of cortisol was blocked using dexamethasone, a synthetic glucocorticoid that acts at hypothalamic level to inhibit the secretion of ACTH (Beaven et al., 1964). With a low background concentration of cortisol, tests using exogenous ACTH to elicit a cortisol response from the adrenal glands become far more predictable, allowing robust comparison of groups of sheep. Each sheep was treated with 0.125 mg/kg (i.v.) dexamethasone, a dose chosen from previous studies showing complete suppression of endogenous ACTH release (Beaven et al., 1964; Espiner et al, 1972). The exogenous ACTH (0.2 iu i.v.) was injected 90 min after the dexamethasone treatment. Blood (3 mL per sample) was sampled 30 min after dexamethasone treatment, then every 30 min until ACTH injection, and then every 20 min until 3 h after the injection. Saline was used as control in an identical protocol for injection and sampling. All blood samples were immediately transferred into 5 mL plastic tubes containing 50 IU of heparin and polystyrene granules (Techno-plas, WA, Australia) before centrifugation at 2000 g for 10 min to obtain plasma for cortisol assay. The plasma was separated and stored at –20°C until assay. The duration and size of the cortisol response were measured.

3.6 Hormone assays for cortisol
Plasma concentration of cortisol was measured in duplicate by antibody radioimmunoassay (RIA) using the ImmuchemTM Coated Tube Cortisol $^{125}$I RIA Kit (MP Biomedicals, Belgium).

**Assay preparation**

1) Standards: 100ug/dL standard from the commercial kit was diluted using a pol of cortisol-free plasma into following concentrations: 50, 25, 12.5, 6.25, 3.125, 1.56, 0.78, 0.39 and 0.2 µg/dL, with cortisol-free plasma used as zero (0 µg/dL).

2) All standards, quality controls, coated tubes and $^{125}$-tracer were brought to room temperature prior to use. Quality was controlled with two sheep plasma samples, obtained from stressed and non-stressed sheep, tht are routinely used as quality control in our laboratories. The quality controls were refrozen (–15°C) between uses, and the standards refrigerated (4°C).

**Assay protocol**

a) 25 µL of each standard, control and unknown samples were pipetted into the coated tubes.

b) 1.0 mL of $^{125}$-tracer was added to all tubes and the tubers vortexed briefly.

c) All tubes were incubated in a water bath (37°C) for 45 min.

d) Tubes were decanted in the same order as they were pipetted; the rim of the test tube was blotted dry on absorbent paper before being turned upright;

e) The tubes were counted in a gamma counter (90 sec).

f) Concentration of cortisol in each unknown sample was estimated from the standard curve using “AssayZap Universal Calculator” software (Elsevier-Biosoft, UK).

3.7 Behavioural tests

It has been reported that the levels of vocalisations and locomotor activities of sheep when exposed to various fear-eliciting situations can be used as the indicator of their levels of fear (Romeyer et al., 1992; Vandenheede et al., 1998).

3.7.1 Arena test

An arena test was used to measure the locomotor activity of sheep exposed to the conflicting challenge of having to pass a human to gain access to a pen of flock mates (Blache and Ferguson 2005). A single test sheep entered a rectangular arena while its flock-mates were kept in a pen at the opposite end of the arena with a human in front of them (Fig. 2.5). Locomotor activity was quantified by counting the number of times the
test sheep crossed line on the floor of the arena (‘TOTAL CROSS’). Each test lasted for 3 min and the test sheep exited through a door located on the side the arena.

3.7.2 Isolation test

Each sheep was isolated without visual communication with its companions by being placed for 1 min in a 1.5×1.5×0.7 m solid enclosed box (Fig. 2.6; Blache and Ferguson 2005). A digital agitation meter that measured vibrations produced by movements and loud vocalisations was used to assess the reactivity of the sheep to isolation. The agitation meter was calibrated before the test at low, medium and high agitation levels using a mechanical device that producing a ‘stomping’ vibration (Blache and Ferguson 2005). An agitation score (‘BOX’) from the digital agitation meter was used as an indicator of the inherent fear and adapt ability exposure to isolation (Blache and Ferguson 2005).

An overall score

An overall selection score was calculated based on the values for TOTAL CROSS and BOX from the above two tests, according to the following equation (Murphy et al., 1994; Murphy 1999).

\[
\text{Selection score} = 100 + \frac{[\text{BOX}_i - \text{BOX}_x]}{\text{BOX}_{sd}} + \frac{[\text{TOTAL CROSS}_i - \text{TOTAL CROSS}_x]}{\text{TOTAL CROSS}_{sd}}
\]

i = individual score, x = flock mean, sd = standard deviation of mean

A high or low overall score was used to classify animals as having high or low reactivity. Sheep with a high overall score were labeled ‘nervous’ and sheep with a low score were labeled ‘calm’.

3.8 Cognitive learning and memory ability tests

To date, studies of cognitive learning and executive function have been done with human and non-human primates (Brozoski et al., 1979; McAlonan and Brown 2003; Dowson et al., 2004; Mehta et al., 2004). Two simple discriminations (visual and spatial) have been used to test the function and impairment of the prefrontal cortex and assess learning and short-term memory abilities in humans, monkeys and rats (Owen et al., 1991; Birrell and Brown 2000; Conrad 2010; Johnson et al., 2012). In my study, two maze systems were utilized: an executive functioning maze apparatus described by
Morton and Avanzo (2011) to assess the learning abilities based on visual cues (visual discrimination - color); and a Y-maze to test short-term memory capabilities based on spatial discrimination.

3.8.1 Visual Discrimination - colour

*Testing apparatus*

Two identical testing units (each unit: 2.4 m × 7.2 m) were used to test whether sheep could learn one color out of a choice of two. The two pens were assembled back to back, so each sheep could be tested multiple times by cycling between the two units. The outsides of the units and testing pen were covered with hessian to isolate the animal from external visual stimuli. Each unit comprised a starting gate located in the middle of the front panel and a testing area divided longitudinally into two sections by a metal hurdle so the sheep could enter into the decision area using a rotating gate. The sheep exit the pen by the doors located at the end of the decision area (Figure 3.1).

![Figure 3.1 Schematics of the testing apparatus for simple discrimination of colour (top: top view, bottom: side view) based on the design of Morton and Avanzo (2011). Two areas (2.4 m × 7.2 m) are adjacent to each other and set in opposite direction (grey area marked the testing area in the top testing pen). Each testing pen comprises a central starting gate, and two discrimination test areas are divided longitudinally by a metal hurdle. One the sheep has made](image-url)
the choice (one side), the gate is closed behind the sheep. After consuming the reward (or after 1 min) the sheep exits through the exit gates located at the end of the pen. The sheep is pushed into the end pen corridor and start the next discrimination test in the adjacent pen. The two discrimination stimuli (S) are located at the end of the pen.

Testing procedure

Testing was conducted using the stimuli shown in Table 3.3, starting with a simple discrimination (SD). The pairs of stimuli were presented at random, and designated S+ (correct) and S- (incorrect). The task required the sheep to discriminate between the colours of blue and yellow, and later, if sheep learnt to learn the reversal. A food reward (a ration of lupin grain) was presented for the correct choice. For each discrimination, a pair of stimuli (blue and yellow card) was placed on either side of the dividing hurdle at the end of each pen, with the correct and incorrect colours (S+ and S-). For all parts of this experiment, the food reward was placed in a feed bucket under the S+ card. The test started when the sheep entered the start gate from where the S+ and S- cards can clearly be seen. The animals were free to move in the test area towards either of the stimuli and, once past a designated line (1 meter from the stimuli), the decision was recorded, together with delay to the decision (seconds). Behavioural observations (bleating, jumping, stomping) were also recorded for all discriminations. Initially, during the training phase if the animal chose incorrectly (S-), they were ushered into the correct side (S+), being allowed one minute to identify the presence of the food reward. The training phase continued until all sheep knew the reward was under the S+ stimulus, after which they were either allowed 1 min to eat the reward if S+ was chosen or had to wait 20 seconds if S- was chosen, before being ushered onto the next discrimination test. Between each discrimination test, we swapped the stimuli (together with the food bucket) so as to control for biases towards the left or right side of the pen. A performance criterion was set at 75% correct over 8 consecutive discriminations, plus 75% of the individuals in each experimental group passing the test. Once sheep reached this criterion, they were considered as having learnt the test and were passed onto the reversal test, where the reward contingency was reversed. All sheep within each experimental group performed the same number of discriminations (4 to 8 discriminations each day).

Table 3.3 The simple discrimination and its reversal, with the designated S+ and S-
### Chapter 3: General Materials and Methods

#### 3.8.2 Y-maze test (spatial discrimination)

**Testing apparatus**

The Y-maze test apparatus comprised a starting gate, leading into a run (2.4 m long; Zone ‘A’, Figure 3.2) that bisected into the two arms of the maze (each 2.4 m long; Zones ‘B’ and ‘C’), with two gates located at the ending of the two arms. All the sides of the Y-maze were covered with hessian to isolate the animal from external visual stimuli.

**Testing procedure**

For all parts of this experiment, the food reward was placed in a feed bucket on the maze arm designated S+, with an empty feed bucket placed on the incorrect arm (S-). The test started when the sheep passed the starting gate into Zone A, from which they had to make a choice within one minute toward the left (B) or right (C) arms of the maze. The animals were free to move around the maze through the different zones. We recorded the time sheep took to find and eat the food and the number of times the sheep crossed the boundaries into different zones until it had found and started eating the food reward in the S+ arm. After 24 trials, the test was reversed by swapping the bucket containing the reward bucket with the empty bucket, so as to control for olfactory recognition of food rather than left-right learning with associated reward. For each group of sheep, one half was trained to find one side of maze and the other half trained to the other side. All trials were video recorded to facilitate the behavioural analysis.

A sheep was considered to have achieved spatial discrimination (or to have learned to take the correct side) once it had made the correct choice over 4 consecutive tests without error. Having met the criteria, the sheep were tested for reversal spatial discrimination. The reversal spatial test was similar to the spatial discrimination test but the side with the food reward (S+) was swapped to the opposite side; sheep previously learned to find rewards on the left were tested with the food reward located on the right.

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<thead>
<tr>
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<th>Relevant dimension</th>
<th>Correct (S+)</th>
<th>Incorrect (S-)</th>
</tr>
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<tr>
<td>Simple Discrimination (SD)</td>
<td>Colour</td>
<td>Blue</td>
<td>Yellow</td>
</tr>
<tr>
<td>Simple Reversal (SR)</td>
<td>Colour</td>
<td>Yellow</td>
<td>Blue</td>
</tr>
</tbody>
</table>
All sheep were subjected to the same total number of trials during the spatial discrimination test.

**Figure 3.2** Schematics of the testing apparatus used to conduct the Y-maze test adapted from the design of Kendrick et al. (1995). The maze is divided into three zones (A, B, C). The feeder with food reward and the empty feeder are placed at the end of the two arms (zones ‘B’ and ‘C’).
Chapter 4

Associations between temperament and gene polymorphisms in the dopaminergic system and adrenal gland in sheep

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4.1 Abstract

Sheep of calm or nervous temperament differ in their physiological (cortisol secretion) and behavioural (motor activity) responses to stressors, perhaps due to variation in genes that regulate glucocorticoid synthesis or brain dopamine activity. Using ewes that had been selected over 20 generations for nervous (n = 58) or calm (n = 59) temperament, we confirmed the presence of a polymorphism in a gene involved for cortisol production (CYP17), and identified polymorphisms in three genes associated with personality and behavioural traits: dopamine receptors 2 and 4 (DRD2, DRD4), and monoamine oxidase A (MAOA). The calm and nervous lines differed in their frequencies of CYP17 SNP628 (single nucleotide A-G mutation at the position 628) (A/A: Nervous 31.0% vs Calm 10.2%, P < 0.01; G/G: Nervous 27.6% vs Calm 47.5%, P < 0.05) and DRD2 SNP939 (single nucleotide T-C mutation at the position 939) (T/T: Nervous 25.9% vs Calm 59.3%, P < 0.01; T/C: Nervous 55.7% vs Calm 33.9%, P < 0.05; C/C: Nervous 19.0% vs Calm 6.8%, P < 0.05), but those for DRD2 SNP483 and the 2 MAOA SNP genotypes did not. We conclude that, for sheep, a combination of the DRD2 SNP939 C allele and the CYP17 SNP628 A/A genotype could be used as a potential genetic marker for nervous temperament prediction, and that a combination of DRD2 SNP939 T/T and CYP17 SNP628 G/G could be used as a potential genetic marker for calm temperament.

Key words: Dopamine receptor, Monoamine oxidase, Glucocorticoid, CYP17, Stress
4.2 Introduction

Humans and animals exposed to a stimulus initially evaluate the situation at brain level on the basis of a variety of parameters such as suddenness, familiarity, pleasantness, controllability, predictability, expectations, and social norms (Boissy 1995). Their subsequent emotional responses involve three components (Corr 2009): 1) a psychological response, such as positive and negative feelings, that cannot be directly assessed in animals; 2) a behavioural response; and 3) a physiological response that involves changes in, for example, adrenaline, noradrenaline, serotonin, dopamine and cortisol (Deckersbach 2006; Haas 2007; Corr 2009). Individuals exposed to the same stimulus can show differences in all three components, and this variability is called temperament (Strelau 1987; Mervielde et al., 2005; Blache and Bickell 2010, 2011; Shiner and DeYoung 2011).

We have been investigating temperament in a flock of sheep that has been genetically selected for 20 generations on the basis of behavioural responses to isolation and human presence. As a result, the two lines were produced, one is hypo-responsive (‘calm’) and the other one is hyper-responsive (‘nervous’) to the stressors (Murphy et al., 1994; Blache and Ferguson 2005), that present a useful model for studying the genetic basis of the relationships between temperament, behaviour and physiology. The temperament of an individual depends on genetic background (Strelau 1987; Saudino 2005; Mervielde et al., 2005; Krueger et al., 2008; Bickell et al., 2009) and life experience (Krueger et al., 2008). Genetic associations with personality, psychological disorders and behavioural traits are supported by an increasing body of evidence from a variety of animal models, including monkeys, birds, rodents, cattle, dogs and horses (Rubinstein et al., 1997; Niimi et al., 1999; Sugiyama et al., 2004; Momozawa et al., 2005; Bailey et al., 2007; Fidler et al., 2007; Glenske et al., 2011). The genes involved could affect the perception or the process of evaluation at brain level, or the modulation of the physiological response at the level of the hypothalamic-pituitary-adrenal (HPA) axis.

At brain level, human personality traits, including temperament, have been linked to genetic differences in neurotransmitter systems (Cloninger 1994). In humans and non-human primates, polymorphisms in genes encoding for tyrosine hydroxylase (TH), dopamine transporter (DAT), dopamine receptors 2, 3 and 4, tryptophan hydroxylase
Gene polymorphisms associated with temperament

(TPH), monoamine oxidase A (MAOA, an enzyme responsible for the catabolism of 5-serotonin and dopamine), serotonin transporter (5-HTT) and serotonin receptors, have all been linked with personality, psychiatric disorders (neuroticism, schizophrenia), substance dependence and abuse, eating disorders, depression, anxiety, child abuse and suicidal behaviour (Reif and Lesch 2003). Moreover, polymorphisms in dopamine receptors 2 and 4 (DRD2, DRD4) and in MAOA (Shih and Thompson 1999) have often been associated with impulsivity, aggression, fear and panic-related behaviours in humans (Noble et al., 1998; Rowe et al., 1999; Deckert et al., 1999; Manuck et al., 2000; Curran et al., 2001; Contini et al., 2006; Eisenberg et al., 2007; Eisenberg et al., 2007; Congdon et al., 2008; Munafo et al., 2008; White et al., 2009; Kramer et al., 2009; Hamidovic et al., 2009; Zai et al., 2012; Pappa et al., 2014). These behavioural phenotypes are similar to those used to define temperament in our ‘calm’ and ‘nervous’ lines (fear, behavioural reactivity in response to the isolation (Murphy et al., 1994; Blache and Ferguson 2005), so it is feasible that the same genes could be involved.

In the HPA axis, cortisol is the final effector and variation in the amplitude of the stressor-induced response in plasma cortisol concentrations has been associated with temperament in humans, monkeys, dogs, cattle, and sheep (Gunnar et al., 1997; Beerda et al., 1998; Kalin et al., 1998; Hennessy et al., 2001; De Palma et al., 2005; Burdick et al., 2010; Hawken et al., 2013). The full range of adrenal steroid hormones are synthesised in the adrenal cortex from the same precursor, and preference for cortisol over mineralocorticoids and androgens is controlled by several enzymes, with CYP17 (cytochrome P450 17α-hydroxylase/17,20-lyase) being a key branch-point enzyme (Nakajin et al., 1981). Polymorphism in the gene for CYP17 affects CYP17 enzyme activity and the cortisol response to a stressor in South African Angora goats and Merino sheep (Storbeck et al., 2007, 2008; Hough et al., 2013). Moreover, the cortisol response to stressors differs between our ‘calm’ and ‘nervous’ sheep (Hawken et al., 2013), so CYP17 is an obvious candidate gene.

Therefore, the present study aimed to identify polymorphisms associated with temperament for potential use in marker assisted selection. The physiological and behavioural differences between our ‘calm’ and ‘nervous’ lines of sheep have been well characterised, so we used an association study (Glazier et al., 2002; Bah Rosman 2008) to look at the association of candidate genes with those differences. This approach is
more appropriate than ‘whole-genome association analysis’ or ‘linkage analysis’, both of which are preferable when the underlying pathophysiological mechanisms are not known and false positive results are likely (Fan et al., 2006; Bah Rosman 2008). Therefore, in this chapter, we tested the hypothesis that polymorphisms of specific genes will be distributed differently between the two lines of sheep that had been selected for calm or nervous temperament by quantifying 4 genes (DRD4, DRD2, MAOA, CYP17) in both phenotypic lines.

4.3 Materials and Methods

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/3/100/1252.

4.3.1 Animals

We used a flock of Merino sheep that had been selected for over 20 generations on criteria that reflect emotional reactivity. At approximately 16 weeks of age, within 2 weeks after weaning, the behavioural reactions of lambs to humans measured in an ‘arena test’ and their reactions to social isolation were measured in an ‘isolation box test’ (Murphy et al., 1994; Blache and Ferguson 2005). The two tests are described in ‘general materials and methods’ chapter. All animals were assigned an overall selection score that combined these two tests, allowing them to be classified as ‘nervous’ or ‘calm’ on the basis of expression of high or low levels of movement or vocalisation. The ‘nervous’ line is more reactive to contact with humans and with isolation, whereas the ‘calm’ line is less reactive to humans and to isolation (Blache and Ferguson 2005). For the first part of this study, we used 58 ewes from the ‘nervous’ line and 59 ewes from the ‘calm’ line, all 16 weeks old and of similar live weight (20 ± 1.6 kg). The animals in the two lines were always managed together except at mating and lambing.

4.3.2 Genomic DNA isolation

Whole blood was sampled by jugular venepuncture into EDTA vacutainers (Greiner Bio-One, Australia). Genomic DNA was isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the instructions provided by the manufacturer. The success of the isolation was confirmed by gel electrophoresis and the concentration of
genomic DNA was measured using BioPhotometer Plus (Eppendorf, Hamburg, Germany).

4.3.3 Detection of polymorphisms of CYP17, DRD4, DRD2 and MAOA

The primers used to amplify the single nucleotide polymorphism (SNP) located on position 628 (SNP628) fragment of CYP17 had previously been described for South African Merino sheep (Hough 2012; Table 4.1). The other primers were designed using Primer Premier software (Version 5.0, PREMIER Biosoft, Palo Alto, CA, USA) to amplify a highly conserved fragment of DRD4, all 7 exons of DRD2, and 3 exons of MAOA, based on sequences obtained from Genebank (Table 4.1). All primers were synthetised by GeneWorks, Australia.

Fragments of the CYP17 and DRD4 genes were amplified by PCR. Amplification reactions (in 10 µL) contained 2 µL of 5×PCR buffer including 0.2 mM dNTPs (Fisher Biotec, Australia), 2.5 mM MgCl$_2$ (Fisher Biotec, Australia), 0.3 µM each of forward and reverse primers (GeneWorks, Australia), 1.1 U Taq DNA polymerase (5.5 U/µL; Fisher Biotec, Australia) and 20 ng genomic DNA. The fragments of CYP17 SNP628 and DRD4 were amplified at an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 15 s, and a final extension at 72 °C for 7 min.

Fragments of the DRD2 and MAOA genes were amplified by PCR in 50 µL: 5 µL of 10×Faststart PCR buffer (Roche, Germany), 2 mM MgCl$_2$ (Roche, Germany), 0.2mM dNTP mix (Roche, Germany), 0.3 µM each of forward and reverse primers (GeneWorks, Australia), 2 U Faststart Taq DNA polymerase (5 U/µL; Roche, Germany) and 80 ng genomic DNA. DRD2 exon2 and exon5 were amplified at an initial denaturation at 95 °C for 6 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 20 s, and a final extension at 72 °C for 7 min. For DRD2, exons 1, 6 and 7 were amplified at an initial denaturation at 95 °C for 6 min, followed by 35 cycles of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 42 s, and a final extension at 72 °C for 7 min. DRD2 exons 3 and 4 were amplified at an initial denaturation at 95 °C for 6 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 25 s, and a final extension at 72 °C for 7 min. MAOA exon 8 was amplified at an initial denaturation at 95°C for 6 min, followed by 35 cycles of 95 °C for 30 s, 60 °C for 30 s, 72 °C for 15 s, and a final extension at 72 °C for 7 min. MAOA exons 1 and 15 were amplified at an initial denaturation at 95°C for 6 min, followed by 35 cycles of 95 °C for 30 s, 68 °C for 30 s, 72 °C for 42 s, and a final
extension at 72 °C for 7 min.

**Table 4.1** Oligonucleotide primers used in PCR amplification for cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), dopamine receptors 2 and 4 (DRD2, DRD4) and monoamine oxidase A (MAOA). Accession numbers from Genebank are NC_019479 for CYP17 mRNA, XM_004016032 for DRD2 mRNA, and XM_004022016 for MAOA mRNA. *Note: CYP17 SNP628 has been reported previously (Hough 2012).*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Fragment</th>
<th>Gene Oligonucleotide sequences (5' - to 3')</th>
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<tr>
<td>CYP17</td>
<td>SNP628</td>
<td>F: CCTGAAGGCCCATACAAA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GGATACTGTCAGGGGTGTG</td>
</tr>
<tr>
<td>DRD4</td>
<td>Conserved fragment</td>
<td>F: TGCTCTGCTGGACGCCCT TCT TC</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: GTGCCG GAACGCCGTTGGAAGAC</td>
</tr>
<tr>
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<td>Exon 1</td>
<td>F: TCCGCTGAACCTGTCTGGTATGAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGAGATGGGACGGCTCTTGAGGGGT</td>
</tr>
<tr>
<td></td>
<td>Exon 2</td>
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<td>R: AAGGTGGTTGTTGCTGGTG</td>
</tr>
<tr>
<td></td>
<td>Exon 3</td>
<td>F: CAAAGTGGGGGTGTCTCTGTGG</td>
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<tr>
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<td></td>
<td>R: CCAGGAGGACAGAGGCAAGACT</td>
</tr>
<tr>
<td></td>
<td>Exon 4</td>
<td>F: GCTTTTCTCTCTCTCTCCCA</td>
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<td></td>
<td>R: CAGAGACATTGGGGGAGAGGTG</td>
</tr>
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<td></td>
<td>Exon 5</td>
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<td></td>
<td></td>
<td>R: GTCCCTGACCTGAACACTTACCAC</td>
</tr>
<tr>
<td></td>
<td>Exon 6</td>
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<td></td>
<td>R: CCTGTATGTGGTGGTGCTG</td>
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<tr>
<td></td>
<td></td>
<td>R: CAGAGACATGGCCGAGGT</td>
</tr>
<tr>
<td>MAOA</td>
<td>Exon 1</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>R: CCCCCAAGCGACCTTCAG</td>
</tr>
<tr>
<td></td>
<td>Exon 8</td>
<td>F: GCTTCCATCGAGGGGAAAACA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TAAACACAGCCTACCTTTTTCTTC</td>
</tr>
<tr>
<td></td>
<td>Exon 15</td>
<td>F: CTCTGATGTGTTGTAGTGCCACT</td>
</tr>
<tr>
<td></td>
<td></td>
<td>R: TGAGCATGTCAACTTTAACTTTCTTG</td>
</tr>
</tbody>
</table>

The polymorphisms for each gene fragment were checked after sanger sequencing by the Australian Genome Research Facility (Western Australia). Each gene fragment was
amplified and sequenced in a subset of 5 nervous and 5 calm sheep and the sequences were aligned using BioEdit Sequence Alignment Editor software (version 7.0.5.2©1997-2007, T. Hall).

4.3.4 Real-time genotyping

The probes used to genotype the SNP628 fragment of CYP17 have also been published for South African sheep (Hough 2012; Table 4.2). The specific primers and Taqman MGB probes for other fragments (Table 4.2) were designed according to the polymorphisms identified above and used to genotype each ewe using a Taqman MGB probe assay. Each sample was genotyped in triplicate using a Vii7™ Real-Time instrument from Applied Biosystems (California, USA). Amplification reactions (10 µL) were performed using 5 µL of 2×TaqMan® Genotyping Master Mix (Applied Biosystems, California, USA), 0.5 µL of custom TaqMan® SNP genotyping Assay (20×) containing two primers and two labelled probes (Applied Biosystems, California, USA) and 20 ng genomic DNA. The thermal cycle included a pre-read stage at 60 °C for 30 s, a hold stage for denaturation at 95°C for 10 min to activate the AmpliTaq Gold®DNA polymerase, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s (with the additional step of cooling to 52 °C for 30 s prior to 60 °C extension for CYP17 SNP628 due to the lower annealing temperature of the primers) and a post-read stage at 60 °C for 30 s. The transition rate between all steps was 1.6 °C/s.

The data were collected using the Allelic Discrimination Plot of Vii7™ software. A no-template control (negative control) was also included in each assay. Accuracy of real-time genotyping results was confirmed by direct sequence analysis by the Australian Genome Research Facility (Western Australia). Sequencing results were analysed with BioEdit Sequence Alignment Editor software (version 7.0.5.2© 1997-2007, T. Hall).
Table 4.2 Nucleotide sequences of primers and probes used in real-time PCR genotyping for cytochrome P450 17α-hydroxylase/17,20-lyase (CYP17), dopamine receptors 2 and 4 (DRD2, DRD4) and monoamine oxidase A (MAOA). *Note: CYP17 SNP628 has been reported previously (Hough 2012).*

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Oligonucleotide sequence (5’- to 3’-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP17</td>
<td>SNP628 Forward primer</td>
<td>CCTGAAGGCCATACAAA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
<td>GGATACTGTCAAGGCTG</td>
</tr>
<tr>
<td></td>
<td>Sensor probe</td>
<td>(VIC®-labelled): CTTCTTTGCTCAGAACC</td>
</tr>
<tr>
<td></td>
<td>Anchor probe</td>
<td>(FAM™-labelled): TCCTTGCCCAAGAC</td>
</tr>
<tr>
<td>DRD2</td>
<td>SNP483 Forward primer</td>
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<tr>
<td></td>
<td>Reverse primer</td>
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</tr>
<tr>
<td></td>
<td>Sensor probe</td>
<td>(VIC®-labelled): TGAAGGACAGGACCCAG</td>
</tr>
<tr>
<td></td>
<td>Anchor probe</td>
<td>(FAM™-labelled): AAGGACAGAACCAC</td>
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<tr>
<td>SNP939</td>
<td>Forward primer</td>
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<tr>
<td></td>
<td>Reverse primer</td>
<td>GGCATGCCCCTCTCT</td>
</tr>
<tr>
<td></td>
<td>Sensor probe</td>
<td>(VIC®-labelled): TCCCACCATTGCCTC</td>
</tr>
<tr>
<td></td>
<td>Anchor probe</td>
<td>(FAM™-labelled): CCACCACGGCCTC</td>
</tr>
<tr>
<td>MAOA</td>
<td>SNP189 Forward primer</td>
<td>GCATGGAGAGTCTGCAGAAGA</td>
</tr>
<tr>
<td></td>
<td>Reverse primer</td>
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<tr>
<td></td>
<td>Sensor probe</td>
<td>(VIC®-labelled): TCGAACATCTGGGCCG</td>
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<td></td>
<td>Anchor probe</td>
<td>(FAM™-labelled): TCGAACATCTGACCG</td>
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<td>SNP219</td>
<td>Forward primer</td>
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<td></td>
<td>Reverse primer</td>
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<td>Sensor probe</td>
<td>(VIC®-labelled): TGAGATGCCGCCTCT</td>
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<tr>
<td></td>
<td>Anchor probe</td>
<td>(FAM™-labelled): TGAGATGCCACCTCT</td>
</tr>
</tbody>
</table>

4.4 Statistical analysis

BioEdit Sequence Alignment Editor software (version 7.0.5.2©1997-2007, T. Hall) was used to identify gene polymorphisms (SNPs) in individual sheep, and Vii7™ software (Applied Biosystems, California, USA) was used to collect the genotypes of each SNP. For each SNP, differences between temperament lines in genotype frequency were compared using Chi-square tests and the reliability of the data was tested using the Hardy-Weinberg equilibrium. Differences were considered significant at P < 0.05 and
very significant at P < 0.01.

4.5 Results

4.5.1 Identification of the gene polymorphisms (5 SNPs).

There was no variation in nucleotide sequence in the conserved fragment of DRD4, but two SNPs were detected in the DRD2 gene: a C/T SNP at position 483 in exon 3 and a T/C SNP in position 939 in exon 6 (Table 4.3). All three genotypes were obtained by genotyping. For the sequenced MAOA gene fragments, two C/T mutations were detected in exon 1, one at position 189 and one at position 219. Again, all three genotypes were obtained by genotyping (Table 4.3). For CYP17, we detected the same A/G mutation, SNP628, that had been described in South African sheep (Storbeck et al., 2008).

Table 4.3 SNPs detected in the three candidate genes that control glucocorticoid synthesis (CYP17), dopamine receptor 2 (DRD2) and monoamine oxidase A (MAOA). Note: for CYP17, this is a confirmation of SNP628 reported previously (Storbeck et al., 2008)

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Oligonucleotide sequence (5'→3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP17</td>
<td>SNP628</td>
<td>AAGGAACGA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>AAGGAACGG</td>
</tr>
<tr>
<td>DRD2</td>
<td>SNP483</td>
<td>CTGGGTCTGTCCCTT</td>
</tr>
<tr>
<td></td>
<td>SNP939</td>
<td>CACCAAGGCCTCCA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CACCAAGGCCTCCA</td>
</tr>
<tr>
<td>MAOA</td>
<td>SNP189</td>
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</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td></td>
<td>GTGATAGGAGGGT</td>
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</tbody>
</table>

4.5.2 Genotypes called by real-time genotyping

Three genotypes (Allele1/Allele1; Allele2/Allele2; Allele1/Allele2) for above detected each SNP were identified according to the Allelic Discrimination Plot (Figure 4.1). The clustering of datapoints along horizontal axis call homozygous Allele1/Allele1 genotype (A/A for CYP17 SNP628; C/C for DRD2 SNP483; T/T for DRD2 SNP939; C/C for MAOA SNP189 and SNP219), the clustering of datapoints along vertical axis call homozygous Allele2/Allele2 genotype (G/G for CYP17 SNP628; T/T for DRD2
SNP483; C/C for DRD2 SNP939; T/T for MAOA SNP189 and SNP219) and the clustering of datapoints along diagonal call heterozygous Allel1/Allel2 genotype (A/G for CYP17 SNP628; C/T for DRD2 SNP483; T/C for DRD2 SNP939; C/T for MAOA SNP189 and SNP219).

Fig 4.1 The Allelic Discrimination Plot results for the three genotypes.
4.5.3 Frequency of polymorphism in each temperament line

Individual genes

The CYP17 SNP628 A/A genotype was about three-fold more frequent (P < 0.01) in the nervous line than in the calm line, whereas the G/G genotype was about twice as frequent (P < 0.05) in the calm line than in the nervous line (Table 4.4). There were no differences between the lines for the frequencies of the A/G genotype of CYP17 SNP628 (Table 4.4) or any of the three genotypes of DRD2 SNP483 (Table 4.4). By contrast, for the DRD2 SNP939, the T/T genotype was twice as frequent in the calm line than in the nervous line (P < 0.01), whereas the T/C and C/C genotypes were more frequent in nervous than in calm animals (P < 0.05; Table 4.4). The frequencies of all three genotypes for MAOA SNP189 and SNP219 were similar for calm and nervous sheep (Table 4.4).

Combinations of genes

The genotype frequencies of only two genes (CYP17 SNP628 and DRD2 SNP939) differed significantly between the calm and nervous lines, so we analysed the relationships between gene frequency combinations and temperament (Table 4.4). In the nervous line, 17/18 sheep (94%) with the CYP17 SNP628 A/A genotype also had the DRD2 SNP939 C/T and C/C genotypes. In the calm line, 15/35 sheep (43%) with the DRD2 SNP939 T/T genotype also carried the CYP17 SNP628 G/G genotype, but only 4/35 sheep (11%) carried the CYP17 SNP628 A/A genotype.
Table 4.4 Frequency distribution of the SNPs of target genes in the control of glucocorticoid synthesis (CYP17), dopamine receptor 2 (DRD2) and monoamine oxidase A (MAOA) in nervous (n = 58) and calm (n = 59) lines of sheep. Different superscripts in the same column within SNP indicate significant difference between lines: P < 0.01 for uppercase, P < 0.05 for lowercase.

<table>
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<tr>
<th>Selection line</th>
<th>Gene</th>
<th>SNP</th>
<th>Genotypes</th>
<th>N</th>
<th>%</th>
<th>N</th>
<th>%</th>
<th>N</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CYP17</td>
<td>SNP628</td>
<td>A/A</td>
<td>18</td>
<td>A</td>
<td>31.0</td>
<td>16</td>
<td>A</td>
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<tr>
<td>Nervous</td>
<td></td>
<td></td>
<td>G/G</td>
<td>16</td>
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<td>28</td>
<td>b</td>
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<tr>
<td>Calm</td>
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<td>9</td>
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<td>15.5</td>
<td>14</td>
<td>a</td>
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<td>11</td>
<td>a</td>
<td>18.6</td>
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4.6 Discussion

Polymorphisms of two specific genes, one controlling glucocorticoid synthesis (CYP17) that has previously been described in South African sheep (Storbeck et al., 2008), and the other controlling dopamine receptor 2 (DRD2), are associated with temperament phenotype (calm or nervous) in Australian Merino sheep.
For CYP17 SNP628, we detected three genotypes (A/A, A/G, G/G) in the two temperament lines whereas, in South African sheep, only two genotypes (A/A, A/G) were detected (Storbeck et al., 2008). Moreover, the frequency of A/A and G/G genotypes differed between our calm and nervous temperament lines whereas, in South African sheep, the frequency of A/A and A/G genotypes were similar in the rearing ability lines (Vander Walt et al., 2009). The presence or absence of the homozygous G/G genotype might be explained by differences in the historical origins of the Australian and South African Merino sheep, or by differences in the genetic selection criteria used in the two studies.

The origin of the South African Merino is not clear but could explain the absence of the homozygous G/G. It has also been suggested that, South African Merino sheep are mixed Merino breed with different Merino types from around the world (Ryder 1984; Mason 1996; Anon. 2005). Two Spanish rams and four Spanish ewes would have been introduced into South Africa in 1789 (Ryder 1984), and then from 1891, American Vermont type Merinos were introduced into South Africa (Mason 1996), and then, Australian Merinos were imported before 1929 (Ryder 1984). South African Merinos are a mixed genetic background which is different from that of Australian Merinos which came from Spanish Merinos only. It was suggested (Cottle 2010) that in 1797, the Spanish Merino sheep were first introduced into Australia, and the Australian Merinos were then introduced into South Africa before 1929 (Ryder 1984). A possibility is that the genotype of CYP17 SNP628 of all the rams or all the ewes introduced into South Africa from Australia was accidently A/A, therefore, whatever was the genotypes of the ewes or rams (A/A, A/G or G/G), the offspring could not be G/G.

Our results show that G/G genotype is strongly associated with the phenotype for calm temperament (in our selected flock) and that its percentage in nervous group was small (27.59%). Since the two temperament lines have been selected for over 20 generations, the probability of a homozygous G/G genotype is higher in animals that are calm (47.46%) than in a random population as suggested by the percentage of homozygous G/G (13.31%) detected in the non selected flock. The sheep used in South African Merino sheep were from two different lines (H-line and L-line) selected for the ability of ewes to rear multiple offspring, not for temperament as such and since probability to detect G/G can be low, the homozygote genotype G/G for CYP 17 SNP628 was not
present in the 136 South African Merino sheep genotyped in the study by Vander Walt et al. (2009).

For the DRD2 gene, we detected two SNPs that are novel for Australian Merino sheep, SNP939 (exon 6) and SNP483 (exon 3), but only SNP939 is associated with temperament phenotype. The difference between the two alleles is not surprising because SNP483 is a synonymous mutation (GTC-GTT) with both versions encoding for valine, whereas SNP939 (ATG-ACG) causes a change from methionine to threonine that has the potential to influence phenotype. Interestingly, a DRD2 polymorphism has also been associated with personality traits and behaviours in humans (Noble et al., 1998; Rowe et al., 1999; Eisenberg et al., 2007; Eisenberg et al., 2007; Hamidovic et al., 2009; White et al., 2009; Zai et al., 2012). These observations also agree with the presence of large numbers of DRD2 in brain tissue (Muly et al., 1998) in dopaminergic pathways that are involved in the expression of behaviours related to fear, aggression and novelty-seeking (Jonsson et al., 2003; Zai et al., 2012).

In contrast to CYP17 and DRD2, we did not detect polymorphisms in the fragments of DRD4 or MAOA that we targeted, so it seems unlikely that DRD4 or MAOA are associated with temperament in sheep. On the other hand, it is feasible that important polymorphisms are located on other parts of the genes. In the absence of a published mRNA sequence for ovine DRD4, we selected a highly conserved fragment that had been published for other species and aligned well with the sheep genome. For the ovine MAOA gene, we investigated only the three longest known exons (exons 1, 8, 15) and neither of the two SNPs detected in exon 1 were associated with temperament. Again, this is to be expected because they were both synonymous mutations, with both variants of SNP189 (GGC and GGT) encoding for glycine and both variants of SNP219 (GTT and GTC) encoding for valine.

The lack of association between MAOA polymorphisms and temperament phenotype agrees with observations on humans where MAOA polymorphisms are associated with psychological disorders and substance addiction, rather than behavioural reactivity (Schulze et al., 2000; Du et al., 2002). However, there is an important difference in methodology. In humans, the phenotype is measured using clinical interview or diagnosis, in marked contrast to the measurement of temperament in sheep using
behavioural challenges. Nevertheless, where an association between MAOA genotype and phenotype has been found in humans, the phenotype was antisocial behaviours that are assessed using methods analogous to our behavioural tests, with scores allowing a segregation between high and low levels of impulsiveness, aggression and irritability (Jorm et al., 2000; Koller et al., 2003). In addition, MAOA activity seems to differ between genders (Murphy et al., 1976) and we only used female sheep, a factor that might also explain the lack of association with temperament. In all but one study of humans, an association between MAOA polymorphism and behavioural reactivity was detected in males rather than females. The exception was the work on females by Deckert et al. (1999), but they assessed phenotype by structured clinical interview. In our breeding flock, we retain very few males so we could not include gender in the design. The present study is the first investigation of MAOA polymorphisms in sheep and their association with temperament, and it was clearly not exhaustive, so the remaining 12 exons need to be explored, as does the issue of gender.

4.7 Conclusion
A total of 5 polymorphisms were identified in sheep that affect glucocorticoid synthesis (CYP17), dopamine receptor 2 (DRD2) and monoamine oxidase A (MAOA). Of these, one for CYP17 (SNP628) and one for DRD2 (SNP939) were distributed significantly different between the calm and nervous lines. The data suggest that DRD2 SNP939 C allele combined with CYP17 SNP628 A/A genotype could be used as a potential genetic marker for nervous temperament prediction, whereas the combination of DRD2 SNP939 T/T plus CYP17 SNP628 G/G genotypes could be used as a potential genetic marker for calm temperament in Merino sheep. The validation of their correctness as the genetic marker will to be explored in next chapter.
Chapter 5

The association of CYP17 SNP628 and DRD2 SNP939 genotypes with behavioural and physiological cortisol responses

Validation of the correctness of the two SNPs as genetic markers

5.1 Abstract
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  5.3.1 Animals
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5.1 Abstract
To validate the two SNPs obtained in Chapter 4 as genetic markers for prediction of sheep temperament, we genotyped a large non-selected flock of 268 ewes of unknown temperament for the DRD2 SNP939 and CYP17 SNP628 polymorphisms. We tested whether these two SNPs were associated with the two phenotypes of temperament, the physiological (cortisol) phenotype and the behavioural phenotype (reactivity). The results showed that behavioural responses to the ‘arena test’ and ‘isolation box’ tests were affected by DRD2 SNP939 genotype and that the response to ACTH challenge was affected by CYP17 SNP628 genotype. The sheep carrying the DRD2 SNP939 calm genotype had lower behavioural scores than those carrying the SNP939 nervous genotype (P < 0.05). After ACTH administration, sheep with CYP17 SNP628 nervous genotype had higher values for maximum cortisol concentration, amplitude and area under the curve (AUCI) than sheep with CYP17 SNP628 calm genotype (P < 0.05).

Therefore, considering the results presented here alongside those in Chapter 4, we conclude that the combination of the DRD2 SNP939 C allele plus the CYP17 SNP628 A/A genotype could be used as a genetic marker for nervous temperament, whereas the combination of the DRD2 SNP939 T/T plus the CYP17 SNP628 G/G genotype could be used as a genetic marker for calm temperament in Merino sheep.

**Key words:** Genetic marker; Validation; Behavioural response; Physiological response
5.2 Introduction

In Chapter 4, we described the detection of DRD2 SNP939 and CYP17 SNP628 genotypes and their association with temperament in sheep: it appears that the DRD2 SNP939 C allele can be combined with the CYP17 SNP628 A/A genotype to provide a genetic marker for prediction of nervous temperament, whereas the DRD2 SNP939 T/T genotype can be combined with the CYP17 SNP628 G/G genotype to provide a genetic marker for prediction of calm temperament. However, this conclusion is a statistical association so we need to validate these two SNPs as genetic markers with a phenotype verification experiment. Therefore, in this chapter, we will use a non-selected flock to test the association of the SNP genotypes with the two phenotypes of temperament, the physiological response (cortisol) and the behavioural response (reactivity).

The CYP17 gene encodes P450c17, an enzyme that is predominantly expressed in the mammalian adrenal cortex where it plays a central role in the production of cortisol (Nakajin et al., 1981). This enzyme catalyses two reactions, 17-hydroxylation and 17, 20-lyase, both of which are important for synthesis of mineralocorticoids, glucocorticoids and androgens. Of all the cytochrome P450 enzymes involved in steroidogenesis, only P450c17 possesses dual hydroxylase and lyase activities, so CYP17 sits at a critical branch point where it determines the synthesis of cortisol in preference to mineralocorticoids and androgens from the same precursors (Nakajin et al., 1981).

A study of South African sheep has suggested that the same SNP in the CYP17 gene is associated with the cortisol response to stressor (Hough et al., 2013). We therefore used the physiological ACTH stimulation test to assess the relationship of the CYP17 SNP628 genotype with the cortisol response in non-selected Merino sheep.

Large numbers of DRD2 are located in the prefrontal cortex (Sesack et al., 1995; Muly et al., 1998) and are involved in the evaluation of stimuli by the brain, a process that precedes any behavioural response. Previous studies have suggested that polymorphisms in the gene for DRD2 are linked to dopaminergic activity, the quantity of brain D2 dopamine receptors, and variation in some personality and behaviour traits such as extraversion, agreeableness impulsivity/sensation-seeking and aggression (Noble et al., 1991, 1994a, 1998; Berman and Noble 1997; Blum et al., 1997; Jonsson et al., 2003; Lee et al., 2007; Ajna et al., 2009; Zai et al., 2012).
Therefore, in this chapter, we investigated the association between DRD2 SNP939 polymorphism and behavioural response to stressors in non-selected merino sheep. We used the same two behavioural challenges (Arena test and Isolation box test; Murphy et al., 1994; Murphy, 1999; Blache and Ferguson, 2005) that measure the reactivities to novelty and isolation that we have been using for more than 20 years to generate the UWA Temperament Flock.

5.3 Materials and Methods
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 (Approval RA/3/100/1252).

5.3.1 Animals
The study described in Chapter 4 indicated that DRD2 SNP939 and CYP17 SNP628 were the most promising genetic markers for calm and nervous temperaments. Here, we genotyped 278 14-month-old ewes of unknown temperament from a commercial flock at UWA Farm Ridgefield (Pingelly, Western Australia). The aim was to identify ewes that represent each of the four possible combinations of the two SNPs then use those animals to assess the association between the possible combinations of SNP genotypes and the behavioural and physiological phenotypes associated with temperament.

5.3.2 Blood sampling for genotypes screening
Whole jugular blood (3 mL) was collected into EDTA-coated tubes (Greiner Bio-One, Australia) from each of the 278 sheep. After genotyping, we selected four groups of 12 ewes with similar live weight (42.8 ± 2.1 kg) that represented the four combinations of the two SNPs: Group 1 carried the combination of polymorphisms most associated with nervous temperament (DRD2 SNP939 C alleles T/C and C/C, plus CYP17 SNP628 A/A); Group 2 carried the DRD2 polymorphism most associated with nervous temperament (SNP939 C allele T/C and C/C) plus the CYP17 polymorphism that was most associated with calm temperament (SNP628 G/G); Group 3 carried the DRD2 polymorphism that was most associated with calm temperament (SNP939 T/T) plus the CYP17 polymorphism that was most associated with nervous temperament (SNP628 A/A); Group 4 carried the combination of polymorphisms that was least associated with both calm and nervous temperaments (DRD2 SNP939 G allele T/G and C/G, plus CYP17 SNP628 A/G).
A/A); Group 4 carried the combination of polymorphisms that were most associated with calm temperament (DRD2 SNP939 T/T plus CYP17 SNP628 G/G).

5.3.3 Phenotyping

The behavioural phenotypic traits of the 48 ewes were measured using the same isolation box and arena tests that have been used routinely for assessment of sheep temperament (Murphy et al., 1994; Blache and Ferguson 2005).

**Isolation Box Test (IBT)**

Each sheep was visually isolated from any other sheep for 1 min in a solid enclosed box (L = 1.5 m, W = 0.7 m, H = 1.5m). Agitations and high-pitched calls were quantified using an electronic agitation meter, fixed on the side of the isolation box (Blache and Ferguson 2005; Brown et al., 2015). The agitation meter had previously been calibrated for reflection of low, medium and high agitation levels using a ‘mechanical sheep’ (Blache and Ferguson 2005; Brown et al., 2015). The agitation score (arbitrary units) read from the meter indicated the individual degree of inherent fear or adaptability to isolation (Blache and Ferguson 2005; Brown et al., 2015). A high score indicating high behavioural reactivity to isolation is considered to reflect nervous temperament, whereas a low score is considered to reflect calm temperament.

**Open-Field Arena Test**

This test measures the locomotor activity and the number of bleats in sheep exposed to a conflicting situation inherent in the need to approach a human to gain access to conspecifics. The test pen was 3.3 m wide and 7.0 m long and divided into four sectors of identical size. Briefly, each sheep entered the test arena from a door situated at the end opposite to a pen containing a group of sheep that was visible from anywhere in the arena. A human stood motionless in front of the pen of conspecifics, and the locomotor activity of the test animal was measured by counting the number of times it entered each sector (‘crosses’) during a 3 min period. A high level of locomotor activity indicated a high level of reactivity to the conflicting situation.

**Cortisol response to ACTH**

The physiological phenotypic trait, adrenal responsiveness, was measured with an ACTH challenge. All 48 sheep were habituated to the indoor conditions and contact with people for one week. The 48 sheep were divided into 3 blocks of 16 with equal numbers of ewes from each of the four genotypes (4 sheep from each genotype). For
each block, one day before the start of the experiment, the animals were fitted with an
in-dwelling jugular cannula made of plastic tubing (ID 18 G, OD 14 G, Techno-plas,
Western Australia) connected to a three-way tap (BD, Mexico). The 16 sheep received
either saline injection or ACTH injection following a latin square design with each
repeat conducted over two days at 2-day intervals. On each repeat, 2 sheep from each
genotype received a 3 mL intravenous injection of either saline (vehicle control) or
saline containing 0.2 iu ACTH (Polypeptide, Strasbourg, France). Every sheep had been
injected with dexamethasone (0.125 mg/kg i.v., Provet, WA, Australia) to suppress
endogenous ACTH release (Beaven et al., 1964) and the saline or ACTH was injected
90 min later (time = 0). Blood (3 mL) was sampled 90, 60, 30 and 0 min before
injection of saline or ACTH and then every 20 min for 3 h. Samples were immediately
transferred into plastic tubes containing 50 i.u. heparin and polystyrene granules
(Techno-plas, Western Australia), and then centrifuged at 2000 g for 10 min. The plasma
was separated and stored at –20 °C until assay.

**Cortisol assay**

Plasma concentrations of cortisol were measured in duplicate by radioimmunoassay
using the Immuchem\textsuperscript{TM} Coated Tube Cortisol \textsuperscript{125}I RIA Kit (MP Biomedicals, Belgium).
The limit of detection was 1.7 ng/mL. Quality control samples (30.5 ng/mL and 13.5
ng/mL) were used to estimate inter- (5.6 % and 5.0 %) and intra-assay (7.9 % and 8.4 %)
coefficients of variation.

**5.4 Statistical analysis**

For the behavioural tests, the data were analysed using the Univariate procedure of the
General Linear Model in SPSS (SPSS 16.0 V), with DRD2 genotype as Factor A
(Nervous genotype-A1, Calm genotype-A2) and CYP17 genotype as Factor B (Nervous
genotype-B1, Calm genotype-B2). Prior to analysis, all data were assessed for normality
using the Shapiro-Wilk test and for variance homogeneity using Bartlett’s test. When
the variance was homogenous, the data were compared using Tukey’s post-hoc tests.
When the variance was not homogenous, the data were compared using Tamhane’s tests.
Differences were considered significant at P < 0.05 and very significant at P < 0.01. For
the cortisol response, data were analyzed using ANOVA for repeated measurements of
the General Linear Model in SPSS (SPSS 16.0 V), with time as the repeated measure
and genotype as the factor. The parameters of the cortisol response that were analysed
included the maximum (peak) concentration, amplitude of response, rate of increase,
delay to peak, and delay in return from peak to baseline concentration (calculated as the average cortisol concentration for samples taken at –30 min and 0 min before injection with ACTH or vehicle) and the area under the response curve calculated relative to baseline (AUCB) as described by Pruessner et al. (2003). The values for cortisol measured at –90 and –60 min were omitted because, with the low dose of dexamethasone, the endogenous secretion of ACTH/cortisol was not fully suppressed until –30 min. Each parameter of the cortisol response was analysed using the Univariate procedure of the General Linear Model in SPSS (SPSS 16.0 V), with DRD2 genotype as Factor A (Nervous genotype-A1, Calm genotype-A2) and CYP17 genotype as Factor B (Nervous genotype-B1, Calm genotype-B2) and order of injection (first or second repeat) or day of ACTH injection as blocking factors. Order and day of ACTH injection did not have an effect on any parameter of the response to ACTH. Prior to analysis, all data were assessed for normality using the Shapiro-Wilk test and for variance homogeneity using Bartlett’s test. The data did not require transformation. Differences were considered significant at $P < 0.05$ and very significant at $P < 0.01$.

5.5 Results

5.5.1 Frequency of each genotype and combination of genotypes in non-selected sheep

In the non-selected sheep, the highest frequencies were for CYP17 SNP628 A/G and DRD2 SNP939 T/C, so the frequency of the combination of these two SNPs was highest among the 6 possible combinations (Table 5.1). For CYP17 SNP628, the A/A genotype was the least frequent. For DRD2 SNP939, the T/C and C/C genotypes were both associated with nervous temperament so they were combined, and the combination was more frequent than the calm T/T genotype. Therefore, when the two genes were considered in combination, the frequency of the combination SNP628 A/A and SNP939 T/T was lowest among the 6 combinations (Table 5.1). For the combinations of DRD2 plus CYP17 genotypes, the frequencies were low for both the calm and the nervous phenotypes (Table 5.1).

| Table 5.1 | Frequencies of individual and combinations of genotypes in 278 non-selected sheep for genes that control glucocorticoid synthesis (CYP17), dopamine receptor 2 (DRD2) and monoamine oxidase A (MAOA). |
## 5.5.2 Behavioural phenotype

There were no interactions between the DRD2 and CYP17 genotypes for any of the variables measured. There was a main effect of the DRD2 SNP939 genotype, with the nervous genotype (T/C + C/C) showing a higher isolation box score and more crosses than the calm genotype (T/T), although the number of bleats was similar (Table 5.2). There was no effect of CYP17 SNP628 genotype on IBT scores and crosses of the behavioural phenotypes (Table 5.2), but there was a tendency for the number of bleats to be affected by CYP17 SNP628 genotype (P = 0.077, Table 5.2).

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype and associated phenotype</th>
<th>T/T (calm)</th>
<th>T/C (nervous)</th>
<th>C/C (nervous)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD2</td>
<td>SNP939</td>
<td></td>
<td>174</td>
<td>91</td>
<td>13</td>
</tr>
<tr>
<td>CYP17</td>
<td>SNP628</td>
<td></td>
<td>100</td>
<td>37</td>
<td>141</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Combined</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD2 + SNP939</td>
</tr>
<tr>
<td>CYP17 SNP628</td>
</tr>
<tr>
<td>N</td>
</tr>
<tr>
<td>%</td>
</tr>
</tbody>
</table>
Table 5.2 The effect of genotypes for glucocorticoid synthesis (CYP17) and dopamine receptor 2 (DRD2) on behaviour (IBT score, Bleats, Crosses) in sheep.

<table>
<thead>
<tr>
<th>Item</th>
<th>IBT score</th>
<th>Bleats</th>
<th>Crosses</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DRD2 SNP939 T/C+C/C</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP17 SNP628 A/A</td>
<td>101.1 ± 6.26</td>
<td>5.2 ± 0.92</td>
<td>8.4 ± 0.66</td>
</tr>
<tr>
<td>CYP17 SNP628 G/G</td>
<td>97.2 ± 8.34</td>
<td>1.4 ± 0.51</td>
<td>7.0 ± 1.22</td>
</tr>
<tr>
<td><strong>DRD2 SNP939 T/T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CYP17 SNP628 A/A</td>
<td>46.5 ± 4.54</td>
<td>4.1 ± 0.79</td>
<td>5.9 ± 0.55</td>
</tr>
<tr>
<td>CYP17 SNP628 G/G</td>
<td>35.3 ± 3.77</td>
<td>1.6 ± 0.47</td>
<td>5.9 ± 0.52</td>
</tr>
<tr>
<td><strong>DRD2 SNP939 T/C+ C/C</strong></td>
<td>100.2 ± 5.10 ^A</td>
<td>4.2 ± 0.74</td>
<td>8.1 ± 0.58 ^a</td>
</tr>
<tr>
<td><strong>DRD2 SNP939 T/T</strong></td>
<td>43.3 ± 3.44 ^B</td>
<td>3.3 ± 0.37</td>
<td>5.9 ± 0.41 ^b</td>
</tr>
<tr>
<td>CYP17 SNP628 A/A</td>
<td>67.2 ± 4.57</td>
<td>4.5 ± 0.60</td>
<td>6.8 ± 0.44</td>
</tr>
<tr>
<td>CYP17 SNP628 G/G</td>
<td>55.9 ± 6.15</td>
<td>1.5 ± 0.35</td>
<td>6.3 ± 0.53</td>
</tr>
</tbody>
</table>

| **P value**                   |           |        |         |
| DRD2 SNP939                   | 0.001     | 0.915  | 0.024   |
| CYP17 SNP628                  | 0.263     | 0.077  | 0.377   |
| DRD2 SNP939×CYP17 SNP628     | 0.592     | 0.498  | 0.381   |

Different uppercase superscripts in the same column indicate difference at P < 0.01.
Different lowercase superscripts in the same column indicate difference at P < 0.05.

5.5.3 Cortisol response

There was no change in plasma concentrations of cortisol following the injection of the saline vehicle (data not shown). There was no genotype×time interaction (DRD2 SNP939 genotype×time: P = 0.461; CYP17 SNP628 genotype×time: P = 0.112). There was a main genotype effect of CYP17 SNP628 (P = 0.039), but no genotype effect of DRD2 SNP939 (P = 0.377). For all parameters of cortisol response, there was no effect of any interaction between DRD2 SNP939 and CYP17 SNP628 genotypes (Table 5.3, P > 0.05), and no main effect of DRD2 SNP939 genotype for any parameter (Table 5.3, P > 0.05), but there was a main effect of CYP17 SNP628 genotype on the maximum value (P = 0.040), amplitude (P = 0.048) and AUCB (P = 0.041) (Table 5.3), with higher values observed in the CYP17 SNP628 A/A nervous genotype (Figure 5.1 a,b) than in the CYP17 SNP628 G/G calm genotype (Figure 5.1 c,d). There was no effect of CYP17 SNP628 genotype on the other parameters (Table 5.3, P > 0.05).
Table 5.3 The effect of the genotype on cortisol response to ACTH administration in unselected sheep. Factor A: DRD2 SNP939 genotypes (Nervous genotype A1, Calm genotype A2). Factor B: CYP17 SNP628 genotypes (Nervous genotype-B1, Calm genotype-B2). All animals were treated with dexamethasone (Dexa) 90 min before ACTH injection. The area under the response curve was calculated relative to ground (AUCG) and to baseline (AUCB).

<table>
<thead>
<tr>
<th>Item</th>
<th>Before Dexa (ng/mL)</th>
<th>Before ACTH (ng/mL)</th>
<th>Peak value (ng/mL)</th>
<th>Amplitude (ng/mL)</th>
<th>Delay to peak (min)</th>
<th>Delay, peak to baseline (min)</th>
<th>Rate of increase (ng/mL/min)</th>
<th>AUCG (ng/mL)</th>
<th>AUCB (ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>28.6 ± 6.76</td>
<td>10.3 ± 1.63</td>
<td>80.0 ± 8.04</td>
<td>70.2 ± 8.04</td>
<td>25.0 ± 2.62</td>
<td>102.5 ± 11.36</td>
<td>2.8 ± 0.32</td>
<td>1697 ± 179.74</td>
<td>1543 ± 189.43</td>
</tr>
<tr>
<td>B2</td>
<td>29.0 ± 6.27</td>
<td>10.2 ± 1.21</td>
<td>65.8 ± 4.82</td>
<td>53.6 ± 4.82</td>
<td>21.7 ± 1.67</td>
<td>90.0 ± 7.70</td>
<td>2.6 ± 0.27</td>
<td>1427 ± 117.29</td>
<td>1175 ± 108.67</td>
</tr>
<tr>
<td>A2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1</td>
<td>20.0 ± 4.51</td>
<td>8.3 ± 0.50</td>
<td>69.4 ± 6.05</td>
<td>61.1 ± 6.05</td>
<td>21.7 ± 1.67</td>
<td>93.3 ± 8.57</td>
<td>2.8 ± 0.32</td>
<td>1444 ± 133.82</td>
<td>1246 ± 133.86</td>
</tr>
<tr>
<td>B2</td>
<td>32.5 ± 8.89</td>
<td>7.0 ± 0.55</td>
<td>58.2 ± 4.54</td>
<td>51.2 ± 4.54</td>
<td>20.0 ± 0.00</td>
<td>80.8 ± 8.65</td>
<td>2.6 ± 0.23</td>
<td>1303 ± 112.26</td>
<td>1015 ± 103.51</td>
</tr>
<tr>
<td>A1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A2</td>
<td>28.8 ± 6.13</td>
<td>10.3 ± 1.38</td>
<td>72.9 ± 4.81</td>
<td>62.9 ± 4.82</td>
<td>23.3 ± 1.55</td>
<td>96.3 ± 6.83</td>
<td>2.7 ± 0.25</td>
<td>1562 ± 120.20</td>
<td>1359 ± 118.16</td>
</tr>
<tr>
<td>B1</td>
<td>26.3 ± 6.34</td>
<td>7.7 ± 0.41</td>
<td>63.8 ± 3.87</td>
<td>56.1 ± 3.83</td>
<td>20.8 ± 0.83</td>
<td>87.1 ± 6.44</td>
<td>2.7 ± 0.20</td>
<td>1374 ± 86.53</td>
<td>1130 ± 86.07</td>
</tr>
<tr>
<td>B2</td>
<td>24.3 ± 5.28</td>
<td>9.3 ± 0.86</td>
<td>74.7 ± 5.04⁹</td>
<td>65.6 ± 5.00⁹</td>
<td>23.3 ± 1.55</td>
<td>97.9 ± 7.02</td>
<td>2.8 ± 0.26</td>
<td>1570 ± 123.88</td>
<td>1395 ± 122.10⁹</td>
</tr>
<tr>
<td>P</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>0.778</td>
<td>0.082</td>
<td>0.136</td>
<td>0.265</td>
<td>0.162</td>
<td>0.335</td>
<td>0.625</td>
<td>0.210</td>
<td>0.114</td>
</tr>
<tr>
<td>B</td>
<td>0.476</td>
<td>0.616</td>
<td>0.040</td>
<td>0.048</td>
<td>0.162</td>
<td>0.191</td>
<td>0.285</td>
<td>0.173</td>
<td>0.041</td>
</tr>
<tr>
<td>A×B</td>
<td>0.506</td>
<td>0.682</td>
<td>0.802</td>
<td>0.695</td>
<td>0.638</td>
<td>0.628</td>
<td>0.949</td>
<td>0.665</td>
<td>0.631</td>
</tr>
</tbody>
</table>

Different lowercase superscripts in the same column indicate difference at P<0.05.
Figure 5.1 Plasma concentrations of cortisol (mean ± s.e.m) before and after intravenous injection of 0.2 iu ACTH\textsubscript{1–24} in Australian Merino sheep carrying: a) both genotypes associated with nervousness (CYP17 SNP628 A/A and DRD2 SNP939 C allele); b) the CYP17 SNP628 genotype associated with nervousness (A/A) and the DRD2 SNP939 genotype associated with calmness (T/T); c) the CYP17 SNP628 genotype associated with calmness (G/G) and the DRD2 SNP939 genotype associated with nervousness (C allele); d) both genotypes associated with calmness (CYP17 SNP628 G/G and DRD2 SNP939 T/T). The animals were injected with dexamethasone (0.125 mg/kg) 90 min before the injection of ACTH to suppress the endogenous secretion of cortisol. The dotted line represents the baseline of cortisol concentrations (see Materials and Methods).

5.6 Discussion

The DRD2 SNP939 was specifically associated with behavioural phenotype, as measured by tests of behavioural reactivity, and the CYP17 SNP628 was specifically associated with physiological phenotype, as measured by the cortisol response to ACTH, thus verifying the association of these two SNPs with temperament in sheep.

In sheep genotyped for CYP17, the amplitude of the response to ACTH was similar to that observed in sheep responding to hypoglycaemic stress (Graybeal and Fang 1985), suggesting that the dose of ACTH we used was within the physiologically-relevant range. The cortisol response to ACTH was linked to polymorphism CYP17 SNP628
We did not challenge the sheep from the calm and the nervous lines in the UWA Temperament Flock with ACTH, but these two lines do present different frequencies for SNP628 A/A and G/G so their responses to ACTH would probably differ in the same way. Indeed, the sheep of the nervous line had a greater cortisol response with higher maximum and longer duration to layered stressors (eg, isolation, human presence, restraint) than the sheep of the calm line (Hawken et al., 2013). The same polymorphism, SNP628, has been shown to affect the cortisol response to stress in South African Merino sheep (Hough et al., 2013). In Angora goats, a different type of CYP17 mutation, that nevertheless causes amino acid substitution, is also associated with variation in the cortisol response to stress (Engelbrecht et al., 2000; Storbeck et al., 2007). Despite differences in the location and the nature of the polymorphisms among our sheep, South African sheep, and goats, the consistent observation is that CYP17 polymorphisms can affect on adrenal responsiveness to exposure to stressors.

In our study, the plasma cortisol concentration of all 4 groups of sheep reached the peak very quickly (20 min) after injection of a low dose of ACTH (3 µg/sheep), then decreased rapidly after 20 min, and had returned to baseline values by about 2 h after ACTH injection. However, in other studies with sheep, there was a longer duration in the cortisol response to ACTH and a later peak value (60 min after ACTH) before the rapid decrease began (Engelbrecht et al., 2000; van Lier et al., 2014). This difference between studies can be explained by the dose-dependency of the cortisol response to ACTH stimulation (Daidoh et al., 1995). In the other studies, the dose was 10 µg/kg or 500 µg per sheep (Engelbrecht et al., 2000; van Lier et al., 2014), both much greater than the dose (around 3 µg/sheep) that we used. It was proposed long ago that the dose of exogenous ACTH required to obtain plasma cortisol levels similar to those recorded after induction of physiological stress (insulin) was 0.2 µg/kg (Graybeal and Fang, 1985), or about 10 µg/sheep. Several investigators have since confirmed the usefulness of low doses of ACTH for estimation of adrenocortical function, and they have defined the lowest dose of ACTH capable of eliciting a maximum response as 500 ng/1.73 m² (Crowley et al., 1991; 1993) or 1 µg per sheep (Dickstein et al., 1991). In fact, all of these doses are within the physiological range. The low dose of ACTH (0.1-5 µg) and the standard dose of 250 µg have been compared and it was found that the low dose of ACTH elicited very rapid cortisol peak level at 20 min after ACTH administration, consistent with our observations, whereas the standard dose did not elicit the cortisol
peak before 60 min. Similar results have been obtained in studies on humans (Park et al., 1999), calves (Safwate et al., 1982; Veissier and Le Neindre 1988; Lay et al., 1996; Negra et al., 2004), pigs (Parrott et al., 1989) and dogs (Vincent and Michell 1992). In general, the cortisol peak is earlier with lower the doses of ACTH than with higher doses, for reasons that are not clear. In summary, the dose of ACTH that we used seems to be the most appropriate for testing adrenal responsiveness.

It is important to note that, although our results suggest that SNP628 can explain differences between the temperament lines in cortisol secretion, other components of the HPA axis, such as ACTH production, adrenal responsiveness to ACTH, and steroidogenic enzymes other than CYP17, could also play a role (Vander Walt et al., 2009; Hough et al., 2015). For example, CYP17 17, 20-lyase activity is strongly affected by cytochrome B5 and this interaction contributes to the production of cortisol in South African sheep (Hough et al., 2013). These issues require further investigation.

In contrast to the link between CYP17 polymorphism and adrenal responsiveness, IBT score and number of crosses were similar between homozygous CYP17 genotypes (calm and nervous). Interestingly, there was a tendency for the number of bleats to be higher in the sheep carrying the CYP17 A/A genotype (nervous) than in the sheep carrying the CYP17 G/G genotype (calm). Similarly, South African Merino sheep carrying the CYP17 A/G genotype exhibited a greater number of bleats and lines crossed in the arena test than the sheep carrying the A/A genotype (Hough 2012). In our study, the sheep were visually isolated from their conspecifics but were potentially able to hear them from a distance, so the test conditions might not have allowed us to detect a difference in bleating between the two homozygous CYP17 genotypes. In addition, we only used female sheep, whereas the South African study used only rams. Gender could affect the relationship between CYP17 SNP628 genotypes and behavioural reactivity, in the same way that it affects the relationship between MAOA polymorphism and behavioural reactivity (Murphy et al., 1976; Deckert et al., 1999). Finally, our Merino sheep could differ from South African Merino sheep, in which the CYP17 genotype is associated with both behavioural and physiological reactivity, but one homozygous genotype appears to be absent. At this stage, therefore, we are assuming that the CYP17 SNP628 polymorphism affects the adrenocortical cortisol response to ACTH whereas the DRD2 polymorphism independently affects behavioural response (agitation).
5.7 Conclusion

DRD2 SNP939 genotypes were specifically associated with behavioural phenotype as measured by tests of behavioural reactivity, and CYP17 SNP628 genotypes were specifically associated with the physiological phenotype as measured by cortisol response to ACTH. These observations clearly validated the statistics-based results presented in Chapter 4 and add great support to the conclusion that DRD2 SNP939 C allele combined with CYP17 SNP628 A/A genotype could be used as a genetic marker for nervous temperament, whereas the combination of DRD2 SNP939 T/T plus CYP17 SNP628 G/G genotypes could be used as a genetic marker for calm temperament in Merino sheep. The heritability of these genotypes as well as their impact on other aspects of temperament (cognition) will be explored in next chapters.
Chapter 6

Heritability of behavioural reactivity to isolation based on DRD2 SNP939 and CYP17 SNP628 genotypes associated with sheep temperament phenotype

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<th>Section</th>
<th>Page</th>
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</thead>
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</tbody>
</table>
6.1 Abstract

The estimate of heritability of temperament in sheep, based on phenotypic measurements, is known to be moderate to high, but we do not have an estimate of heritability based on genotypic measurement. In a previous Chapter, we have described 2 SNP genotypes (DRD2 SNP939 and CYP17 SNP628) that are associated with temperament. Therefore, in the present study of a small number of animals, we used these two SNPs genotypes to make a preliminary estimate of heritability for temperament as measured by reactivity to isolation (IBT). We studied 116 parents and offspring from 3 farms, including 56 phenotypic nervous and 60 phenotypic calm sheep, and 7 phenotypic nervous and 6 phenotypic calm sires. The heritability of reactivity to social isolation, measured by the agitation score (IBT), was 0.45 based on the phenotypic selection, 0.50-0.60 for sheep carrying the DRD2 SNP939 genotypes, 0.36-0.48 for sheep carrying the CYP17 SNP628 genotypes, and 0.62-0.76 for sheep carrying the combination of the two SNP genotypes. These results suggest that the heritability of reactivity to social isolation could be improved by selection based on the 2 SNP genotypes, but validation using a larger number of animals is needed.

Keywords: Sheep temperament; Phenotype; Genotype; Heritability; CYP17 SNP628; DRD2 SNP939
6.2 Introduction

Animal temperament can be defined as the variability of emotional or behavioural activity in response to a stimulus (Blache and Bickell 2010, 2011; Dodd et al, 2012). In livestock, behavioural activity is related to the efficiency of routine farm practices, animal welfare and productivity (Turner et al., 2011). Temperament is heritable (Burrow et al., 1988; Krueger and Johnson 2008; Quinn et al., 2009; Bickell et al., 2009b; Turner et al., 2011) and, in sheep, a cross-fostering experiment has suggested that temperament is mainly determined by genetic inheritance (Bickell et al., 2009b). Similar conclusions have been drawn for cattle using similar approaches (Phocas et al., 2006). However, the current methods used to select for temperament are based behavioural tests, such as the isolation box test or flight speed, that measure phenotypic traits. They are not always repeatable and they are very time-consuming (Murphy et al., 1994; Blache and Ferguson 2005; Dodd et al., 2012, 2014) so the challenge is to find an easier method for assessing temperament that can be used as a selection tool (Turner et al., 2011).

The estimate of heritability of temperament, based on phenotypic measurement (the reactivity to isolation, IBT), is moderate to high in sheep ($h^2 = 0.41$, Blache and Ferguson 2005; $h^2 = 0.48$, Boissy et al., 2005; $h^2 = 0.31$, Zambra et al., 2015; $h^2 = 0.30-0.36$, Brown et al., 2015). Recently, we have shown that a single nucleotide polymorphism (SNP) located at nucleotide position 939 of DRD2 (DRD2 SNP939) and a single nucleotide polymorphism located at nucleotide position 628 of CYP17 (CYP17 SNP628) are associated with the behavioural response and the cortisol response, and can define temperament in sheep (chapter 4 and 5). However, we still do not have an estimate of heritability of temperament based on genotypic measurements, or a comparison of the heritabilities from between phenotypic and genotypic measurements.

Here we describe a preliminary study, using the two SNPs genotypes associated with temperament (DRD2 SNP939 and CYP17 SNP628), with a small number of animals from two generations of sheep, in which we have calculated the heritability of reactivity to isolation (IBT) based on these two SNPs genotypes and compared the outcome with the estimate of phenotypic heritability. To estimate the heritability, we used two common methods: the parent-offspring regression and the paternal half-sib correlation (Smith and Kinman 1965; Van Vleck and Bradford 1965; Dickerson 1969).
6.3 Materials and Methods

6.3.1 Animals
Samples used in the present study were obtained from animals generated in another project (Blache and Ferguson 2009). Ewes and lambs were selected on the basis of the accuracy of their parentage. Briefly, the F0 was generated from a flock of 800-1000 ewes on 3 farms (Billandri, Merinotech and Grindon) located in Western Australia and the animals were tested for temperament using the isolation box test (IBT); the 200-300 ewes with the lowest and highest IBT scores were selected as the good and poor temperament groups, respectively. Blood (3 mL) was sampled from the ewes using EDTA vacutainers (BD Diagnostics, Franklin Lakes, NJ, USA) and later analysed for DNA to assign the parentage of their lambs. A total of 1609 ewes were subsequently artificially inseminated to selected sires. An associative mating program was used where 802 ewes from the good temperament group were mated to one of 6 sires with low estimated breeding values (Mean EBV (± SD): 40.3 ± 14.18) for IBT agitation score (ie. good temperament). Similarly, 7 sires with high EBVs for the trait (EBV: –34.3 ± 5.74; ie. poor temperament) were mated to the 807 poor temperament ewes. The behavioural reactivity of the subsequent lambs to social isolation was measured using the Isolation Box Test, within two weeks of weaning (14 weeks of age; Murphy et al., 1994; Blache and Ferguson 2005). Blood was sampled after the behavioural test, as described above. Live weight was similar for the two temperament lines in both F0 (50.7 kg ± 5.9 kg) and F1 (18.4 ± 2.0 kg).

6.3.2 Parentage assignment
DNA was extracted using a DNeasy Blood and Tissue Kit (Qiagen, Germany) according to the procedures of the manufacturer. A sample of blood from each ewe and lamb was used for parentage assignment using the Shepherd® DNA-based Parentage System from Catapult Genetics (Dunedin, New Zealand). Only a subset of animals was used in this project because blood samples were no longer available for all animals at the time of the study. In total, 116 parents (F0) and offspring (F1) were identified including 56 phenotypic nervous and 60 phenotypic calm sheep.

6.3.3 Genomic DNA isolation
Genomic DNA was isolated from the whole blood samples using the DNeasy Blood and
Chapter 6  Heritability of behavioural reactivity to isolation based on the 2 SNPs

Tissue Kit (Qiagen, Germany) according to the instructions provided by the manufacturer. DNA concentration was determined by visualization after gel electrophoresis.

6.3.4 Genotyping

DRD2 SNP939 and CYP17 SNP628 genotypes were genotyped in F0 and F1 using the method described in Chapter 4, using Taqman MGB probe assays (Table 6.1). All of the study sheep were genotyped using a Vii7™ Real-Time instrument (Applied Biosystems, California, USA). Amplification reactions (10 µL) were performed using 5 µL TaqMan® Genotyping Master Mix (2×) (Applied Biosystems, California, USA), 0.5 µL custom TaqMan® SNP genotyping Assay (20×) containing two primers and two labelled probes (Applied Biosystems, California, USA) and 20 ng genomic DNA. The thermal cycle included a pre-read stage at 60 °C for 30 s, a hold stage for denaturation at 95°C for 10 min to activate the AmpliTaq Gold®DNA polymerase, followed by 40 cycles of 95 °C for 15 s, 60 °C for 60 s (with the additional step of cooling to 52 °C for 30 s prior to 60 °C extension for CYP17 SNP628 due to the lower annealing temperature of the primers) and a post-read stage at 60 °C for 30 s. The transition rate between all steps was 1.6 °C/s. The data were collected using the Allelic Discrimination Plot of Vii7™ software (Applied Biosystems, California, USA). A no-template control (negative control) was also included in each assay.

Table 6.1  Nucleotide sequences of primers and probes used in real-time PCR genotyping. Note: The primers and probes for CYP17 SNP628 were from Hough (2012).

| Gene  | SNP   | forward primer  | reverse primer  | sensor probe  | anchor probe  | Oligonucleotide sequence (5’- to 3’-)
<table>
<thead>
<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP17</td>
<td>SNP628</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CGGTCATGATCGCCATCGT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GAGCATCGGCGAGGAGAT</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>VIC®-labelled)</td>
<td>TGAAGGACAGGACCCAG</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td>FAM™-labelled)</td>
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</tr>
<tr>
<td></td>
<td>SNP939</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>ACCCTCCCCGACCCCA</td>
</tr>
<tr>
<td>DRD2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>GGCATGCCCCTTCTCTCT</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td>VIC®-labelled)</td>
<td>TCCACCATGGCCTCT</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>FAM™-labelled)</td>
<td>CCACCACCGCCCT</td>
</tr>
</tbody>
</table>

Accuracy of real-time genotyping results was confirmed by direct sequence analysis.
Chapter 6  Heritability of behavioural reactivity to isolation based on the 2 SNPs

(AGRF). Sequencing results were analyzed with BioEdit Sequence Alignment Editor software (version 7.0.5.2©1997-2007, T. Hall).

6.4 Data analysis

Frequency distribution of the 2 SNP genotypes in the temperament lines

The frequency distributions of each genotype were compared for the Nervous (n = 56) and Calm lines (n = 60), in both the F0 and F1 generations, using Chi-square test. The reliability of the data was tested using the Hardy-Weinberg equilibrium.

Effects of farm and genotype on temperament phenotype

The associations of farm of origin and genotype with temperament (IBT scores) were tested in both F0 and F1. Our previous study (Chapter 5) has shown a main effect of the DRD2 SNP939 genotype but no effect of CYP17 SNP628 genotype on IBT score. We therefore only tested the association of temperament (IBT) with DRD2 SNP939 genotype and farm of origin. The data were analysed using the Univariate procedure from the General Linear Model in SPSS (SPSS 16.0 V), with DRD2 genotype as a factor (Calm genotype, Nervous genotype) and farm of origin as a factor (Billandri, Merinotech, Grindon). Prior to analysis, all data were assessed for normality using the Shapiro-Wilk test and for variance homogeneity using Bartlett’s test. The data in F0 and F1 did not require transformation. Differences were considered significant at P < 0.05 and very significant at P < 0.01.

Heritability estimates of the two SNP genotypes for offspring temperament

The heritability of reactivity to isolation, as measured by isolation box test (IBT) score (temperament phenotype), was analyzed according to the selection lines or on the basis of the individual or combined genotypes for the DRD2 and CYP17 SNPs. Parent-offspring regression and paternal half-sib correction were used to estimate the heritability in the narrow sense.

Parent-offspring regression

\[ b_{OP} = \frac{\Sigma(O - \bar{O})(P - \bar{P})}{\Sigma(O - \bar{O})^2} = \frac{N \Sigma OP - (\Sigma O)(\Sigma P)}{N \Sigma (\bar{P})^2 - (\Sigma \bar{P})^2} \]

\[ b_{OP}: \text{parent-offspring regression} \]
\[ O: \text{values of offspring} \]
\[ \bar{P}: \text{mid-parent value} \]
\[ h^2 = b_{OP} = 2h_{OP} (\text{Dam-offspring regression}) \]
Chapter 6  Heritability of behavioural reactivity to isolation based on the 2 SNPs

Paternal half-sib correction

\[ t = \frac{MS_b - MS_w}{MS_b + (k_0 - 1)MS_w} \]

\[ k_0 = \frac{1}{n-1} \left( \sum k_i - \frac{\sum k_i^2}{\sum k_i} \right) \]

\[ MS_b = \frac{\sum (\sum x_i^2) - C}{df_{\text{inter-group}}} \]

\[ MS_w = \frac{\sum x^2 - MS_b}{df_{\text{intra-group}}} \]

\[ C = \frac{\sum (\sum x_i^2)}{N} \]

\[ h^2 = 4t \]

- \( t \): paternal half-sib correction
- \( MS_b \): inter-group mean square
- \( MS_w \): intra-group mean square
- \( k_0 \): weighted average
- \( n \): number of groups
- \( k \): number of offspring in each group  \( i \): 1,2,..,n
- \( N \): total number of offspring
- \( C \): corrected value
- \( df \): degree of freedom
- \( df_{\text{total}} = N-1 \)
- \( df_{\text{inter-group}} = n-1 \)
- \( df_{\text{intra-group}} = df_{\text{total}} - df_{\text{inter-group}} = N-n \)

6.5 Results

6.5.1 Distribution of the two SNPs genotypes in the temperament lines

The distributions of sheep carrying each genotype in both the F0 and F1 generations were similar for all 3 farms, so we compared genotype frequencies for the two temperament lines across farms (Tables 6.2 and 6.3). In the F0, except for DRD2 SNP939 C/C for which the frequency was significantly higher in nervous group than that in calm group, the frequencies of the other genotypes were similar between two temperament lines (Tables 6.2 and 6.3). In the F1, the DRD2 SNP939 T/T genotype
frequency in the calm line was double that in nervous line, and the T/C and C/C genotype frequencies in the nervous line were both significantly higher than that in calm line (Tables 6.2 and 6.3). The combined percentage of T/C and C/C genotypes was around 70% (57.1%+14.3%) in nervous group. The CYP17 SNP628 A/A genotype frequency in the nervous line was double that in calm line (Tables 6.2 and 6.3).

**Table 6.2** The distribution of sheep with different genotypes for DRD2 SNP939 and CYP17 SNP628 in F0 calm and nervous phenotype groups from 3 different farms. Different lowercase superscripts in the same line indicate difference at P < 0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Overall</th>
<th>#1 Farm</th>
<th>#2 Farm</th>
<th>#3 Farm</th>
<th>Overall</th>
<th>#1 Farm</th>
<th>#2 Farm</th>
<th>#3 Farm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>DRD2</td>
<td>SNP939</td>
<td>T/T</td>
<td>19</td>
<td>33.93a</td>
<td>5</td>
<td>8</td>
<td>6</td>
<td>28</td>
<td>46.67a</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/C</td>
<td>23</td>
<td>41.07a</td>
<td>6</td>
<td>7</td>
<td>10</td>
<td>29</td>
<td>48.33a</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>14</td>
<td>25.00a</td>
<td>6</td>
<td>5</td>
<td>3</td>
<td>3</td>
<td>5.00a</td>
<td>0</td>
</tr>
<tr>
<td>CYP17</td>
<td>SNP628</td>
<td>A/A</td>
<td>19</td>
<td>33.93a</td>
<td>6</td>
<td>6</td>
<td>7</td>
<td>14</td>
<td>23.33a</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A/G</td>
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<td>34</td>
<td>56.67a</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>G/G</td>
<td>8</td>
<td>14.29a</td>
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<td>3</td>
<td>2</td>
<td>12</td>
<td>20.00a</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 6.3** The distribution of sheep with different genotypes for DRD2 SNP939 and CYP17 SNP628 in F1 calm and nervous phenotype groups from 3 different farms. Different lowercase superscripts in the same line indicate difference at P<0.05.

<table>
<thead>
<tr>
<th>Gene</th>
<th>SNP</th>
<th>Genotype</th>
<th>Overall</th>
<th>#1 Farm</th>
<th>#2 Farm</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
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<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td></td>
<td>T/T</td>
<td>16</td>
<td>28.57a</td>
<td>6</td>
<td>5</td>
<td>5</td>
<td>36</td>
<td>60.00b</td>
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<td>DRD2</td>
<td>SNP939</td>
<td>T/C</td>
<td>32</td>
<td>57.14a</td>
<td>10</td>
<td>12</td>
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<td>23</td>
<td>38.33b</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>C/C</td>
<td>8</td>
<td>14.29a</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1.67b</td>
<td>0</td>
</tr>
<tr>
<td>CYP17</td>
<td>SNP628</td>
<td>A/A</td>
<td>20</td>
<td>35.71a</td>
<td>5</td>
<td>9</td>
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<td>11</td>
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<td></td>
<td></td>
<td>A/G</td>
<td>30</td>
<td>53.57a</td>
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<td>1</td>
<td>3</td>
<td>2</td>
<td>15</td>
<td>25.00b</td>
<td>7</td>
</tr>
</tbody>
</table>
6.5.2 Effects of farm and genotype on behavioural reactivity (isolation box test) in the F0 and F1 generations

There were no interactions between the DRD2 SNP939 genotype and farm of origin for the isolation box test result (IBT) in either the F0 or F1 generation (P > 0.05; Table 6.4). There was also no effect of farm of origin on IBT score in either F0 or F1 (P > 0.05; Table 6.4). There was no main effect of DRD2 genotype in the F0 generation (P > 0.05; Table 6.4) but there was a main effect of the DRD2 SNP939 genotype in the F1, with higher isolation box scores in the sheep carrying nervous genotype (T/C + C/C) than the sheep carrying calm genotype (T/T) (P < 0.05; Table 6.4).

Table 6.4 Effect of farm of origin and genotype on reactivity to isolation (IBT, mean ± s.e.) in two generations of sheep (n = 116).

<table>
<thead>
<tr>
<th>Item</th>
<th>F0</th>
<th>F1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DRD2 SNP939 genotype</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Calm</td>
<td>26.7 ± 3.6</td>
<td>22.6 ± 2.7</td>
</tr>
<tr>
<td>Nervous</td>
<td>32.1 ± 3.1</td>
<td>60.1 ± 3.3</td>
</tr>
<tr>
<td><strong>Farm</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Billandri</td>
<td>28.9 ± 3.2</td>
<td>35.5 ± 4.1</td>
</tr>
<tr>
<td>Merinotech</td>
<td>28.7 ± 3.3</td>
<td>41.6 ± 4.1</td>
</tr>
<tr>
<td>Grindon</td>
<td>30.6 ± 3.0</td>
<td>47.0 ± 4.6</td>
</tr>
<tr>
<td><strong>P value</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genotype</td>
<td>0.263</td>
<td>0.010</td>
</tr>
<tr>
<td>Farm</td>
<td>0.944</td>
<td>0.175</td>
</tr>
<tr>
<td>Genotype × Farm</td>
<td>0.227</td>
<td>0.144</td>
</tr>
</tbody>
</table>

Different lowercase superscripts in the same column indicate difference at P < 0.05.

6.5.3 Heritability estimates

The heritability of reactivity to social isolation measured by the agitation score (IBT) based on the phenotypic selection was 0.45. The heritability of reactivity to social isolation was 0.50-0.60 for sheep carrying the DRD2 SNP939 genotype, 0.36-0.48 for sheep carrying the CYP17 SNP628 genotype, and 0.62-0.76 for sheep carrying the combination of the two SNPs (Table 6.5). Because all the sires were carrying the CYP17 A/G genotype that is not associated with the temperament (Chapter 4), we only estimated the heritability of DRD2 SNP939 genotypes from a sire perspective (Table
6.6), and found that it was similar to estimates from a dam perspective (Table 6.5).

Table 6.5 Heritability, estimated by Dam-Offspring Regression, of the reactivity to social isolation measured by the agitation score (IBT) based on the DRD2 SNP939 and CYP17 SNP628 genotypes.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Gene</th>
<th>Gene</th>
<th>N</th>
<th>ΣOP</th>
<th>ΣO</th>
<th>ΣP</th>
<th>Σp²</th>
<th>h²</th>
</tr>
</thead>
<tbody>
<tr>
<td>DRD2 T/T (calm)</td>
<td></td>
<td></td>
<td>24</td>
<td>23396</td>
<td>785</td>
<td>623</td>
<td>26234</td>
<td>0.60</td>
</tr>
<tr>
<td>DRD2 C/T or C/C</td>
<td>SNP939</td>
<td></td>
<td>41</td>
<td>117403</td>
<td>2119</td>
<td>2177</td>
<td>134781</td>
<td>0.50</td>
</tr>
<tr>
<td>DRD2 T/T (calm)</td>
<td>SNP939</td>
<td></td>
<td>12</td>
<td>23138</td>
<td>593</td>
<td>427</td>
<td>31407</td>
<td>0.24</td>
</tr>
<tr>
<td>DRD2 C/T (nervous)</td>
<td>SNP939</td>
<td></td>
<td>11</td>
<td>12493</td>
<td>269</td>
<td>485</td>
<td>32263</td>
<td>0.12</td>
</tr>
<tr>
<td>CYP17 A/A (nervous)</td>
<td>SNP939</td>
<td></td>
<td>17</td>
<td>62092</td>
<td>1253</td>
<td>797</td>
<td>55559</td>
<td>0.36</td>
</tr>
<tr>
<td>CYP17 G/G (calm)</td>
<td>SNP939</td>
<td></td>
<td>8</td>
<td>1128</td>
<td>94</td>
<td>113</td>
<td>2439</td>
<td>0.48</td>
</tr>
<tr>
<td>SNP939 C/T or C/C</td>
<td>SNP628</td>
<td></td>
<td>17</td>
<td>90430</td>
<td>1068</td>
<td>1278</td>
<td>122548</td>
<td>0.76</td>
</tr>
<tr>
<td>+ SNP939 C/T or C/C</td>
<td>SNP628</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ SNP939 A/A (nervous)</td>
<td>SNP628</td>
<td></td>
<td>10</td>
<td>6355</td>
<td>239</td>
<td>215</td>
<td>8585</td>
<td>0.62</td>
</tr>
<tr>
<td>+ SNP939 C/T (nervous)</td>
<td>SNP628</td>
<td></td>
<td>6</td>
<td>4787</td>
<td>93</td>
<td>294</td>
<td>19904</td>
<td>0.08</td>
</tr>
<tr>
<td>+ SNP939 A/A (nervous)</td>
<td>SNP628</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T/T (calm) +</td>
<td></td>
<td></td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>G/G (calm) +</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Table 6.6 Heritability, estimated by Paternal half-sib correction, of the reactivity to social isolation measured by the agitation score (IBT) based on the DRD2 SNP939 genotype.

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>Sires</th>
<th>F1</th>
<th>n</th>
<th>N</th>
<th>ΣΣx</th>
<th>ΣΣx²</th>
<th>Σ (Σx)²/k_i</th>
<th>h²</th>
</tr>
</thead>
<tbody>
<tr>
<td>T/T (calm)</td>
<td>T/T (calm)</td>
<td>5</td>
<td>33</td>
<td>676</td>
<td>20640</td>
<td>15462</td>
<td>0.60</td>
<td></td>
</tr>
<tr>
<td>C/T or C/C (nervous)</td>
<td>C/T or C/C (nervous)</td>
<td>6</td>
<td>45</td>
<td>2596</td>
<td>163876</td>
<td>150508</td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>T/T (calm)</td>
<td>C/T (nervous)</td>
<td>5</td>
<td>14</td>
<td>570</td>
<td>40562</td>
<td>29640</td>
<td>0.40</td>
<td></td>
</tr>
<tr>
<td>C/T (nervous)</td>
<td>T/T (calm)</td>
<td>6</td>
<td>19</td>
<td>597</td>
<td>21229</td>
<td>19464</td>
<td>0.36</td>
<td></td>
</tr>
</tbody>
</table>

6.6 Discussion

This study supports our general hypothesis that the phenotypic heritability of the reactivity to social isolation, as measured by the agitation score (IBT), can be improved by genetic selection based on the two SNP genotypes associated with temperament.

There was no effect of farm of origin on temperament (IBT) in either the F0 or F1 generations, an observation that is consistent with previous results showing little influence of early management in extensive or semi-intensive farm systems on the behaviours of lambs in the arena test (Goddard et al., 2000). In addition, environmental influences during early rearing have been showed to have little effect on emotional reactivity as measured by the isolation box test and arena test (Boissy et al., 2005). However, early environmental conditions, particularly routine farm management procedures such as shearing, vaccination, herding and handling, are known to be stressful to gregarious livestock (Boissy and Neindre 1990; Bouissou et al., 2001; Fisher and Matthews 2001) and can elicit fear and anxiety (Hargreaves and Hutson 1990a, 1990b; Wohlt et al., 1994; Boissy et al., 2005) during the procedures. They can also influence the emotional reactivities or behaviours of sheep to other stressors (Hargreaves and Hutson 1990b; Mateo et al., 1991; Markowitz et al., 1998). The absence of a farm effect might reflect the fact that the livestock management systems were essentially similar among the three farms. Moreover, previous studies have shown that temperament is driven by genetic inheritance rather than by the environmental influence in sheep (Bickell et al., 2009b) and also cattle (Phocas et al., 2006).

The estimated heritability of the reactivity to social isolation measured by the agitation
score (IBT) based on the phenotypical selection \( (h^2 = 0.45) \) was similar to that found previously in animals that had also been selected for phenotype divergence in a temperament trait in sheep \( (h^2 = 0.41, \) Blache and Ferguson 2005), dairy cattle \( (h^2 = 0.40: \) O’Blesness et al., 1960, 0.45: Sato 1981, 0.53: Dickson et al., 1970), and beef cattle \( (0.40: \) Shrode and Hammack 1971, 0.44-0.48: Stricklin et al., 1980). However, other studies have reported lower values for sheep \( (h^2 = 0.16: \) Hocking Edwards et al., 2011, 0.20: Plush et al., 2011; Dodd et al., 2014) and cattle \( (0.22: \) Morris et al., 1994; Neindre et al., 1995, 0.30-0.35: Burrow and Corbet 2000), mostly based on unselected groups of animals.

This variation among studies in the heritability estimates could be explained by differences in experimental design, behavioural tests and scoring systems, previous experience of the animals, and the breeds used (Broucek et al., 2008). For example, in the study by Boissy et al. (2005), the heritability estimate for the number of vocalizations by sheep in response to isolation was 0.48 – a value close to our result \( (h^2 = 0.45) \) which is based on an agitation score that also includes vocalizations in response to isolation. Some of the variation in the estimates of heritability could be explained by the impact of environmental factors on the behavioural responses of the animals. For example, the heritability of fear of humans was estimated to be 0.35 if the fear of humans was measured by the flight speed upon exit from a handling chute, whereas the heritability was 0.30 when fear of human was measured by the agitation score in the chute (Burrow and Corbet 2000). Finally, the estimate heritability can be affected by the age of the animal – in cattle, for example, the heritability of flight speed in response to the fear of humans was 0.54 at 6 months and only 0.26 at 18 months of age (Burrow et al., 1988).

To our knowledge, there have been no estimates of the heritability of temperament traits based on SNP genotype. The present study suggests that genotypic selection can further improve the heritability of this trait (agitation score, response to isolation). Indeed selection based on the combination of DRD2 SNP939 and CYP17 SNP628 genotypes was associated with the highest heritability. This outcome is not surprising because the results verified yet again that DRD2 SNP939 and CYP17 SNP628 genotypes are associated with the two temperaments. However, the heritability estimates in this study were calculated on a small number of animals, so a larger study will be needed for full
validation.

6.7 Conclusion

Compared to the phenotypic method for selection for temperament in sheep, the genotypic method based on CYP17 SNP628 and DRD2 SNP939 appears to improve the estimates of heritability. Our preliminary results support the conclusion that CYP17 SNP628 and DRD2 SNP939 could be used as the potential genetic markers for sheep temperament selection.
Chapter 7

Cognitive learning ability in Merino sheep with different DRD2 SNP939 genotypes

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7.2 Introduction
7.3 Materials and Methods
  7.3.1 Animals
  7.3.2 Color visual discrimination
  7.3.3 Spatial discrimination
7.4 Data analysis
7.5 Results
  7.5.1 Color visual discrimination
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  7.5.3 Behavioural reactivity during the tests
7.6 Discussion
7.7 Conclusion
7.1 Abstract

Polymorphism in the D2 dopamine receptor (DRD2) has been implicated in variability in cognitive function, such as behavioural flexibility and decision-making in humans. Similarly, cognition is known to vary with emotional reactivity (temperament). However, the effect of the interaction between emotional reactivity and DRD2 polymorphism on cognitive function is poorly understood. Using a sheep model in which animals of ‘calm’ and ‘nervous’ temperament carry two different DRD2 SNP939 polymorphisms, we assessed cognitive capacity by simple visual discrimination, simple spatial discrimination, and reversal learning of spatial discrimination. Neither genotype was able to learn color visual discrimination within 48 discriminations, but both were able to learn left-right spatial discrimination within 10 discriminations. Sheep with DRD2 SNP939 T/T (‘calm’) genotype performed better in reverse spatial discrimination, taking less time to learn the reversal task and committing significantly fewer errors, than the sheep with DRD2 SNP939 C/C+C/T (‘nervous’) genotypes. The results suggest that, in sheep, DRD2 SNP939 is involved in the behavioural flexibility that underpins spatial learning, but is not involved in visual learning.

**Key words:** Merino sheep; DRD2 polymorphism; cognitive learning; DRD2 SNP939
7.2 Introduction

In a challenging and continuously changing environment, a vital determinant of survival is the ability to learn and memorize associations between a stimuli and actions, to make appropriate decisions, and to adapt with appropriate behaviours (Provenza 1995; Shettleworth 2001). The mechanisms that underpin these cognitive functions are complex but there is increasing knowledge of the location of the brain regions and neuronal pathways involved (Keil and Wilson 2000). The prefrontal lobe and striatum are mainly responsible for attention and for directing the behaviour toward a given goal in a given situation (Posner and Petersen 1990), so dys-regulation of these brain regions is associated with cognitive disorders (Rubia et al., 1999; Castellanos and Tannock 2002; Johansen et al., 2002). With respect to the neurotransmitters involved, dopamine is considered important because it exerts a strong regulatory effect on neuronal activity in the prefrontal cortex and striatum (Missale et al., 1998; Haber et al., 2000; Schultz 2002). Moreover, in humans, monkeys and rats, midbrain dopaminergic neurons that project to the prefrontal cortex and striatum have been implicated in a variety of cognitive and executive functions, including working memory (Cai and Arnsten 1997; Floresco and Phillips 2001), behavioural flexibility (reversal learning and shifting of attentional set; Owen et al., 1991, 1993; Birrell and Brown 2000) and decision-making (Bechara et al., 2001; Winstanley et al., 2006). In the prefrontal cortex and striatum, dopamine acts via several subtypes of receptor, with each subtype suggested to play a specific role in various aspects of cognitive learning ability (Floresco and Magyar 2006). In particular, subtype DRD2 and its polymorphisms have been linked to variability amongst individual human and mice in decision-making, attention shifting, cognitive learning and reversal learning (Smith et al., 1999; Kruzich and Grandy 2004; Lee et al., 2007; Jocham et al., 2009).

Cognition is also thought to depend on emotional reactivity (or ‘temperament’; Mendl 1999; Erhard et al., 2004; Lansade and Simon 2010; Butts et al., 2013), with lower levels of emotional activity associated with better performance in cognitive tasks such as spatial learning, discrimination and recognition. However, the role of the dopamine pathways in this interaction has never been tested. In the present study, we have addressed the problem with the sheep model because it has: 1) a large brain with human-like basal ganglia and a well-developed cerebral cortex (Jacobsen et al., 2010); 2)
good visual and spatial discrimination and memory (Kendrick et al., 2001; Lee and Fisher 2006; Morton and Avanzo 2011); and 3) a polymorphism in DRD2 that is associated with temperament (Chapter 4 and 5).

In sheep, temperament is assessed with tests that quantify the behavioural responses to the presence of a human and to social isolation (Murphy et al., 1994; Blache and Ferguson 2005). These tests have allowed the definition of two phenotypes: ‘calm’ animals with a low behavioural reactivity and ‘nervous’ animals with high behavioural reactivity (Murphy et al., 1994; Blache and Ferguson 2005; Beausoleil et al., 2008). We have shown that the DRD2 SNP939 T/T genotype is preferentially associated with ‘calm’ temperament and the DRD2 SNP939 C/C and C/T genotypes are preferentially associated ‘nervous’ temperament (Chapter 4 and 5), but we have not yet investigated the effects of these polymorphisms on cognitive function.

We have therefore tested whether, for sheep carrying the DRD2 SNP939, the T/T genotype, associated with ‘calm’ temperament, is better at learning than the ‘nervous’ C/C+C/T genotype. We used three paradigms: simple visual discrimination, simple spatial discrimination, and reverse spatial learning. All of these tests have previously been used to assess learning and short-term memory abilities in rats as well as sheep (Conrad et al., 1996; Morton and Avanzo 2011; Johnson et al., 2012).

### 7.3 Materials and Methods

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/3/100/1252.

#### 7.3.1 Animals

We used female Merino sheep (age 14 months; body mass 42.8 ± 2.1 kg) with known DRD2 SNP939 genotypes: 12 sheep carried the DRD2 SNP939 C/C+C/T genotypes associated with nervous temperament and 12 sheep carried the DRD2 SNP939 T/T genotype associated with calm temperament. The sheep were housed at the UWA Farm Ridgefield in a paddock-feedlot system where they had free access to food and water
7.3.2 Color visual discrimination

An apparatus comprising two identical testing units, previously described by Morton and Avanzo (2011), was used to measure the capacity of an animal to learn one color out of a choice of two (Figure 7.1). Each unit measured $2.4 \times 7.2$ m and comprised a starting gate located in the middle of the front panel, and a testing area divided longitudinally into two decision areas by a metal hurdle. The sheep could be locked in the decision area using a rotating gate. The sheep exit the pen by the doors located at the end of each decision area. At the end of each decision area, an A4 colored sheet of cardboard was displayed 10 cm above a small feed trough ($20 \times 15 \times 20$ cm). The two identical testing units were assembled back to front, so each sheep could be tested multiple times by circling through the two units using corridors located at each end of the block of units. The outsides of the units and testing pen were covered with hessian to isolate the animal from external visual stimuli (Figure 7.1).

Figure 7.1 Top and side views of the design of the apparatus for testing the discrimination of colour (based on Morton and Avanzo 2011). Two of the $2.4 \times 7.2$ m areas were constructed adjacent to each other but the testing areas (the grey in the top schema) were set at opposite ends. Each testing pen comprised a central starting gate and two discrimination test areas divided...
longitudinally by a metal barrier. The two discrimination stimuli (S) are located at the end of the pen. After a sheep has made its choice (one side), the gate was closed behind it. After consuming the reward (or after 1 min), the sheep exited through the exit gates located at the end of the pen and was pushed into the end corridor so it start the next discrimination test in the adjacent pen.

During the visual discrimination testing procedure, a pair of colored stimulus cards (blue and yellow) was used, as previously described by Morton and Avanzo (2011). For each individual sheep, one color was randomly associated with a food reward (25 g lupin grain). Between discrimination trials, the stimulus cards were swapped to control for biases towards the left or right side of the pen. The test began when the sheep entered the start gate from where both stimuli could clearly be seen. The animals were free to move in the test area towards either of the stimuli and, once past a designated line (1 m from the stimulus cards), their decision was recorded, together with the delay taken to the decision (seconds). Each sheep was tested for one minute (Morton and Avanzo 2011) during which all behaviours (bleating, jumping, stamping) were recorded.

During the first 8 discrimination trials, animals that chose incorrectly (not the rewarded color) were allowed to make a correction and then gently ushered to the correct side so they could collect the food reward. In the following trials, once the choice had been made, the gate was closed behind the sheep it was either allowed to eat the reward, if the correct choice was chosen, or wait 20 seconds if the incorrect choice was chosen, before being ushered onto the next discrimination trial. All sheep in both experimental groups were subjected to 6 testing sessions of 8 discriminations. Sheep were considered to have learned to discriminate when they reached 75% correct choice over 8 consecutive discriminations (one testing session), and at least 75% of the animals in each group reached this criterion before commencing the reversal.

7.3.3 Spatial discrimination
This test made use of a Y-maze apparatus (Figure 7.2) with a starting gate leading into a straight run (2.4 m long; Zone ‘A’) that split into two arms (each 2.4 m long; Zone ‘B’ and ‘C’), each with an exit gate located at the end.
The maze is divided in 3 zones (A, B and C). Feeders, either empty or containing the food reward, were placed at the end of the two arms (zones ‘B’ and ‘C’).

Every side of the Y-maze was covered with hessian to isolate the animal from external visual stimuli. The food reward was always placed in a feed bucket on the maze arm designated S+, with an empty feed bucket placed on the incorrect arm (S−).

The test started when the sheep passed the starting gate into the run (A), from which it had to make a choice within 1 min toward the left (B) or right (C) arms of the maze. The animals were free to move around the maze through the different zones. We recorded the time taken by the sheep to find and eat the food reward and the number of times the sheep crossed the boundaries into different zones until it had found and started eating the reward. One half of each treatment group was trained to find the reward on one side of maze and the other half trained to find it on the other side. A sheep was considered to have achieved spatial discrimination (or to have learned to take the correct side) when it had made 4 consecutive correct choices. Each animal was allowed a maximum of 24 attempts. After at least 80% of each group had reached the criterion for success, the animals were tested for reverse spatial discrimination by swapping the location of the reward. The same criterion was used to assess success in the reversal discrimination.

### 7.4 Data analysis

Data were analyzed using SPSS (16.0 V). A two-factor ANOVA for repeated measurements in the General Linear Model was used to analyze the data with trials as
the repeated measure, genotype as a factor and order and day of trial as blocking factors. 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 normally distributed and where variance was homogenous, the data were compared using Tukey’s post-hoc tests. The data for total time (s) in the spatial discrimination test was transformed using a cosine function and the other sets of data (average delays (s) to decision in the simple visual discrimination test, total time in the spatial reversal test, number of errors in the spatial and reversal tests) were transformed using Blom’s formula in the transformation program in the software. Given the observed differences in standard deviations across the conditions, Maulchy's test of sphericity was used to test whether variances and covariances of the within-subject variables were equal. If the test was significant (P < 0.05) for each factor and for the interactions, it indicated a violation of the ANOVA assumption for compound symmetry, and a more conservative Greenhouse-Geisser (G-G) F-test was used. Differences were considered significant at P < 0.05 and highly significant at P < 0.01.

7.5 Results

7.5.1 Color visual discrimination

Neither group reached the criterion of 75% correct choice within 48 discriminations (Figure 7.3) so, under the 75% criterion, neither would be classified as successful at color visual discrimination, although the percentages of correct choices for the two groups differed (53 ± 2.6% vs 39 ± 2.1%; F_{1,10} = 5.188; P = 0.035).
Figure 7.3 Performance of sheep either carrying the DRD2 variant C/C+C/T (black circle) or the T/T (open circle) at nucleotide position 929 in the two-choice colour discrimination task. Each point represents the mean (± S.E.; N = 12) number of correct choices made in a testing sessions comprising 8 discrimination trials.

The average delays to decision were similar for the two genotypes in each session of 8 discrimination trials (P > 0.05; Table 7.1). There was no genotype effect ($F_{1,142}$ (Greenhouse-Geisser) = 0.000; $P = 0.997$) and no interaction between time (session) and genotype ($F_{5,138}$ (Greenhouse-Geisser) = 0.565; $P = 0.685$). However, there was an effect of time (trials) within group (C/C+C/T: $F_{5,66} = 4.628$, $P = 0.041$; T/T: $F_{5,66} = 4.021$, $P = 0.045$; Table 7.1), with both groups performing the task significantly faster in the last session (Session 6) than in the first session.

Table 7.1 The effect of DRD2 SNP939 genotype on the average delay (s) to decision during the visual discrimination test (mean ± S.E.; N = 12). Lowercase subscripts that differ between testing sessions within group (in the same column) indicate $P < 0.05$ (each session = 8 discriminations).


<table>
<thead>
<tr>
<th>Test session</th>
<th>DRD2 SNP939 Genotype</th>
<th>( \text{T/T} )</th>
<th>( F_{1.22} )</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 8.6 \pm 1.99^a )</td>
<td>( 8.0 \pm 1.83^a )</td>
<td>0.178</td>
</tr>
<tr>
<td>2</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 5.3 \pm 0.73^{ab} )</td>
<td>( 5.9 \pm 1.00^{ab} )</td>
<td>0.262</td>
</tr>
<tr>
<td>3</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 4.5 \pm 0.75^{ab} )</td>
<td>( 5.1 \pm 0.75^{ab} )</td>
<td>0.608</td>
</tr>
<tr>
<td>4</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 4.7 \pm 0.75^{ab} )</td>
<td>( 6.3 \pm 1.55^{ab} )</td>
<td>1.182</td>
</tr>
<tr>
<td>5</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 4.7 \pm 0.99^{ab} )</td>
<td>( 4.4 \pm 1.03^{ab} )</td>
<td>0.031</td>
</tr>
<tr>
<td>6</td>
<td>( \text{C/C+/-C/T} )</td>
<td>( 2.8 \pm 0.11^b )</td>
<td>( 2.9 \pm 0.21^b )</td>
<td>0.402</td>
</tr>
<tr>
<td>( F_{5.66} )</td>
<td></td>
<td>( 4.628 )</td>
<td>( 4.021 )</td>
<td></td>
</tr>
<tr>
<td>( P )</td>
<td></td>
<td>( 0.041 )</td>
<td>( 0.045 )</td>
<td></td>
</tr>
</tbody>
</table>

### 7.5.2 Spatial discrimination

Two sheep from each group did not reach the criterion of spatial discrimination so we analyzed the data for 10 sheep per group. There was no genotype effect (\( F_{1.198} \) (sphericity assumed) = 1.366; \( P = 0.265 \)) and no interaction between time (trial) and genotype (\( F_{9.190} \) (sphericity assumed) = 0.373; \( P = 0.946 \)) for the total time (s) taken to complete the initial spatial discrimination test. With all trials combined, there were no differences between the two genotypes in the time taken to complete the test (\( P > 0.05 \); Table 7.2). However, there was an effect of trial within genotype (\( C/C+/-C/T \): \( F_{9.90} = 5.208, P = 0.033 \); \( T/T \): \( F_{9.90} = 6.081, P = 0.025 \); Table 7.2) in both groups, with less time taken to complete the task in Trial 4 than in Trial 1 (Table 7.2). There was no genotype effect (\( F_{1.198} \) (Greenhouse-Geisser) = 0.028; \( P = 0.870 \)) and no interaction between time (trial) and genotype (\( F_{9.190} \) (Greenhouse-Geisser) = 2.361; \( P = 0.071 \)) for the number of choice errors in the test. Apart from the first trial, there were no differences between genotypes in the number of error choices (\( P > 0.05 \); Table 7.2). In Trial 7, sheep with \( C/C+/-C/T \) genotype reached the learning criterion (error choice = 0) because they ran straight toward the correct side (Table 7.2), whereas sheep with the \( T/T \) genotype reached the learning criterion in Trial 6 (Table 7.2),
Table 7.2 The effect of DRD2 SNP939 genotype (C/C+/C/T versus T/T) on the total time (s) taken to complete the spatial discrimination test and the number of choice errors (mean ± S.E.; N = 10). Different uppercase superscripts in the same line (between the two groups for each trial) indicate P < 0.05. Different lowercase superscripts in the same column (between trials within each group) indicate P < 0.05.

<table>
<thead>
<tr>
<th>Trial</th>
<th>C/C+/C/T</th>
<th>T/T</th>
<th>F_1,18</th>
<th>P</th>
<th>C/C+/C/T</th>
<th>T/T</th>
<th>F_1,18</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>19.7 ± 4.74a</td>
<td>25.1 ± 5.43a</td>
<td>0.498</td>
<td>0.491</td>
<td>0.6 ± 0.16ba</td>
<td>1.3 ± 0.26ab</td>
<td>5.188</td>
<td>0.035</td>
</tr>
<tr>
<td>2</td>
<td>11.2 ± 2.99ab</td>
<td>14.0 ± 6.43ab</td>
<td>0.126</td>
<td>0.728</td>
<td>0.5 ± 0.27b</td>
<td>0.5 ± 0.34b</td>
<td>0.000</td>
<td>1.000</td>
</tr>
<tr>
<td>3</td>
<td>11.6 ± 4.78ab</td>
<td>14.0 ± 9.12ab</td>
<td>0.052</td>
<td>0.823</td>
<td>0.4 ± 0.31b</td>
<td>0.1 ± 0.10b</td>
<td>0.871</td>
<td>0.363</td>
</tr>
<tr>
<td>4</td>
<td>7.4 ± 1.89b</td>
<td>7.7 ± 2.05b</td>
<td>0.012</td>
<td>0.915</td>
<td>0.0 ± 0.00b</td>
<td>0.1 ± 0.10b</td>
<td>1.000</td>
<td>0.331</td>
</tr>
<tr>
<td>5</td>
<td>4.1 ± 0.16b</td>
<td>5.2 ± 0.51b</td>
<td>1.531</td>
<td>0.235</td>
<td>0.2 ± 0.13b</td>
<td>0.1 ± 0.10b</td>
<td>1.110</td>
<td>0.306</td>
</tr>
<tr>
<td>6</td>
<td>4.6 ± 0.27b</td>
<td>4.4 ± 0.12b</td>
<td>0.792</td>
<td>0.388</td>
<td>0.2 ± 0.13b</td>
<td>0.0 ± 0.00b</td>
<td>2.250</td>
<td>0.151</td>
</tr>
<tr>
<td>7</td>
<td>4.8 ± 0.25b</td>
<td>5.5 ± 0.37b</td>
<td>1.612</td>
<td>0.220</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>2.250</td>
<td>0.151</td>
</tr>
<tr>
<td>8</td>
<td>4.9 ± 0.22b</td>
<td>5.9 ± 0.34b</td>
<td>2.723</td>
<td>0.116</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>2.250</td>
<td>0.151</td>
</tr>
<tr>
<td>9</td>
<td>4.9 ± 0.42b</td>
<td>5.9 ± 0.79b</td>
<td>1.282</td>
<td>0.272</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>2.250</td>
<td>0.151</td>
</tr>
<tr>
<td>10</td>
<td>4.7 ± 0.31b</td>
<td>4.7 ± 0.21b</td>
<td>0.003</td>
<td>0.956</td>
<td>0.0 ± 0.00b</td>
<td>0.0 ± 0.00b</td>
<td>2.250</td>
<td>0.151</td>
</tr>
</tbody>
</table>

Reverse spatial discrimination

Two sheep from each group failed to reach the criterion for the reverse spatial discrimination test, so we analyzed the data for 8 sheep per group. The sheep with DRD2 SNP939 T/T genotype learned ran straight to the correct side with no error by Trial 10, one trial earlier than the sheep with C/C+C/T genotype (Trial 11; Table 7.3). There was a main effect of genotype (F_1, 286 (Greenhouse-Geisser) = 8.423; P = 0.027) and an interaction between time (trial) and genotype (F_17, 270 (Greenhouse-Geisser) = 3.517; P = 0.035) for the time (s) taken to complete the task. Before Trial 10, in Trials 4, 5, 8 and 9, the two genotype groups differed in the time taken to complete the task (P < 0.05; Table 7.3).

There was an effect of trial within genotype (P < 0.05; Table 7.3): compared to the first 3 trials, the time taken to complete the task was shorter by Trial 5 in the SNP939 T/T genotype, but did not become shorter until Trial 10 for the SNP939 C/C+C/T genotype. There was a main effect of genotype (F_1, 286 (Greenhouse-Geisser) = 6.569; P = 0.023) and interaction between time (trial) and genotype (F_17, 270 (Greenhouse-Geisser) = 2.646;
P = 0.031) for the number of error choices in the test. The genotypes differed in the number of errors in Trials 5, 6 and 7 (P < 0.05; Table 7.3). Compared the first 3 trials, the number of errors fell by Trial 5 in the DRD2 SNP939 T/T genotype (P < 0.05; Table 3) but not until Trial 10 in the C/C+C/T genotype (P < 0.05; Table 7.3).

**Table 7.3** The effect of DRD2 SNP939 genotype (C/C/+C/T versus T/T) on the time (s) taken to complete the reverse spatial discrimination test and the number of error choices (mean ± S.E.; N = 8). Different uppercase superscripts in the same line (between the two groups for each trial) indicate P < 0.05. Different lowercase superscripts in the same column (between trials within each group) indicate P < 0.05.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Time (s)</th>
<th>Number of Errors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C/C/+C/T</td>
<td>T/T</td>
</tr>
<tr>
<td>1</td>
<td>35.9 ± 2.06&lt;sup&gt;a&lt;/sup&gt;</td>
<td>25.2 ± 3.80&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>20.7 ± 4.55&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.0 ± 2.68&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>15.7 ± 3.26&lt;sup&gt;b&lt;/sup&gt;c</td>
<td>14.5 ± 3.22&lt;sup&gt;b&lt;/sup&gt;c</td>
</tr>
<tr>
<td>4</td>
<td>11.9 ± 1.03&lt;sup&gt;bced&lt;/sup&gt;A</td>
<td>7.9 ± 1.34&lt;sup&gt;cdf&lt;/sup&gt;d</td>
</tr>
<tr>
<td>5</td>
<td>10.2 ± 1.24&lt;sup&gt;cde&lt;/sup&gt;A</td>
<td>5.8 ± 0.94&lt;sup&gt;dis&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>9.6 ± 1.43&lt;sup&gt;cd&lt;/sup&gt;d</td>
<td>7.1 ± 1.29&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>7</td>
<td>10.5 ± 1.37&lt;sup&gt;bced&lt;/sup&gt;d</td>
<td>6.1 ± 0.86&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>8</td>
<td>11.9 ± 1.60&lt;sup&gt;bced&lt;/sup&gt;d</td>
<td>6.1 ± 0.88&lt;sup&gt;dis&lt;/sup&gt;</td>
</tr>
<tr>
<td>9</td>
<td>11.7 ± 2.07&lt;sup&gt;bced&lt;/sup&gt;d</td>
<td>5.4 ± 1.04&lt;sup&gt;dis&lt;/sup&gt;</td>
</tr>
<tr>
<td>10</td>
<td>5.5 ± 0.44&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.3 ± 0.35&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>11</td>
<td>4.9 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.8 ± 0.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>5.4 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.7 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>13</td>
<td>4.8 ± 0.36&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.9 ± 0.48&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>14</td>
<td>4.7 ± 0.37&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.6 ± 0.21&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>4.5 ± 0.27&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.8 ± 0.27&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>16</td>
<td>4.5 ± 0.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.5 ± 0.33&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>17</td>
<td>4.1 ± 0.17&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.7 ± 0.39&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>18</td>
<td>4.4 ± 0.38&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4.6 ± 0.30&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>P</td>
<td>0.008</td>
<td>0.012</td>
</tr>
</tbody>
</table>
7.5.3 Behavioural reactivity during the tests

The C/C+C/T genotype paced significantly more than the T/T genotype during the visual discrimination test (P < 0.05; Table 7.4) and bleated significantly more during the reverse spatial discrimination test (P < 0.05; Table 7.4). Both genotype groups showed higher frequencies of jumping, stamping, pacing and, in particular, bleats during the visual discrimination test than during both the initial spatial discrimination test and the reverse spatial discrimination test (P < 0.05; Table 7.4). Compared to the initial spatial discrimination test, sheep in the reverse spatial discrimination test showed more staring, stamping, pacing and, in particular, more bleating (P < 0.05; Table 7.4). In the reverse spatial discrimination test, there were more bleats in the C/C+C/T genotype than in the T/T genotype (P < 0.05; Table 7.4), but the genotypes were similar in the initial spatial discrimination test (P > 0.05; Table 7.4).

Table 7.4 The mean (± S.E.) frequency of behaviours in the two genotypes during the visual discrimination test (N = 12), the initial spatial discrimination test (N = 10) and the reverse spatial discrimination test (N = 8). Different lowercase superscripts in the same line (between tests for each behaviour) indicate P < 0.05. Different uppercase superscripts in the same column (between genotype groups within each test) indicate P < 0.05.

<table>
<thead>
<tr>
<th>Behaviours (items/min)</th>
<th>Visual Discrimination</th>
<th>Spatial discrimination</th>
<th>Reverse spatial discrimination</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>DRD2 C/C+C/T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleating</td>
<td>7.9 ± 1.98&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.7 ± 0.33&lt;sup&gt;b&lt;/sup&gt;</td>
<td>3.2 ± 0.81&lt;sup&gt;Aa&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jumping</td>
<td>1.5 ± 1.06</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Stamping</td>
<td>1.3 ± 1.16</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Staring</td>
<td>0.3 ± 0.25</td>
<td>0.0 ± 0.00</td>
<td>0.3 ± 0.16</td>
</tr>
<tr>
<td>Pacing</td>
<td>3.0 ± 0.69&lt;sup&gt;Aa&lt;/sup&gt;</td>
<td>0.3 ± 0.21&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.5 ± 0.26&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td><strong>DRD2 T/T</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bleating</td>
<td>5.3 ± 1.89&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.0 ± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.00 ± 0.86&lt;sup&gt;Bb&lt;/sup&gt;</td>
</tr>
<tr>
<td>Jumping</td>
<td>0.8 ± 0.32</td>
<td>0.3 ± 0.30</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Stamping</td>
<td>0.4 ± 0.33</td>
<td>0.0 ± 0.00</td>
<td>0.1 ± 0.12</td>
</tr>
<tr>
<td>Staring</td>
<td>0.5 ± 0.41</td>
<td>0.0 ± 0.00</td>
<td>0.0 ± 0.00</td>
</tr>
<tr>
<td>Pacing</td>
<td>0.5 ± 0.23&lt;sup&gt;B&lt;/sup&gt;</td>
<td>0.2 ± 0.13</td>
<td>0.4 ± 0.18</td>
</tr>
</tbody>
</table>

7.6 Discussion
The SNP DRD2 (SNP939) receptor appears to influence learning ability in animals with different phenotypes for temperament. Both DRD2 genotypes were able to learn the initial left-right spatial discrimination and the reverse spatial discrimination tasks, and they both performed similarly in left-right spatial discrimination test, but sheep carrying a T/T (calm) genotype performed better in reverse spatial discrimination, taking less time to learn the task and committing fewer errors, than the sheep with C/C+C/T (nervous) genotype. On the other hand, neither genotype was able to learn color visual discrimination within 48 attempts. In no situation did the C/C+C/T (nervous) genotype perform better than the T/T (calm) genotype, but the fact that the sheep were not able to learn visual discrimination, yet were able to learn spatial discrimination, suggests that we need to consider the adequacy of some of the tests in the context of this animal model.

Similar differences in capacity to learn spatial and visual discrimination have been reported for studies with pigs, cattle and horses (Wesley and Klopfer 1962; Rehkamper and Gorlach 1997; Moustgaard et al., 2004; Murphy and Arkins 2007), and well as earlier studies for sheep (Tanaka et al., 1995; Johnson et al, 2012). On the other hand, several other studies from a variety of laboratories, including our own, have shown that sheep can discriminate colors (Bazely and Ensor 1989; Bickell et al., 2011; Morton and Avanzo 2011; Howery et al., 2013; Sena et al., 2013). The discrepancy among these studies might be explained by: i) differences in behavioural reactivity when exposed to stressors; ii) relevance of the stimulus; iii) attention towards the reward; iv) training conditions. Below we consider each of these possibilities.

1. Behavioural reactivity when exposed to stressors

The behaviour of individuals, including cognitive learning, can be disrupted when they are exposed to high levels of stress (Lyons et al., 1993; Boissy and Bouissou 1995; Kleen et al., 2006; Conrad 2010; Simitzis et al., 2012; Sena et al., 2013). During testing in the present study, the sheep were isolated from their flock-mates, one of the most stressful situations for a social species and a factor known to influence performance in cognitive tasks (Lyons et al., 1993; Boissy and Bouissou 1995). In addition, the sheep in the present study had some contact with human investigators, another known stressor (Romeyer and Bouissou 1992; Lyons et al., 1993; Boissy and Bouissou 1995). Moreover, there could be an interaction between these two sources of stress because
Lyons et al. (1993) found that sheep are more likely to react to human presence when they are isolated from their conspecifics. It therefore seems likely that, during our visual discrimination test, the sheep perceived significant potential threats, an explanation supported by the locomotion and vocalization behaviours that we observed: jumping, stamping, pacing and, especially, the large number of bleats. This raises the question of why the same two groups were able to learn spatial discrimination. The spatial discrimination testing followed visual discrimination testing, allowing the sheep a few weeks of contact with the human (the same investigator), perhaps increasing familiarity and reducing fear of both human presence and the novel environment. There is evidence in the present study for this adaptation in the fear-related behaviours: in both genotype groups, there was far less jumping, pacing, and bleating in the spatial discrimination tests than in the visual discrimination test. Therefore, the high behavioural reactivities elicited by the exposure to novelty (human, surroundings) and isolation from their conspecifics is probably the main reason for their inability to learn the visual discrimination. Further study is therefore needed with a modified test conducted in the presence of conspecifics, in familiar surroundings, and thus without stress.

\textit{ii) Relevance of the stimulus}

Many studies have suggested that sheep can visually discriminate among stimuli that are highly relevant to survival, such as toxic and non-toxic food, and the distinctive characteristics of their mates (Nowak and Lindsay 1992; Kendrick et al., 2001; Bickell et al., 2011; Howery et al., 2013). It is also known that sheep can live in a small area without the need for confinement by fences and then return to that area instinctively after lambing and shearing (Hunter 1962; Gray 1996), suggesting that they have good spatial memory. Good spatial memory is probably linked to survival and could explain why the sheep in the present study were able to learn spatial discrimination within 10 trials, regardless of their DRD2 genotype. By contrast, the blue and yellow cards used in the visual discrimination task might not be very relevant cues for sheep so an association with the food reward was difficult for them to learn. However, in the study by Morton and Avanzo (2011), the sheep could discriminate between blue and yellow, the test colors used in the present study, and also green and purple, even when presented in different shapes. The disagreements between the two studies could be explained by differences in the breed. Morton and Avanzo (2011) used Welsh Mountain sheep while we used Merino sheep and the breeds often differ in their behavioural characteristics (Scott and Fuller 1965). Sheep breeds also differ in cognition, including the ability to
discriminate between ewes and lambs (Shillito and Alexander 1975; Alexander 1977; Nowak et al., 1987; Walser and Walters 1987; Nowak and Lindsay 1990).

**iii) Attention towards the reward**

It has been suggested that the amount of attention paid to a stimulus plays a vital role in the learning of a cognitive task (Mendl 1999; Moustgaard et al., 2004). The key element of any reward-based discrimination task is the motivation of the animal to obtain the reward, so a highly motivational stimulus with little risk of distraction is essential (Conrad 2010; Sena et al., 2013). Furthermore, the effectiveness of a food reward as motivation can be diminished when the animal experiences stress or fear (Hutson 1985). Mendl (1999) proposed that the most fearful animals shift their attention away from their tasks, resulting in poor performance in those tasks. During the visual discrimination test in the present study, the animals were agitated, judging from their behaviour, so their attention appeared to be more focused on stressors (isolation, human presence) than on the food reward. We were unable to control external auditory stimuli because of the design of the test facility so the sheep were visually isolated from their conspecifics but were nevertheless able to hear them in the background. This distraction might have limited the test animal’s focus on the stimulus and the food reward.

**iv) Training conditions**

It seems that sheep need a great deal of training if they are to learn a visual discrimination task, and that they might never learn. Johnson et al. (2012) found that even 5 weeks of training was insufficient for them to learn visual discrimination, and Tanaka et al. (1995) found that sheep needed 7 sessions of 30 discrimination trials (210 in total) to pass a criterion for shape discrimination. We trained our sheep for 48 trials, so a longer training period might be needed to, for example, assess DRD2 genotypes. In addition to the duration of training, habituation to the test environment might play a role. Morton and Avanzo (2011) handled their sheep intermittently for 4 months and habituated them to the test apparatus before they began formal testing, so their testing environment was not novel. We could not follow this protocol because of the potential effect of the DRD2 polymorphisms on novelty-seeking and fear-related behaviour. Perception of our testing apparatus as novel by our sheep might explain their inability to learn visual discrimination.

DRD2 polymorphism affected performance in the reversal of spatial learning test but not the initial spatial memory task. This outcome could be explained by the specific
association of different types of cognition (memory and reversal learning) with DRD1 and DRD2 subtypes of dopamine receptors, and the effect of a challenging environment on individual differences in cognition learning.

DRD1 receptors and their polymorphisms have been linked to memory ability in primates (Sawaguchi and Goldman-Rakic 1991, 1994; Williams and Goldman-Rakic 1995; Murphy et al., 1996; Muller et al., 1998). By contrast, in rodents, DRD2 receptors and their polymorphisms are mainly associated with reversal learning (Ridley et al., 1981; Smith et al., 1999; Kruzich and Grandy 2004; Lee et al., 2007; Cools et al., 2001, 2007; Jocham et al., 2009). Where DRD2 receptor polymorphisms have been shown to be linked with working memory in mice and humans (Kellendonk et al., 2006; Xu et al., 2007), the polymorphism loci differed from those described in our study. It seems that the polymorphisms that we have been studying induce greater differences in reversal learning capacity than in memory ability. In addition, individual differences in behavioural response to environmental stimuli often become more evident when individuals are presented with a more challenging environment (Broom 2001; Koolhaas et al., 2010; Carere et al., 2010). The learning task in our spatial memory test was not as challenging as the reverse spatial test, and the effect of polymorphisms on cognitive function might have been expressed only during the more challenging reversal situation. This would suggest that DRD2 polymorphisms might be involved in behavioural flexibility (ie, the reversal task) in sheep, as indicated for humans (Jocham et al., 2009).

The sheep with SNP939 T/T genotype performed better in the spatial reversal task, taking less time to learn and committing fewer errors, than the sheep carrying the SNP939 C/C+C/T genotype. We interpret this outcome as being caused by the specific association between the polymorphisms and temperament because temperament can affect cognitive function (Mendl 1999; Erhard et al., 2004). The SNP939 T/T genotype is associated with a lower level of behavioural reactivity to isolation and to presence of a human (‘calm’), compared to the SNP939 C/C+C/T genotype (‘nervous’ sheep; Chapter 4 and 5). The better learning performance of the SNP939 T/T genotype could be due to their relative calmness since lower levels of emotional activity are associated with better performance on cognitive tasks such as spatial learning, discrimination and recognition (Mendl 1999; Erhard et al., 2004; Lansade and Simon 2010; Bickell et al., 2011; Butts et al., 2013).
In a stressful situation, nervous individuals may adopt an ‘active’ strategy characterized by higher levels of locomotor activity and vocalizations, and higher activation of the sympathetic-adrenal medullary and hypothalamic-pituitary-adrenal (HPA) systems, than calm individuals (Clark et al., 1997; Beausoleil et al., 2008). The activation of the HPA system under stress results in increased vigilance and, for example, increased scanning of the environment (Dukas and Clark 1995; Boissy et al., 2005). Compared to the calm T/T genotype sheep, the nervous C/C+C/T genotype sheep appeared to show a higher level of activity and poorer ability to maintain attention on the task, as indicated by longer time to learn the reversal task and the commitment of more errors.

The difference between the two genotypes in the activation of the HPA system could have also contributed to the difference in performance during the reversal task because an increase in cortisol secretion is associated with impaired memory and learning in both humans and animals (de Quervain et al., 1998; Heffelfinger and Newcomer 2001; Erhard et al., 2004). We did not measure cortisol during the present study because we needed to avoid the disturbance of blood sampling, but we have previously shown that nervous sheep have a greater cortisol response to exposure to accumulated stressors than calm sheep (Hawken et al., 2013).

Finally, temperament involves the way in which individuals perceive and react to fear-eliciting events (Boissy and Bouissou 1995; Broom 2001; Carere et al., 2010), so it is logical that a similarly challenging environment is needed to reveal differences between temperament groups in cognitive ability. In the present study, the reverse spatial test fits the classification of ‘challenging’, as evidenced by the ‘nervous’ C/C+C/T genotype sheep emitting more bleats than the ‘calm’ T/T genotype sheep, whereas the two genotypes emitted similar numbers of bleats in the initial spatial discrimination test.

In recent years, polymorphisms in two other genes, BDNF (brain-derived neurotrophic factor) and COMT (catechol-O-methyltransferase), have been associated with cognitive function in humans (Rybakowski et al., 2003; Barnett et al., 2008; Miyajima et al., 2008; de Frias et al., 2010). BDNF is a potent modulator of synaptic transmission and plasticity in the central nervous system (Barnett et al., 2008) and COMT is an enzyme.
involved in the metabolic degradation of released dopamine (Rybakowski et al., 2003). Our study strongly suggests a role for DRD2 polymorphism in reversal learning, but roles for polymorphisms of COMT and BDNF, and possibly other genes, in cognition in sheep still remain to be investigated.

7.7 Conclusion
In sheep, it was technically difficult to measure or demonstrate differences between DRD2 SNP939 genotypes with our simple test of visual discrimination, but our test of reverse spatial learning, a challenging situation, clearly indicated an association between DRD2 SNP939 genotype and cognitive learning ability. Sheep with the DRD2 SNP939 T/T genotype, associated with a ‘calm’ temperament, adapt better to spatial challenges in their environment than sheep with the DRD2 SNP939 C/C+C/T genotype, associated with a ‘nervous’ temperament.
Chapter 8

General Discussion

This thesis supports the general hypothesis that polymorphisms of specific genes are associated with differences in behavioural reactivity and physiological response (cortisol secretion) in sheep of calm and nervous temperament. First, in the UWA Merino temperament flock, for the four candidate genes, I detected five SNPs and identified two of them are associated with temperament. One SNP controls glucocorticoid synthesis (CYP17 SNP628) and has previously been described in South African sheep (Storbeck et al., 2009); the other SNP controls dopamine receptor 2 (DRD2 SNP939). Using a flock of sheep that had not been selected on temperament phenotype, I verified that these two SNPs are actually associated with differences in behavioural reactivity and physiological response. Finally, I estimated the heritability of the reactivity to isolation (one measurement of temperament) based on these two SNPs and looked at the association of DRD2 SNP939 with cognitive learning ability, and was able to further verify that the two SNPs can be used as genetic markers for prediction of temperament in sheep.

Temperament affects reproduction, production and welfare in sheep, so selection for temperament could improve the productivity of the wool and sheep-meat industries. Compared to the current phenotypic selection method, a complex and time-consuming process, a genotypic method is quicker and easier and will therefore save time and labour. Moreover, genotype selection will provide useful contributions to our understanding of animal breeding, welfare, and conservation, for animals in the wild as well as those in production systems. However, before launching into an industry-wide breeding program, some issues need to be clarified. Most importantly, the results of genetic association study can be affected by uncontrolled factors, such as gender, methodology, gene-gene and gene-environment interactions.

Gender has always been seen as an important factor because most studies have shown that gender can affect personality and temperament, and behavioural reactivity to stressors, as shown for humans and rats (Cailhol and Mormede 1999; Costa Jr et al.,
2001; Louvart et al., 2005; Slotten et al., 2006; Else-Quest et al., 2006). Some studies disagree (Desautes et al., 1997; McManis et al., 2001; Weiss et al., 2004) but the general consensus is that gender must be considered. In our breeding program, we retain very few males so we could not include gender in the design. We thus only investigated female sheep and this might explain we failed to find an association between our MAOA SNPs and temperament. Indeed, MAOA activity seems to differ between genders (Murphy et al., 1976): except for one study (Deckert et al., 1999), an association between MAOA polymorphism and behavioural reactivity has been detected in males but not females (Hsu et al., 1996; Manuck et al., 2000; Caspi et al., 2002; Saito et al., 2002; Cohen et al., 2003; Newman et al., 2005; Jin et al., 2006; Nilsson et al., 2006, 2007; Roohi et al., 2009). Gender could also affect the association between CYP17 SNP628 genotypes and behavioural reactivity in the same way, thus explaining the lack of such association in our female sheep and its presence in rams (Hough 2012). Therefore, it is very important to test the effect of gender on the association of the two SNPs with behavioural reactivity in future.

We detected our five SNPs only on basis of the mRNA sequence of the candidate genes. Other regions of each gene, including the promoter region, the 5’- and 3’- untranslated regions, and the introns could also affect the phenotype. For example, the previously mentioned SNP218 (A/C) in Intron 7 and the SNPs in the promoter region of the TPH gene, the -141C insertion/deletion in the promotor of DRD2 gene, the 30 bp repeats polymorphism in the promoter, and a 128-bp allele in Intron 4 of the MAO A gene, have all been found to be associated with substance addiction, aggressiveness, impulsiveness, panic disorder, depression and antisocial behaviours (Nolan et al., 2000; Jonsson et al., 2003; Newman et al., 2005; Liu et al., 2006; Wendland et al., 2006; Hong et al., 2008b; Beaver et al., 2013; Gallardo-Pujol et al., 2013). Our study is a major step towards an understanding of the association of polymorphisms in the candidate genes with the temperament, but polymorphisms in other regions of the same genes now need to be investigated.

In humans, temperament is thought to be an important sub-system of personality (Corr 2009) and is conceptualized in nine broad dimensions: level of activity, approach/withdrawal, intensity, threshold, adaptability, rhythmicity, mood, persistence of attention span, and distractibility (Thomas and Chess 1977). A similar sub-system
could exist in animal personality (Reale et al., 2007; Gosling 2008; Weinstein et al., 2008). Temperament has complex traits that include perception, emotion and behaviours in both humans and animals. With respect to the effect of genes on emotional disorders or behaviours, except for Huntington’s disease which is known to be single-gene effect (Gusella et al., 1983), polygenic effects determine emotional disorders or behaviours, including attention deficit hyperactivity, depression, substance addiction, aggression, impulsiveness and novelty seeking, all of which are included under the temperament ‘umbrella’ (Bah Rosman 2008; Corr 2009).

In this thesis, we used an association study to investigate the four candidate genes that were most likely to be associated with the well-characterised physiological and behavioural differences between the ‘calm’ and ‘nervous’ lines that we have been studying for many years. We found only two SNPs that were associated with the temperament phenotype, so an obvious question is whether there are other genes or gene polymorphisms associated the temperament. In addition to the genes in the HPA axis and central neural system that we have focused on, others have been shown to be involved in personality or temperament (Reif and Lesch 2003), but they have rarely been studied in detail:

1) Noradrenergic genes – dopamineβ-hydroxylase (DBH; responsible for norepinephrine synthesis), adrenergic receptor (ADRA2A) and norepinephrine transporter (NET1), all suggested to play a role in ‘fight or flight’ response (Major et al., 1980);

2) GABAergic genes – the GABA-A receptor gene has been shown to be involved in anxiety disorders (Crestani et al., 1999);

3) Nitric oxide (NO) synthase – the gene has been proposed to be associated with aggressive and sexual behaviour (Nelson et al., 1995);

4) Human transcription factor (AP-2β) has shown to be related to aggression (Damberg et al., 2000).

So these genes remain attractive as candidates for further research. As an approach, it would probably be best to use a genome-wide association study (GWAS) to target the whole genome as a means to identify and investigate more functionally relevant polymorphisms. This is our future goal for improving our knowledge of the specific genes associated with temperament.
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There is an increasing number of studies investigating the relative contributions of genes, environment and other factors to phenotype variance. Gartner (1990) was able to show that some non-genetic variability was due to an epigenetic modification. Epigenetic mechanisms, in which genes are modified by methylation and acetylation without changing the DNA sequence, might also change gene expression in a way that affects temperament phenotype. Evidence of the epigenetic mediated sources of variation in genetically identical organisms can be found in the examples of human twins (Fraga et al., 2005) and in isogenic mice (Morgan et al., 1999; Rakyan et al., 2003). Traditionally, it has been thought that the epigenetic modification is set up during cell differentiation and embryonic morphogenesis before birth, and after birth, during the maturation of the germ line, gametes re-program their epigenetic status by erasing the old and re-establishing a new epigenetic profile (Monk et al., 1987; Oswald et al., 2000; Mayer et al., 2000; Surani 2001; Wong et al., 2005). Therefore, both prenatal and postnatal challenges could induce epigenetic changes (Jablonka and Lamb 1995; Surani 2001; Sutherland and Costa 2003; Waterland and Jirtle 2003). A study by Weaver et al. (2004) indicated that maternal behaviour can induce the epigenetic modification (DNA methylation) of the gene for the cortisol receptor and therefore affect the stress response in the offspring. Other studies have produced evidence that epigenetic regulation of the expression of genes for the key steroidogenic enzymes (e.g. CYP17) can affect steroid hormone biosynthesis and action (Martinez-Arguelles and Papadopoulos 2010). In addition, there are indications that epigenetic modification (DNA methylation) of the DRD2 gene plays a role in the regulation of DRD2 expression (Popendikyte et al., 1999;Frieling et al., 2010) and that such modifications are associated with the psychiatric disorders and behaviours, such as schizophrenia, bipolar disorder, eating disorder (Abdolmaleky et al., 2005, 2008; Frieling et al., 2010). Therefore, we need to explore whether epigenetic modification of the two genes associated with temperament in our sheep, CYP17 and DRD2, is associated with the cortisol response and behavioural reactivity (temperament).

In conclusion, CYP17 SNP628 and DRD2 SNP939 are associated with temperament in sheep. The DRD2 variant is specifically associated with behavioural reactivity and cognitive learning ability, and the CYP17 variant is specifically associated with the physiological cortisol response. These observations lead to the conclusion that the
combination of DRD2 SNP939 C allele and the CYP17 SNP628 A/A genotype could be used as a genetic marker for prediction of nervous temperament, whereas the combination of DRD2 SNP939 T/T and CYP17 SNP628 G/G could be used as a genetic marker for calm temperament. With these two SNPs, calm animals could be selected and potentially be better adapted to intensive conditions whereas nervous animals could be selected as better adapted to an extensive environment. Therefore, it is possible to select sheep for temperament using a genotypic method instead of the complex and time-consuming phenotypic method. Future studies using GWAS are needed to investigate the association of other regions of these candidate genes, or other genes along the HPA axis and central nervous systems, with temperament in sheep so we can better understand the genetic association with temperament.
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