THE ROLE OF CYTOCHROME P450 17α-HYDROXYLASE/17,20-LYASE (CYP17) IN THE STRESS COPING ABILITY OF A DIVERGENTLY SELECTED MERINO SHEEP POPULATION

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SUMMARY

South African Merino sheep were selected divergently from the same base population for their ability to rear multiples. Two distinct populations were formed over a period of more than 20 years of selection. Reproduction (and therefore presumably fitness) in the line selected in the upward direction (H-line) was substantially improved compared to the line selected in the downward direction (L-line). In the present study, it was demonstrated that the H-line was more stress-tolerant than the L-line in terms of their glucose and cortisol response when challenged with insulin. Sheep from the breeding program were genotyped according to one of two cytochrome P450 17α-hydroxylase/17-20 lyase (CYP17) alleles, as these genotypes were previously linked to the ability of Angora goats to cope with external stressors. However, no association was found between CYP17 genotype and selection line. The difference in insulin induced stress response between the H- and the L-line can therefore not be attributed to CYP17 genotype.

INTRODUCTION

Fitness of farm animals (defined as reproduction and survival) has long been identified as being of economic importance. Yet fitness traits have seldom been incorporated in selection programs for livestock (Goddard 2009). These fitness traits can be linked to genotypic markers that can be identified within livestock breeding programmes. These genetic targets can then be recorded and included in selection criteria to ultimately improve livestock fitness. In this study we look at cytochrome P450 17α-hydroxylase/17-20 lyase (CYP17) genotype as a possible genetic target to link to stress coping ability, a fitness trait.

CYP17 plays a critical role in the production of mineralocorticoids, glucocorticoids and androgens by the adrenal cortex in mammals (Vander *et al.* 2001). These steroid hormones are involved in fitness, since they play a vital role in the control of water and mineral balance, stress management and reproduction, respectively. CYP17 catalyses two distinct reactions, namely: a 17α-hydroxylation and a C17-C20 lyase reaction (Nakajin *et al.* 1981). This dual enzymatic activity places CYP17 at key branch points in the biosynthesis of adrenal steroid hormones.

Cortisol and corticosterone are the glucocorticoid hormones produced in the adrenal gland, which play an important role in stress management (Vander *et al.* 2001). As in humans, cortisol is by far the main glucocorticoid in sheep that counters a stress stimulus. Cortisol production is stimulated when the adrenal gland receives a "stress-signal" through the hypothalamus-pituitary-adrenal axis, via adrenocorticotropic hormone. The decreased ability of an animal to produce cortisol will lead to a reduced ability to counteract stress associated with the environment. Such an example was observed by Engelbrecht and Swart (2000) in Angora goats. These animals had a decreased ability to produce cortisol compared to Merino sheep and Boer goats, and accordingly exhibited a reduced ability to cope with insulin-induced stress.

Stress has been shown to reduce fitness, as reflected by growth, reproduction and survival of farm animals. Divergent selection for number of lambs weaned in Merino sheep, an example of a composite fitness trait, has resulted in marked differences in responses between the lines in this

trait (Cloete *et al*. 2004). Differences between the lines in survival of lambs and behavioural adaptations conducive to lamb survival were also observed (Cloete and Scholtz 1998).

Two CYP17 alleles have previously been identified in Merino sheep (Genbank accession no. L40335/WT1 and AF251388/WT2) and confirmed by Storbeck *et al.* (2008). However, it has not been established in this species whether a specific CYP17 genotype would enhance cortisol production, and thus stress coping ability, relative to the other. In this study, we investigated whether the observed divergence in fitness (as reflected by number of lambs weaned) observed in a Merino selection experiment can be related to the genotypic composition of ovine CYP17.

MATERIALS AND METHODS

Breeding program. A Merino sheep breeding program has been undertaken since 1986 in which sheep have been divergently selected for their ability to rear multiples (alternatively defined as number of lambs weaned per mating). The selection lines were derived from the same base population and selection within each line based on maternal ranking values for number of lambs weaned per lambing opportunity (Cloete *et al*. 2004). Number of lambs weaned per mating in the line selected in the upward direction (H-line) has been proved to be near to double that of the line selected in the downward direction (L-line) (Cloete *et al.* 2004).

Stress test. Stress coping ability was tested on 24 rams from this breeding program (13 H-line and 11 L-line sheep), housed at the Elsenburg Research farm near Stellenbosch, South Africa. These rams were injected intravenously with human insulin (Actrapid® HM, Novo Nordisk, Johannesburg, South Africa) after which 6 blood samples of each animal were collected over a 2 hour period and placed on ice. Blood samples were centrifuged at 2 500x*g* for 10 minutes (4^{o}C) to acquire representative plasma samples from each animal. Plasma glucose and cortisol levels were determined by PathCare Reference Laboratory (PathCare Park, N1 City, Goodwood, Cape Town, South Africa). Ethics approval for this stress test was obtained from the Departmental Ethics Committee for Research on Animals (DECRA reference R08/21).

Genomic DNA isolation. Blood samples of both H- (n=105) and L-line (n=31) sheep were collected in EDTA treated collection tubes (BD Vacutainer® Blood Collection Tubes; Pronto™ Quick Release Holder and Eclipse™ Blood Collection Needles). Blood samples were also acquired from the heart chamber of 36 lambs that had died during the 2008 lambing season. Genomic DNA was isolated using the Wizard® Genomic DNA isolation kit (Promega, Madison, Wisconsin) according to the instructions provided by the manufacturer.

CYP17 genotyping with real time polymerase chain reaction (RT-PCR). All 172 sheep were genotyped using the RT-PCR method developed by Storbeck *et al*. (2008). The primers and hybridisation probes (TibMolBio, Berlin, Germany) were as follows: LCLP, 5'-CCTGAAGGCCATACAAA-3'; LCRP, 5'-GGATACTGTCAGGGTGTG-3'; fluorescein-labelled CYP17 sensor probe, 5'-TTCTGAGCAAGGAAATTCTGTTAGA-FL; LC640-labelled CYP17 anchor probe, 640-TATTCCCTGCGCTGAAGGTGAGGA-3'. RT-PCR was carried out using a LightCycler® 1.5 instrument. Amplification reactions (20µl) contained 2 µl LightCycler® FastStart DNA Master HybProbe Master Mix (Roche Applied Science, Mannheim, Germany), 3 mM $MgCl_2$, 0.5 µM of each CYP17 primer, 0.2 µM fluorescein-labelled CYP17 sensor probe, 0.2 µM LC640-labelled CYP17 anchor probe and 10 to 100 ng genomic DNA. Following an initial denaturation at 95°C for 10 min to activate the FastStart *Taq* DNA polymerase, the 35-cycle amplification profile consisted of heating to 95°C with a 8 s hold, cooling to 52°C with a 8 s hold and heating to 72°C with a 10 s hold. The transition rate between all steps was 20°C/second. Data

were acquired in single mode during the 52°C phase using the LightCycler® software (version 3.5). Following amplification, melting-curve analysis was performed as follows: denaturation at 95°C with a 20 s hold, cooling to 40°C with a 20 s hold and heating at 0.2°C/s to 85°C with continuous data acquisition.

The sensor probe was designed to be a perfect match for the WT1 sequence and dissociated at 58°C when bound to the perfectly matched WT1 sequence. However, when bound to the mismatched sequence (WT2) dissociation occurred at 54°C. A no-template control (negative control) was also included in each assay.

Statistical analysis. For the stress test, plasma glucose and cortisol response over time for the H- and L-lines were analyzed with a regular two-way ANOVA with selection line and the period that passed since the insulin injection as factors. Differences between breed lines at specific time points were examined with Bonferonni's post-test (glucose: 95% confidence; cortisol: 94% confidence). The Chi-square test was used to analyze CYP17 genotype frequencies. GraphPad Prism (version 4) software (GraphPad Software, San Diego, California) was used for all statistical analysis.

RESULTS AND DISCUSSION

Stress test. Plasma glucose (mmol/L; which served to monitor the progress of the stress response) and plasma cortisol (log nmol/L) responses to insulin-induced stress are depicted in Figure 1. The H-line reached a hypoglycaemic state earlier than the L-line, with glucose levels of 1.9 mmol/L 30 min post insulin challenge, and recovered to baseline glucose (3.7 mmol/L) 2 hours post insulin challenge (3.3 mmol/L). Cortisol levels increased rapidly from 30 min post insulin challenge (60.3 log nmol/L), reached maximum at 60 min (120.2 log nmol/L) and returned to baseline concentrations after 2 hours. The stress response of H-line animals was completed after 2 hours with both glucose and cortisol concentrations having recovered to baseline levels.

The L-line reached maximum hypoglycaemic state at 60 min post insulin challenge with glucose levels of 2.1 mmol/L, but did not recover to baseline concentrations (3.6 mmol/L) by 2 hours post insulin challenge (2.7 mmol/L). L-line cortisol levels increased from 30 min post insulin challenge (53.3 log nmol/L), but at an apparently slower rate than the H-line. Maximum cortisol in the L-line was observed 90 min post insulin challenge (100.5 log nmol/L).

The interaction between breed line and time of measurement was highly significant ($P<0.0001$) for glucose ($F=9.22$, dfn=5, dfd=132), but not for cortisol ($P=0.3028$, $F=1.22$, dfn=5, dfd=132) responses to insulin-induced stress.

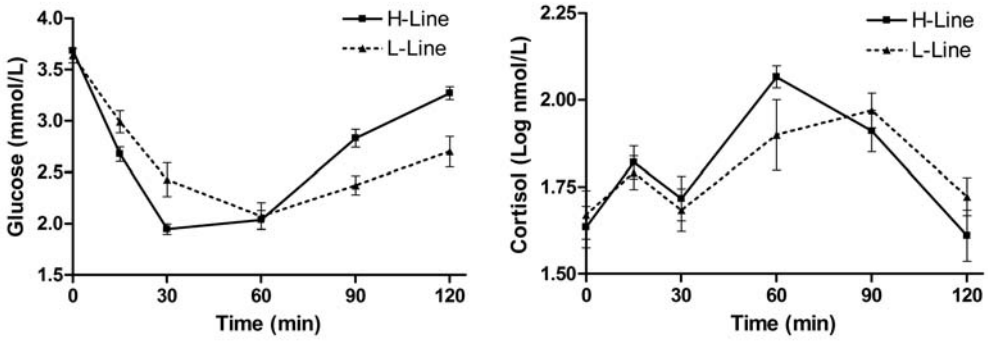

Figure 1. Merino sheep plasma glucose and cortisol response to insulin challenge.

Glucose levels were lower in the H-line at 30 minutes post insulin challenge (P<0.01), the opposite trend being observed after 90 (P<0.01) and 120 min (P<0.001). Cortisol levels were higher (P=0.0590) in the H-line 60 min post insulin challenge. The H-line animals thus had an improved ability to cope with insulin induced hypoglycaemia than the L-line, as reflected by their quicker glucose recovery to baseline and earlier peaking of cortisol at 60 min. An improved cortisol collection and detection method might limit variation in future tests.

CYP17 genotyping. Interestingly, no homozygous WT2 sheep were detected in either the H- or L-lines or among the lambs that died in 2008. Relative DNA copy number determination of sheep CYP17 has previously been done (Storbeck *et al.* 2008), indicating that the two CYP17 genetic sequences are two alleles of one gene. This finding thus warrants further investigation.

Table 1 summarizes the genotyping results obtained for the H-, L-line and lamb mortalities. There was no significant association (P=0.7617, Chi-square=0.5444, df=2) between CYP17 genotypes and designation of sample population (H-, L-line or lamb mortalities). On average 83.4 % sheep in the breeding program was heterozygous, while 16.6 % were homozygous WT1.

Table 1. Frequency distribution of CYP17 genotype in the Merino sheep breeding program

Merino flock	Homozygous WT1		Heterozygous WT1/WT2		Homozygous WT2	
	Number of sheep	Percentage	Number of sheep	Percentage	Number of sheep	Percentage
H-line	15	14.3	90	85.7	0	0
L-line	5	16.1	26	83.9	0	0
Lamb mortalities	7	19.4	29	80.6	0	0

CONCLUSIONS

The divergent breeding program was shown to result in differences in insulin-induced stress coping ability, the H-line having a higher stress tolerance than the L-line. This difference in stress tolerance could not, however, be ascribed to CYP17 genotype, since there was no association between CYP17 genotype and selection lines. One CYP17 isoform is not more advantageous for cortisol production in the adrenal gland than the other. This study rules out CYP17 as possible genotypic marker to use during selection, and suggest investigating other factors along the HPA axis or adrenal steroidogenesis that could be implicated in the stress response difference observed.

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