IDENTIFICATION OF QUANTITATIVE TRAIT LOCI AFFECTING WET CARCASS SYNDROME IN SHEEP USING HIGH DENSITY SNP GENOTYPES

by

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DECLARATION

I, Lené van der Westhuizen, declare that this thesis that I herewith submit for the degree, Doctor of Philosophy with specialisation in Animal Breeding at the University of the Free State is my own work and that I have not previously submitted it for a qualification at another institution of higher education.

I furthermore, cede copyright of the thesis in favour of the University of the Free State.

Dated at _____on this_____day of _____2018.

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Me Lené van der Westhuizen

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The Animal Ethics Committee of the University of the Free State (UFS), Faculty of Natural and Agricultural Sciences approved the project and procedures for sampling of biological material, and assigned the approval number UFS-HSD2017/1495.

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CHAPTER 1

INTRODUCTION TO WET CARCASS SYNDROME

1.1 BACKGROUND INFORMATION ON WET CARCASS SYNDROME

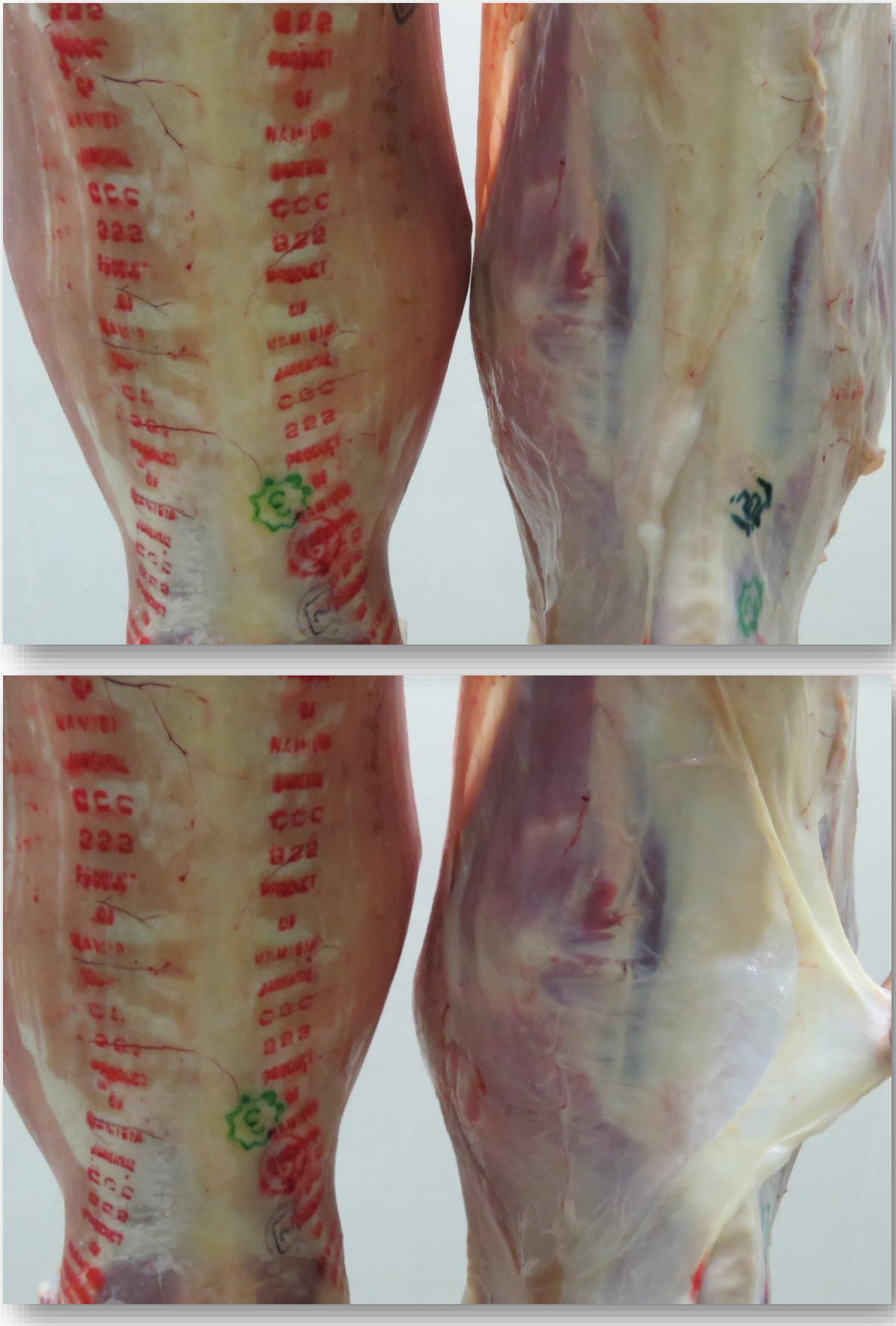
1.1.1 History

Wet carcass syndrome (WCS) was identified in both ovine and bovine species and both are histologically similar (Steven, 1984 – unpublished; Brock, 1984 – unpublished). However, its frequency in bovines has been substantially less than in ovines (Jansen, 1991). Thus, WCS is a condition predominantly found in sheep and which negatively affects the quality of their carcasses. While WCS has been recognized and has been the subject of research since 1981 (Brock *et al*., 1983; Hattingh *et al*., 1983), its etiology remains undetermined. The first incidence of WCS was recorded in January of 1981 at Chambor abattoir in Krugersdorp, Gauteng, South Africa (Jansen, 1991). Since the first incident, difficulties in terms of diagnoses and identification of the condition have been observed. It was initially referred to as oedema (Vleisraadverslag, 1982 – unpublished; Van der Veen, 1986 – unpublished) and subsequently as cachexia or emaciation (Vleisraadverslag, 1982 – unpublished; Meyer, 1985 – unpublished; Van der Veen, 1986 – unpublished). Hattingh *et al*. (1983) called it subclinical oedema and Newsholme (1982 – unpublished) called it hydrosis. It later became known as wet sheep carcass (Lloyd – undated and unpublished) or wet sheep syndrome. The condition was thereafter observed in beef carcasses, hence the name changed from wet sheep syndrome to wet carcass syndrome *circa* 1986. Personal communication by the author with abattoir management indicates the seriousness of the condition to communities in the Northern Cape. However, farmers in other provinces including the Western Cape, Eastern Cape, Free State and Mpumalanga claim to have never heard of this phenomenon.

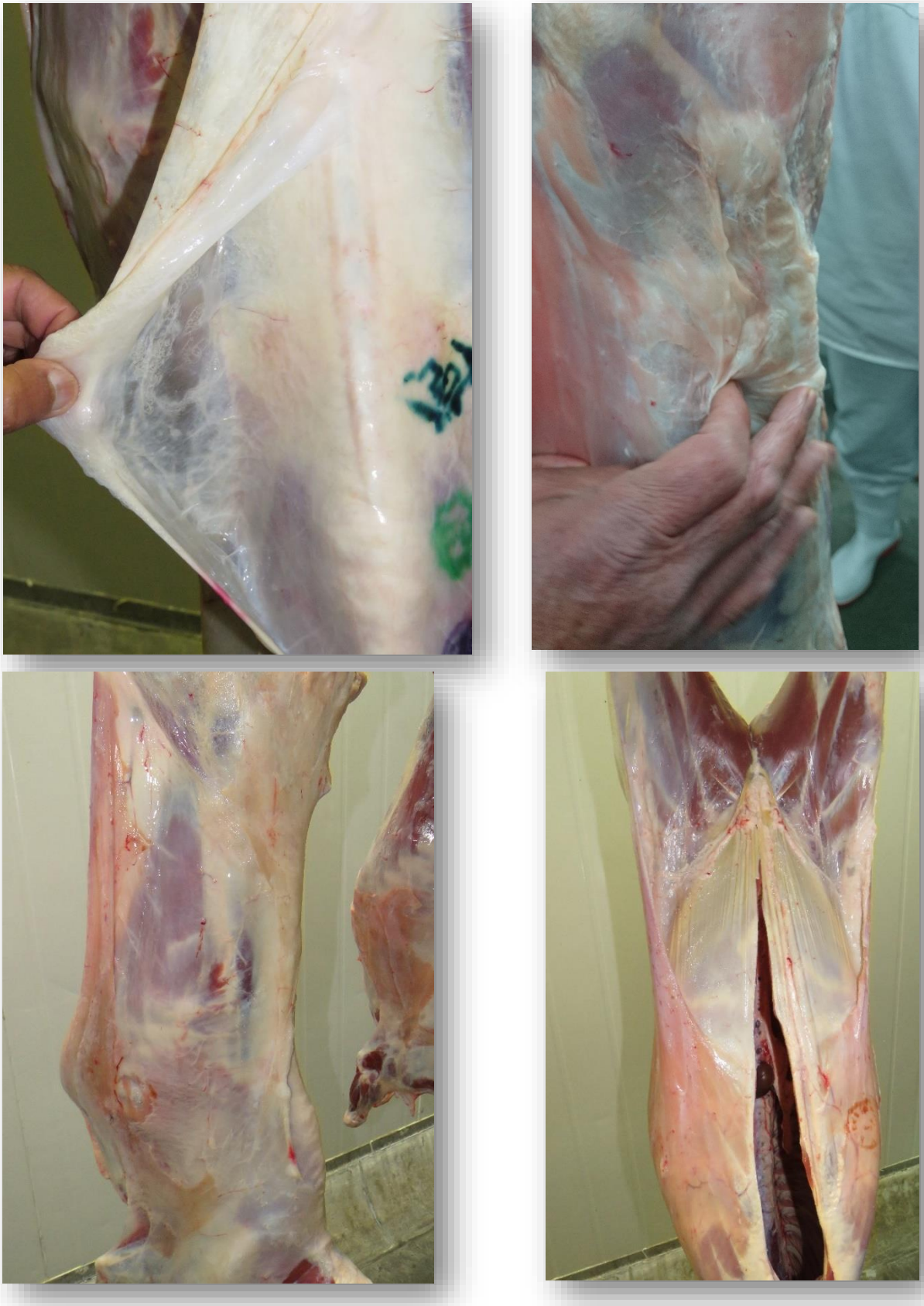
1.1.2 Phenotypic Characterization

During the pre-slaughter period, the animal appears clinically normal, showing no symptoms of abnormality (Brock *et al*., 1983; Hattingh *et al*., 1983). However, post-slaughter when the skin is removed the carcass appears to be "wet" (Hattingh *et al*., 1983). The condition is described phenotypically as a subcutaneous accumulation of watery fluid (Brock *et al*., 1983). The areas on the carcass most affected are the brisket, flanks, hindquarters, sides, and back (Hattingh *et al*., 1983; Brock *et al*., 1983). The watery fluid is also found in the intramuscular connective tissue layers of both the flank and subscapular area. Afflicted carcasses do not dry off with overnight cooling (Joubert *et al*., 1985). Visual representations of WCS afflicted carcasses are shown in Figure 1.1. Figure 1.1.a demonstrates the comparison between a normal- (left) and WCS carcass (right), while Figures 1.1.b, 1.1.c, and 1.1.d illustrate the elastic and soft fat layer. Figures 1.1.e and 1.1.f illustrate the typical shiny and "wet" look of an afflicted carcass. Jansen (1991) described different levels of "wetness" by which severity of WCS can be classified.

WCS results in lamb carcasses that are predominantly deemed to be unacceptable by the end consumer both in appearance and due to reduced shelf life (Brock *et al*., 1983; Joubert *et al*., 1985). The most probable explanation for the reduced shelf life is the surface of the meat being a favourable environment for the growth of microorganisms (Jansen, 1991). In addition, there is an occupational hazard associated with cutting wet carcasses in that a band saw pulls more on the meat (especially the flank) which may result in injury to the operator (Jansen, 1991). These observations further illustrate how potentially detrimental WCS is to the sheep meat industry in South Africa.



Figures 1.1 a and b A comparison between a normal and a wet carcass syndrome carcass (adipose tissue can be pulled away from the carcass).



Figures 1.1 c, d, e, and f Illustrate the shiny and wet look, typically seen in an afflicted wet carcass.

1.1.3 Geographical Distribution

The Northern Cape Province in South Africa and the southern part of Namibia (Kalahari dunes and sandy veld) have been identified as geographic regions where WCS occurs most frequently (Brock *et al*., 1983). These regions are characterized as being extremely arid relative to other regions of South Africa (AGIS, 2007). Extensive production of small stock (sheep and goats) is the primary livestock industry in these areas (Cloete and Olivier, 2010).

Unofficial slaughter statistics from WCS afflicted areas (Figure 1.2) reveal that certain abattoirs have a higher prevalence of WCS sheep, whereas other abattoirs in the same region will have no recorded incidences. Abattoirs often have specific geographical areas from which they receive sheep. Consequently, specific areas could be identified through abattoirs as WCS affected areas. It should be stated, however, that accurate information regarding the incidence rate relating to the number of cases etc. is not readily available.

1.1.4 Economic Impact

Lamb producers are very concerned about this condition and are, therefore, actively participating in research to find solutions or to identify management procedures to alleviate their economic losses which may collectively rise to 10's of millions of Rand annually. Carcasses that exhibit WCS are generally rejected at the abattoir, and thereafter are not sold for human consumption. Although the incidence of WCS varies from 0.2 to 1.5 percent (%) annually, the economic losses can be significant for individual breeders (Webb and Van Niekerk, 2011). Abattoirs do not pay the farmer that consigned the sheep for those that are afflicted. If wet carcasses are delivered to a supermarket, they will again be rejected. It is possible that the entire truckload of carcasses may be rejected (Webb and Van Niekerk, 2011). Between 1981 and 1985, approximately 60 000 to 90 000 carcasses were rejected due to WCS (Brock *et al*., 1983; Vleisraadverslag, 1985, – unpublished). Taking carcass prices and inflation into account, the Rand values lost accounted to a minimum of R45 696 774. During 2010 alone an estimated R27 010 387 was lost due to WCS (Webb and Van Niekerk, 2011; Le Roux, 2012).

Figure 1.2 A map illustrating the geographical distribution of wet carcass syndrome based on historical and current data.

CHAPTER 2

LITERATURE REVIEW

2.1 PAST RESEARCH ON WET CARCASS SYNDROME

2.1.1 Physiological Characteristics

Hattingh *et al*. (1983) proposed that subclinical oedema, reflected as an increase in the volume of the free fluid in the interstitial space, predisposed sheep to affliction with WCS. This increase in the free fluid may be due to factors including the increase in mean capillary pressure, the decrease of plasma protein concentration, the increase of resistance to lymph flow, and changes to the capillary permeability (Hattingh *et al*., 1983). They compared various properties of the plasma- and interstitial fluids between normal carcasses and those presenting with WCS including (i) sodium-, potassium-, and chloride concentrations; (ii) osmolarities; (iii) albumin and globulin ratios (A:G); (iv) total protein concentration; and (v) colloid osmotic pressure. No evidence of right-sided heart failure caused by an increase in capillary pressure was found and, as a result increased venous hydrostatic pressure was ruled out as a causative factor (Hattingh *et al*., 1983). There was also no evidence of lymph obstruction in the afflicted carcasses, and thus the increase in free fluid could not have been caused by reduced drainage. Hattingh *et al*. (1983) also observed that the interstitial space contained a mixture of free fluid and gel. The free fluid was present in small volumes and the largest part of the tissue fluids was in the gel phase. Carcasses that were afflicted by WCS had low interstitial protein concentrations, normal colloid osmotic pressure, normal plasma protein concentrations, increased A:G ratios as well as increased plasma- and interstitial potassium concentrations when compared to unafflicted carcasses (Hattingh *et al*., 1983). The ratio of A:G suggested an increase in capillary permeability to albumin. The authors concluded that a combination of raised mean capillary pressure and the selective increase in permeability of the capillary blood vessels would result in WCS. Furthermore, they also indicated that histamine or histamine-like substances will cause the above-mentioned symptoms and that a possible mild allergic process may also occur.

Brock *et al*. (1983) observed results similar to those of Hattingh *et al*. (1983), especially in terms of total protein concentration and higher albumin concentrations. Brock *et al*. (1983) also studied the electrolyte-, Vitamin A- and Vitamin E levels and concluded that the vitamin A levels were normal, but identified significantly increased levels of vitamin E in WCS afflicted carcasses. Brock *et al.* (1983) ruled out bacterial- or viral microorganisms, macroscopic pathological lesions, cachexia, inflammatory processes, cardiac failure, glycogen storage, lymphoedema, and myxoedema as probable causes contributing to WCS.

Jansen (1991) investigated properties of meat, mineral and protein concentrations of the organs, blood serum, and interstitial fluid from lambs afflicted by WCS. The muscles, *M. Longissimus* and *M. adductor*, of WCS afflicted lambs extruded more moisture than muscles from unafflicted carcasses. The excess fluid was found, not only in the subcutaneous- and intramuscular tissue, but also in the muscle itself. Carcasses rejected for WCS also have been observed to lose vast amounts of moisture in the refrigerator (Sawcsuk *et al*., 1986). Therefore, the lamb grades with low fat content are more predisposed to WCS carcasses (Webb and Van Niekerk, 2011), may be due to poor water holding capacity. However, both carcasses with a very low fat content and poor conformation (A0 / A1, according to South African standards) and carcasses with a grade of SuperLamb and Grade1 (according to the previous carcass grading system) have also exhibited the WCS phenotype. Therefore, the fat content of the carcass as potentially reflecting pre-harvest nutritional levels appears not to be implicated in predisposition to WCS (Brock *et al*., 1983). When freeze drying results were compared between unafflicted and afflicted carcasses, meat from the afflicted WCS indicated a greater loss of moisture i.e. low water holding capacity (WHC) (Jansen, 1991). Carcasses that were afflicted with WCS also had reduced pH compared to their unafflicted counterparts (Jansen, 1991). The rate in which the pH declines and the ultimate pH value will greatly affect meat quality characteristics such as meat colour, meat tenderness, WHC, shelf-life and carcass yields (Wierbicki and Deatherage, 1958; Bray *et al*., 1992; Gispert *et al*., 2000; Gardner *et al*., 2005; Simela, 2005). However, no significant differences were found between the colour of muscles and the meat when it was cooked between WCS afflicted and normal animals (Jansen, 1991). Urea content was less in WCS afflicted carcasses than in normal carcasses. Conversely, the plasma protein and plasma glucose concentrations were greater in carcasses that were afflicted by WCS than in normal carcasses. Greater levels of phosphorus, potassium, calcium, magnesium, sodium, and copper were found in carcasses that exhibited WCS than in normal carcasses (Jansen, 1991). However, no significant differences were observed in mineral content

of kidney tissue between the WCS- and normal carcasses. Despite comprehensive comparison of carcasses that exhibited WCS and normal appearing contemporary carcasses, Jansen (1991) found the results less than conclusive in establishing a physiological basis for WCS. However, the results did indicate significant differences between WCS- and normal carcasses in urea concentration, plasma protein, and plasma glucose. The reduced plasma glucose and urea concentrations may be interpreted to suggest nutritional stress in WCS afflicted lambs; while the increased plasma protein may reflect tissue mobilization. However, histological studies using both light microscopy and electron microscopy revealed no structural differences when the muscles of WCS- and normal carcasses were compared (Jansen, 1991).

2.1.2 Putative Environmental Causes

Jansen and Pretorius (1986) isolated areas where the manifestation of WCS was apparent in South Africa. They concluded that a clear relationship existed between WCS, pasture condition and pasture composition. It was reported that the presence of lick blocks in the holding pens at the abattoir caused a dramatic marked increase (from seven to 270 carcasses) relative to the prevalence of wet carcasses in both sheep and cattle (Jansen, 1991). This increase was also observed when a farmer divided one flock into two groups and each group was sent to a different abattoir. One group was given lick block in the holding pens (Group A) and the other group was given teff hay on arrival (Group B). Twelve percent of the sheep in Group A had wet carcasses while no sheep in group B were similarly afflicted (Joubert *et al*., 1985). Burroughs (1985 – unpublished) reported that the incidence rate of WCS would drastically decrease when lucerne was given instead of 'lick blocks'.

Another outbreak of WCS occurred at the Beaufort West abattoir in 1984 where two different groups were slaughtered. It was suspected that the over-hydration subsequent to dehydration was due to salt intake which caused the high incidence rate of wet carcasses (Joubert *et al*., 1985). Joubert *et al*. (1985) then tested the theory of water deprivation subsequent to over-hydration. A trial was conducted with three groups of sheep: 1) a control, 2) animals deprived of water for 52 hours with lick was provided 18 hours pre-slaughter, and 3) animals deprived of water for 25 hours with lick provided 18 hours pre-slaughter. Post-slaughter, the carcasses were classified according to their degree of wetness. The imposed dehydration resulted in the volume of the free fluid within the interstitial space to decrease. Subsequently excessive intake

of water led to over-hydration and a abnormal amount of free fluid accumulated within the interstitial space (Joubert *et al*., 1985). Thus, WCS was attributed to dehydration subsequent to over-hydration with high salt content feeds exacerbating the condition. However, Jansen and Pretorius (1988) subsequently found no association of salt intake, types of feeding, transport or dehydration followed by over-hydration with WCS. Abattoir-related conditions including washing of carcasses under high pressure, transport stress, feed in holding pens and defective cooling systems also had no effect on the occurrence of WCS (Brock *et al*., 1983).

2.1.3 Superficial Causes

Due to WCS being speculatively associated with over-hydration of thirsty sheep on arrival at abattoirs, Jansen (1991) completed several trials in an attempt to stimulate WCS in lambs. In general, water was withheld from sheep 24 hours before transport as well as during transport. However, pasture and/or feed were freely available to the animals during the period that water was withheld. Thus, the period in which water was not available to the sheep was approximately 48 hours and feed was unavailable approximately 24 hours pre-slaughter. Trial one tested the dehydration-over-hydration theory and no wet carcasses were observed. Trial two was similar to Trial one except the sheep were slaughtered at different times after re-hydration. Only a few carcasses were identified with WCS with the largest number of afflicted carcasses observed at 48 hours after rehydration with the second largest number occurring at 72 hours after rehydration. Trial three utilised a large number of animals (81 510 animals) that was slaughtered during February 1986 at a commercial abattoir (City Deep, Johannesburg) (Jansen, 1991). As animals arrived at the abattoir, they were subjected to one of three treatments, i) no water and no feed; ii) both water and feed; and iii) water but no feed. Results from this large Trial were inconclusive. Trial four described the effect of different types of feed provided to the sheep as well as the effect that pasture stress has due to poor natural pasture conditions, and again no carcasses were identified as being afflicted with WCS. Interestingly, Terrill (1968) reported that the amount of water intake was influenced by the quality of feed consumed. Thus, feed such as lucerne with high a protein concentration may increase water intake. However, Jansen (1991) observed that when lucerne hay was provided to the animals the incidence rate of WCS decreased. Trial five was similar to Trial four, except that some of the sheep were force-fed seven litres of water. This ensured that these animals were over-hydrated. Once again, the results did not support the theory of dehydration and subsequent

over-hydration as causing WCS. Additional Trials which investigated lick intake, transport stress, and Fusarium toxicity also yielded no causal mechanism for WCS (Jansen, 1991).

Industry partners (Meat Board) in South Africa have previously provided funds to investigate possible causative factors of WCS. This research aimed to mimic the nutritional factors which were believed to result in wet carcasses (Le Roux, 2012). As with all other trials conducted during the 1980's (see Jansen, 1991), the trial from Le Roux (2012) was unsuccessful in inducing the condition. The latter author recommended that time should be spent at the abattoirs obtaining data, indicating the history and origins of WCS, and taking samples from WCS carcasses for a histological examination. Instead of working towards the mimicking of conditions leading to WCS, a retrospective approach was recommended. This would entail tracing information from WCS afflicted carcasses backward towards the environmental conditions to which the live animal was exposed. However, this approach may only be fruitful if WCS is a reaction to environmental stimuli (Le Roux, 2012). Similarly to Jansen and Pretorius (1986), Le Roux (2012) proposed that in geographical areas where a high prevalence of WCS existed, an abundance of forage was available, but it was of poor quality. Furthermore, an increased incidence of WCS was observed when high rainfall was experienced during spring but was followed by poor rainfall in summer with consequent effects on body condition (Le Roux, 2012). Le Roux (2012) experimentally simulated the nutritional level of veld after a poor rainfall season in the Upington area, Northern Cape Province, South Africa. A low quality diet of lucerne:wheat straw (30:70) was provided to the animals to cause nutritional stress and a control group received just lucerne throughout the trial. Both treatment and control groups were tested simultaneously and therefore exposed to the same other environmental conditions. No difference in the incidence of WCS was observed. Thus, these results suggested that nutritional stress alone could not cause WCS. In addition, the theory of Joubert *et al*. (1985), that dehydration and over-hydration (before and after transport) also did not cause WCS was confirmed.

2.1.4 Farming Management and Systems

It has also been reported that the incidence of WCS decreases when the animals are handled more frequently at farm level (Jansen and Pretorius, 1986). Wet carcass syndrome is generally limited to geographical areas where extensive sheep production is practiced (Jansen

and Pretorius, 1988). The latter authors observed that two farms situated in the same area may have very different incidence rates of WCS. Jansen (1991), and Jansen and Pretorius (1986) stated that commercial breeders that have little contact with their sheep experience a greater incidence rate of WCS when compared to seed stock breeders that implement intensive production systems and management practices. Of course, better management may also lead to sheep being less stressed in their interactions with humans (Jansen and Pretorius, 1986). This finding, together with the occasionally observed relationship to various nutritional stressors, leads to the speculation that practices that induce stress may play an important role in triggering WCS (Jansen and Pretorius, 1986; Jansen, 1991). Thus, the authors suggested that a combination of factors caused WCS. They contended that the condition is similar to that of dark-, firm- and dry (DFD) meat found in beef, where a combination of factors is the probable cause. It should also be noted that pale-, soft and exudative (PSE) pork results from the coincidence of a genetic predisposition and stress (Hall *et al*., 1980; MacLennan and Phillips, 1992).

2.2 A GENETIC RATIONALE FOR WET CARCASS SYNDROME

2.2.1 Characterization of the Dorper Breed

The Dorper is the most numerous breed in the studied areas. The northwestern part of South Africa experiences low rainfall, limited natural resources, consequently, has a low production potential. Thus, it was necessary to develop a sheep breed suitable for these arid grazing conditions (Bonsma, 1944). It was thought that characteristics of such a breed should include: 1) good carcass characteristics; 2) ability to lamb in autumn; 3) production of lambs that would be slaughter-ready at four to five months of age when raised under veld conditions; 4) being adapted to extreme weather conditions including low and high ambient temperature and radiation; 5) ability to utilize low quality grass and shrub veld; 6) reproductively efficient and early maturing; 7) easy handling and without lambing or shearing problems (due to animals having predominantly hairy coats and not wool) and; 8) appropriate pigmentation (Nel, 1993; Coetzer *et al*., 1995; Milne, 2000). Its development started in the 1930's at Grootfontein Agricultural College as a 50/50 composite of Black Head Persian and Dorset Horn (Nel, 1993; Milne, 2000). These breeds were chosen due to the adaptation of the Black Head Persian to harsh environmental conditions and the extended breeding season of the Dorset Horn (Milne,

2000). At present, two types of Dorper sheep exist, i.e. Blackhead Dorper and White Dorper. Genotypically the breeds are similar, but their heads differ in colour and pigmentation. Selection of the Dorper favours a hairy coat but some animals have fleeces that are made up of wool and hair fibres (Cloete *et al*., 2000), medium sized body frame (Cloete and De Villiers, 1987; Cloete *et al*., 2000; De Waal and Combrinck, 2000; Milne, 2000) and speculation concerning unselective grazing (Brand, 2000). Carcass characteristics of the Dorper are ideal for South African carcass classification systems (Cloete *et al*., 2000). The average birth weight of Dorper lambs is 4.4 kilogram (kg) while the average weaning weight can be 29.6 kg at 100 days (Schoeman, 2000). During 2003, the Dorper was one of the most numerous sheep breeds in South Africa, with a count of more than seven million (Snowder and Duckett, 2003). Dorper sheep are also found in United States, Israel, Kenya, Mauritius, Malawi, Saudi Arabia, Zambia and Zimbabwe, among other countries (Elias *et al*., 1985; Nel, 1993).

Wet carcass syndrome is predominantly observed in hairy-type Dorper sheep and crosses of Dorper with indigenous and locally developed breeds of South Africa and Namibia (Brock *et al*., 1983; Webb and Van Niekerk, 2011). The author could not find any literature or evidence on WCS occurring outside of this area. The apparent manifestation of breed differences in the incidence of WCS provides an initial motivation to propose a genetic basis for the condition. However, to date, there have been no studies in which a genetic basis for WCS has been investigated.

2.2.2 Pre-slaughter Stress and Effects on Meat Quality

The physiological and behavioural response animals have to short-term stressors are mediated through the production- and release of catecholamines, such epinephrine and norepinephrine (Mellor and Stafford, 2000). However, the Hypothalamic-pituitary-adrenal (HPA) axis is activated as a response to long-term stress (Mellor and Stafford, 2000). Neuroendocrine hormones such as corticotropin-releasing hormone (CRH) (released by the hypothalamus), adrenocorticotropic hormone (ACTH) (produced by the pituitary gland) and glucocorticoids (produced and released by the adrenal cortex) is released by the HPA axis. Furthermore, cortisol is a glucocorticoid steroid hormone that is produced as an end product to a stress reaction (Adams *et al*., 1999; Mellor and Stafford, 2000; Tsigos and Chrousos, 2002). Long-term stress experienced by animals in production systems can lead to the continues dysfunction

of the HPA axis, and in return causes adverse physiological effects (Adams *et al*., 1999; Mellor and Stafford, 2000; Narayan and Parisella, 2017).

The influences of pre-slaughter handling have been well documented (Moss, 1980; Shorthose, 1978; Warriss *et al*., 1990; Gardner *et al*., 1999). Carcass and meat quality is rigorously influenced 1 to 48 h before slaughter. In this time, loading and transportation to the abattoirs occurs and thereafter slaughtering of the animals. Live weight loss, carcass weight loss, carcass yield and carcass quality are affected pre-slaughter. At abattoirs, tissue dehydration (Rousel, 1990; Degen & Kam, 1992) due to pre-slaughter stress which is related to the enforced feed and water withdrawal (Warriss *et al*., 1987) will further increase the adrenocortical response to stress (Matthews & Parrot, 1991). Undesirable effects on sheep meat quality due to pre-slaughter transport and stressors such as restraint or isolation have been observed (Warriss *et al*., 1987; Apple *et al*., 1993). However, Warriss *et al*. (1987) suggested that sheep is less susceptible to stress than pigs and cattle. Moreover, body fluids from tissue plays a role when weight loss occurs pre-slaughter in both cattle and sheep (Gortel *et al*., 1992; Cole 1995). Water withdrawal also results in both body mass and carcass losses (Degen & Kam, 1992) with the interstitial space playing a large role for these losses (Gortel *et al*., 1992). In addition, lamb meat quality is severely affected by the rate of reduction of the muscle pH post-slaughter, where the rate of reduction effects the time of rigor inception and the incidence of cold-shortening (Chrystall & Devine, 1985). The final pH of meat is generally described as the most frequent indicator of meat quality (Newton & Gill, 1980-1981; Tarrant, 1981).

2.2.3 Comparative Genetics

Two conditions that are phenotypically similar to WCS have been observed in pork, which arises from mutations at single loci. One of the genetic defects is porcine stress syndrome (PSS) which results in pale, soft and exudative (PSE) meat (Ludvigsen, 1957; Wismer-Pedersen, 1959). The second is reddish, soft and exudative meat (RSE) (Le Roy *et al*., 1990). Meat with visible characteristics of being pale, soft and exudative (PSE) are undesirable and unattractive to the consumer. Ludvigsen (1957) and Wismer-Pedersen (1959) were the first to describe characteristics in meat similar to PSE. Cassens *et al*. (1975) reported that selecting swine for leaner and heavier muscles resulted in some animals having greater susceptibility to stress and meat that is of poor of quality. With RSE meat in pork, high levels of glycogen are found in

the muscle, a low protein concentration and a higher than normal degree of protein denaturation (Estrade *et al*., 1993; Lundström *et al*., 1996; Enfält *et al*., 1997).

2.2.3.1 RYR1 Gene

High susceptibility to stress in swine is today referred to as porcine stress syndrome (PSS). Responses to stress, specifically in swine, include: (i) an increase in aerobic and anaerobic metabolism; (ii) increase in heat, carbon dioxide (CO_2) and lactic acid, and (iii) contracture of skeletal muscles (Lee and Choi, 1999). PSS was first observed and described by Topel *et al*. (1967). Patterson and Allen (1972) describe PSS as acute death induced by stressors such as exercise, service, fighting, high ambient temperatures, birth, stocking density, loading, transport, overcrowding in the lairage, use of electric prodders and abuse (Oliver *et al*., 1988; McKee *et al*., 1998; Guàrdia *et al*., 2004; Adzitey and Nurul, 2011).

Under stressful conditions, PSS is caused by a single autosomal recessive gene i.e. *halothane* gene (*HAL* gene), *stress* gene, *PSS* gene or *ryanodine receptor 1* (*RYR1*) gene (Hall *et al*., 1980; MacLennan and Phillips, 1992). In the pig, this gene is located on Chromosome 6 (Harbitz *et al*., 1990) (map position on *Sus scrofa* Sscrofa11.1: 47 339 759-47 458 457 (https://www.ncbi.nlm.nih.gov/genome/?term=pig)). Fujii *et al*. (1991) found a single point transition mutation, at Nucleotide 1843 that is responsible for the condition. A susceptible animal has a nucleotide replacement of thymine instead of cytosine in the complementary deoxyribonucleic acid (cDNA). The latter caused an alteration in the amino acid sequence where arginine is replaced with cysteine at Position 615. This mutation affects the sarcoplasmic reticulum (SR) calcium ion (Ca^{2+})-release channel and results in the channel opening, but closing is inhibited (Endo *et al*., 1983; O'Brien, 1986; Fill *et al*., 1990). Malignant hyperthermia (MH), a disorder also seen in humans, is also elicited by stress and/or application of anesthetics such as halothane (Mickelson and Louis, 1996). In the past, a reaction to halothane was used as a diagnostic test to identify animals with PSS, hence the name, *halothane* or *HAL* gene (Smith and Bampton, 1977). Furthermore, even if the best pre-slaughter handling procedures are followed, the animal carrying this gene will be predisposed to exhibit PSS and PSE characteristics (Lee and Choi, 1999). Therefore, when the physical response to stress does not cause death, it can be assumed with more than 80 % certainty that PSE meat will manifest itself (Fisher *et al*., 2000a).

Pale, soft and exudative meat is caused by the rapid glycolysis early post-slaughter where the pH of the carcass meat is lower than six (< 6) at 45 minutes'(min) post-slaughter (Wismer-Pedersen, 1959; Bendall, 1973; MacDougall, 1982; Aalhus *et al*., 1998; Schaefer *et al*., 2001). The rate at which glycolysis occurs is influenced by factors like the physiological state of the animal, the genetic predisposition (presence or absence thereof), environmental stressors and stunning method used (Heffron and Dreyer, 1975). The process of glycolysis will stop when residual glycogen is present (Van Laack and Kauffman, 1999; Immonen and Puolanne, 2000; Copenhafer *et al*., 2006).

An increase in the denaturation of muscle proteins and subsequently a reduction in the WHC of the muscle will also occur (Bendall and Wismer-Pedersen, 1962; Briskey, 1964; Alvarado and Sams, 2002; Adzitey and Nurul, 2011). The reduction in WHC is evident in both fresh (Fisher *et al*., 2000a) and processed products (Fisher *et al*., 2000b). Temperatures of the deep muscular tissue remain high when the cooling rate of the carcass is slow. Therefore, when the carcass reaches its ultimate pH, the carcass is still warm (Ludvigsen, 1954; Wismer-Pedersen, 1959; Lawrie, 1960; Aalhus *et al*., 1998).

Even though PSE exhibits negative effects such as lowering the carcass quality (Pommier and Houde, 1993; Webb, 1996), reduced meat tenderness and it also exhibits favourable effects to swine producers (Touraille and Monin, 1982; Boles *et al*., 1991; Guéblez *et al*., 1996). Ollivier *et al*. (1991) suggest that the high incidence rate of PSS and PSE in swine can be attributed to both the intensive selection for muscle development, improved feed conversion and selection against fat deposition. Pigs with heterozygous genotypes for the *PSS* gene also present carcass characteristics such as improved lean tissue growth, greater carcass yield, a higher lean content (McLaren and Schultz, 1992) and carcass weight (Zhang *et al*., 1992; Leach *et al*., 1996). Murray *et al*. (2001) reported that under certain conditions the pH and colour of pork meat may be improved by feed withdrawal pre-slaughter. This, however, results in debilitated carcass yield. There have been reports on PSE meat in other species including cattle (Aalhus *et al*., 1998), ostriches (Van Schalkwyk *et al*., 2000), turkeys (McCurdy *et al*., 1996; McKee *et al*., 1998; Owens *et al*., 2000) and chickens (Swatland, 2008). Adzitey and Nurul (2011) state that conditions such as PSE can be found in all species, but that expression is dependent on pre-slaughter handling.

2.2.3.2 RN⁻ Gene

Another gene causing characteristics similar to the *PSS* is the *PRKAG3* gene or the *Rendement Napole (RN⁻)* gene. The *RN⁻* gene is an autosomal dominant gene (Le Roy *et al*., 1990). Le Roy *et al*. (1990) and Warner *et al*. (1997) proposed that the *RN⁻* allele is responsible for red, soft and exudative (RSE) meat. Warner *et al.* (1997) also proposed that the low pH and high glycogen content in the muscle results in low processing yields, which is also typical characteristics in the presence of the *RN⁻* genotype.

The *RN⁻* gene (*R200Q*) is located between markers SW120 and SW936 on Chromosome 15 in the porcine genome (Milan *et al*., 1995; Mariani *et al*., 1996). The *PRKAG3* gene mechanism encodes for a muscle isoform of the regulatory γ-subunit that forms part of the adenosine monophosphate-activated protein kinase (AMPK). The latter is an enzyme essential in the regulation of energy metabolism. In total five substitutions within the *PRKAG3* gene have been identified and include *I199V, R200Q, T30N, L53P* and *G52S* (Milan *et al*., 2000; Ciobanu *et al*., 2001; Chen *et al*., 2008). Milan *et al*. (2000) proposed that the mutation is most likely the result of selection pressure on growth and lean carcass yield.

During the post-slaughter period, glycogen is altered to lactic acid. The lactic acid (Lundström *et al*., 1996; Enfält *et al*., 1997) in return causes a low ultimate pH (Miller *et al*., 2000; Josell *et al*., 2003; Lindahl *et al*., 2004; Škrlep *et al*., 2010). Therefore, RSE- or acid meat exhibits a high drip loss and therefore a very low WHC (Le Roy *et al*., 1996; Le Roy *et al*., 2000; Škrlep *et al*., 2010).

2.2.3.3 Dark, Firm and Dry Meat

Meat from carcasses exhibiting dark cutting (DC) characteristics causes high economic losses in the beef cattle industry (Tarrant, 1981). Dark cutting beef presents as meat with a dark colour and a firm consistency, and it displays a sticky surface and on occasion slime formation causes premature bacterial spoilage (Lawrie, 1998; Gardner *et al*., 1999; Gardner *et al*., 2001; De la Fuente *et al*., 2010). Therefore, the more common name is dark, firm and dry (DFD) meat. The dark colour of the meat is due to the high intracellular water content, which reflects decreased amounts of light. The dryness of the meat is caused by the muscle that has the ability to bind to water and, consequently, the meat has a high WHC (Apple *et al*., 2005; Zhang *et al*.,

2005). The high WHC also results in the meat having a firm consistency. The bacterial spoilage is caused largely by both the high WHC and high pH (Lister, 1988; Warriss, 2000). The sticky-like surface does not only make the meat unattractive to the consumer but also causes the preparation of the muscle to be challenging (Sornay *et al*., 1981; Viljoen *et al*., 2002). Muscles in beef carcasses that are most frequently affected include the *M. longissimus dorsi*, *M. semimembranosus* and *M. biceps femoris* (Viljoen, 2000). The primary cause of DFD meat is reduced levels of muscle glycogen at slaughter which results from long-term stress (Ashmore *et al*., 1973; Warriss, 1990; Viljoen *et al*., 2002; Apple *et al*., 2005). This negatively affects the carcass and its value, resulting in a large economic loss (Fabiansson *et al*., 1989).

The prevalence of DFD meat is influenced not only by long-term stress but also by many other factors such as nutrition (on-farm and during lairage), breed, gender, age, peculiar pigmentation, transport, temperament, mixing of cattle, behaviour, climate, lairage time, delayed bleeding, muscle type and season (Guilbert, 1937; Ashmore *et al*., 1973; Tarrant, 1981; Bartoš *et al*., 1993; Sanz *et al*., 1996; Hoffman *et al*., 1998; Scanga *et al*., 1998; Silva *et al*., 1999; Geay *et al*., 2001; Gardner and Thompson, 2003; O'Neill *et al*., 2003; Guàrdia *et al*., 2005). However, to this day, no genetic component has been found to influence DFD (Ponnampalam *et al*., 2017). Selecting for growth in cattle has resulted in animals being more susceptible to generate DFD meat (Webb and Casey, 2010). Howard and Lawrie (1956) stated that nutritional stress and exercise alone cannot produce DFD meat but together can increase its prevalence. Pre-slaughter stressors activate the adrenergic mechanisms (includes the release of adrenaline) (Tarrant, 1989). Animals susceptible to these stressors and which exhibit DFD meat do not have enough lairage time to replenish their muscle glycogen to normal levels. This process can take a few days to two weeks (Tarrant, 1989). Warriss *et al*. (1990) suggest that sheep experiencing transport stress will produce in carcasses exhibiting DFD meat. Contrary, studies by Díaz *et al*. (2003) and De la Fuente *et al*. (2010) demonstrated average pH values in sheep carcasses, indicative of normal meat.

Smaller animals tend to tolerate stress better than larger animals, an observation which suggests that the DC condition would be more prevalent in cattle than in sheep (Puolanne and Aalto, 1981; Gardner and Thompson 2003; King *et al*. 2006). However, Gardner *et al*. (1999) show that even though sheep have the ability the handle stress better, stress still has a significant effect on their glycogen levels. Puolanne and Aalto (1981) and Önenç (2004) found that, when the breed effect is taken into account, cattle kept in overnight lairage caused an increase in the

prevalence of DFD meat. When the lairage time was increased, the prevalence decreased. The physical and emotional stress the animals go through pre-slaughter is enough to cause DFD meat (Puolanne and Aalto, 1981). Furthermore, during lairage where high levels of aggressiveness occurred, the prevalence of DFD meat increased (Grandin, 1979; Lacourt and Tarrant, 1985; Bartoš *et al*., 1988). Bartoš *et al*. (1993) and Sanz *et al*. (1996) suggest that simply changing handling procedures can also cause DFD meat. A breed effect regarding glycogen levels repletion has been suggested (Young *et al*., 1993; Hopkins *et al*., 1996; Gardner *et al*., 1999). This has also been observed in different breed lines (Bray *et al*., 1992). Gardner *et al*. (1999) also found a strong relationship between high-stress slaughter conditions and breed type. They found a breed effect when the final pH in muscles was investigated.

In Australia, the perception exists that Merino lambs exhibit more DFD cases than any other breed (Gardner *et al*., 1999). In addition, the condition is predominantly seen in grass-fed cattle in Australia, during the autumn and winter months. Because the muscle glycogen varies between seasons, Knee *et al*. (2004; 2007) found a strong linkage between the glycogen levels and the quality and quantity of forage available during these months. Furthermore, when glycogen reached its maximum levels (in summer months) the total ME intake was also at its highest. Therefore, when the intake decreased due to environmental stressors (low quantity and low quality of forage), the glycogen levels decreased and produced DFD meat. Similarly, Viljoen (2000) suggested a link between seasons and DFD meat, and Grandin (1992) and Scanga *et al*. (1998) indicated DFD meat occurs during very cold temperatures along with precipitation and very high fluctuating temperatures.

The pH of the muscle of DFD meat is generally high due to depleted muscle glycogen levels (Lawrie, 1958; Hedrick *et al*., 1959; Ashmore *et al*., 1973; Scheffler *et al*., 2013). The percentage of glycogen within DFD meat is less than half of what normal meat contains (Davey and Graafhuis, 1981; Viljoen, 2000). This results in meat having abnormally high pH values. These values range from 5.9 to 6.8. The drop in pH decline of DFD meat occurs at a more discrete rate than meat with PSE characteristics (Scheffler *et al*., 2013).

2.3 GENOMIC METHODS FOR THE DETECTION OF GENETIC DEFECTS

Study of common diseases falls under two categories i.e. population- and family-based studies. Population-based studies generally use a case-control study design to focus on candidate gene regions (Miyagawa *et al*., 2008). During the 2000's until presently, genome-wide association studies (GWAS) using high-density single nucleotide polymorphisms (SNPs), had become the conventional method to identify statistically significant loci, underlying both common and complex diseases, but with no prior information on the gene function (Hirschhorn and Daly, 2005; Miyagawa *et al*., 2008; Clarke *et al*., 2011). In sheep, several studies using the GWAS approach to determine quantitative trait loci (QTL) and possible candidate genes in wool (Wang *et al*., 2014), meat (Zhang *et al*., 2013), milk production, body weight (Al-Mamun *et al*., 2015), body size (Kominakis *et al*., 2017), rickets (Zhao *et al*., 2011), polyceraty (Greyvenstein *et al*., 2016), horns (Johnston *et al*., 2011), litter size (Demars *et al*., 2013), chondrodysplasia (Zhao *et al*., 2012) have been conducted. For a GWAS, populations consisting of afflicted and unafflicted individuals are compared with each other using the frequencies of alleles or genotypes, i.e. a case and control study. If a higher frequency of an SNP variant exists in the afflicted group (afflicted individuals with a specific phenotype), or are statistically more common in the afflicted group, the alleles or genotypes are considered to be associated with the disease (Hirschhorn *et al*., 2002). Each single nucleotide polymorphism (SNP), approximately evenly spaced across the genome, is tested independently for an association of a specific trait (Kemper *et al*., 2012) or phenotype (Balding, 2006).

Both linkage analysis (LA) and GWAS has been universally accepted as methods of gene mapping. These methods differ in the experimental design; however, both use genetic markers (Kemper *et al*., 2012). In GWAS, a correlation between the marker and the phenotype is found across a population whereas LA depends on segregation of alleles within the family of a population (Kemper *et al*., 2012). By comparing the two methods one significant difference comes to light: the accuracy of which the location of the QTL is mapped. The difference lies in the confidence interval for the position of the specific QTL, where linkage analysis has a fairly large interval due to solely using recombination events within the recorded pedigree (Darvasi *et al*., 1993). Genome-wide association studies in contrast relies on linkage disequilibrium (LD) between the QTLs and the markers to detect polymorphisms (Clark *et al*., 2003).

The concept of homozygosity mapping was first introduced by Lander and Botstein in 1987. The authors described a method to detect recessive genetic diseases in humans using regions in the genome that is homozygous by descent. Thus, an individual will have adjacent regions in the genome that is homozygous (Broman and Weber, 1999; Gibson *et al*., 2006; Li *et al*., 2006), that is today termed as runs of homozygosity (ROH). In sheep, several studies have assessed ROH (eg. Al-Mamun *et al*., 2015; Muchadeyi *et al*., 2015; Mastrangelo *et al*., 2017; Purfield *et al*., 2017). To detect ROH, each chromosome is scanned separately. Therefore, a fixed size window moves along each chromosome to search for regions in the genome that consist of consecutive homozygous SNPs (Purcell *et al*., 2007). Consequently, if for example a genetic defect is an autosomal recessive inherited disorder, then the genomes of all afflicted individuals should be homozygous at the causative locus.

An illustration by Kijas (2013) (Figure 2.1) explains overlapping homozygous segments (spanning across 25 Megabase pairs (Mbp, 1 000 000 base pairs (bp)) between three cows. The homozygous segments for each cow are given in black boxes, with the number of SNPs in each segment given at the right-hand side. The dashed lines signify the total distance of the ROH from all the animals (UNION) Table. The solid lines (consensus region (CON)) signify the shared ROH region between the animals.

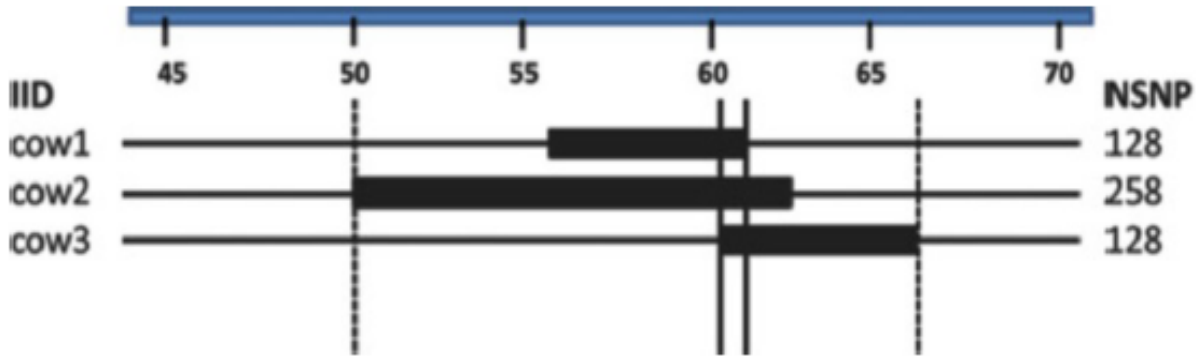


Figure 2.1 Illustration adapted from Kijas (2013) where the overlapping homozygous segments (spanning across 25 Mbp) are shown in three cows. The homozygous segments for each cow are given in black boxes, with the number of SNPs in each segment are given at the right-hand side. The dashed lines signify the total distance of the ROH from all the animals (UNION). The solid lines signify the shared ROH region between the animals (CON).

Observed associations between the genotyped SNPs and disease/trait can be described in two ways: firstly, the association observed can be caused by causal variants each with a small effect

but is in high LD with the SNPs or *vice versa*, where single or multiple causal variants each with large effects is however in low LD with the SNPs. When the allele frequencies of the SNPs and the unidentified causal variants are very different from each other, then low LD will occur (Visscher *et al*., 2012).

With the use of LD, the mapping of genetic disorders has also been successful (e.g., Hästbacka *et al*., 1992). With LD, a chromosomal region, possibly carrying the disease gene, that is identical-by-descent (IBD) in afflicted individuals are identified. This region, IBD, is thereafter detected by the loci closely linked to each other and carries the identical alleles in each of the afflicted individuals (Pritchard *et al*., 1991; Houwen *et al*., 1994). Linkage disequilibrium is suitable to find the marker bracket, which is the region between two markers that contain the disease QTL (Meuwissen & Goddard, 2000). Further, LD is useful in the estimation of QTL positions whereas linkage mapping is useful when a genome scan is used to detect QTL (Meuwissen & Goddard, 2000).

Some previous research has used a candidate gene approach targeting specific regions of the genome due in part to its low cost (Zhu and Zhao, 2007). However, this approach is handicapped by the inability to select appropriate candidates. Candidate genes are selected based on existing knowledge (in other species or breeds) relating to their biochemical-, molecular- and physiological functions that are either directly or indirectly associated with the trait of interest (Andersson and Georges, 2004; Zhu and Zhao, 2007). However, one main constraint to such an approach is limited knowledge one might have on the trait or phenotype that is being investigated (Zhu and Zhao, 2007). Therefore, a difference between the candidate gene approach and the GWAS approach that is worth mentioning is that the latter collects and interprets data without any prior knowledge or set hypothesis about the genes, their functions and role in biological pathways, therefore allowing the development of a new hypothesis.

Complete genome sequences are readily available for several species including the chicken, pig, cattle, sheep and horse (Andersson and Georges, 2004; Bai *et al*., 2012). Deoxyribonucleic acid sequencing allows for small regions of interest, such as genes, to be explored at a nucleotide level. Comparatively, next-generation sequencing (NGS) allows for large-scale sequencing of whole genomes (WGS) (Schuster, 2008) and whole exomes (WES; coding regions; Ku *et al*., 2012; Rabbani *et al*., 2013). The coding region (exon) of a gene is responsible for the sequence of amino acids in a protein. Mutations can result in substitutions, duplications or deletions of nucleotides. This can lead to frameshift, premature termination of

translation, changes in amino acid sequence or the removal of exons (Cartegni *et al*., 2002). The regulatory or non-coding region of a gene can affect transcription and translation and alter gene expression (Ibeagha-Awemu *et al*., 2008). Furthermore, gene expression studies aim to identify differentially expressed proteins and genes within different tissues and cells. Ribonucleic acid (RNA)-sequencing (RNA-Seq) is currently the most widely used method to study gene expression by isolating the RNA and thereafter sequencing which is based on NGS of the cDNA (Shendure and Ji, 2008; Wang *et al*., 2009; Finotello and Di Camillo, 2015). Such studies will allow the comparison between different tissues and diseases to determine which genes are expressed in cells and thereby determining the cause of the phenotype (Finotello and Di Camillo, 2015). Studies such as those of Clark *et al*. (2017) used RNA-Seq gene expression to provide functional annotation to genes previously unknown in the ovine genome. Finally, RNA-Seq data can also be used for the detection of transcripts, detection of alternatively spliced genes and the detection of allele-specific gene expression (Wang *et al*., 2009; Kukurba and Montgomery, 2015).

2.4 PATTERNS OF INHERITANCE

Genetic maps of livestock species have facilitated the detection of genomic regions which contribute to the genetic variation of both polygenic (e.g. Bidanel *et al*., 2001; Malek *et al*., 2001a, 2001b; MacNeil and Grosz, 2002) and Mendelian inherited traits (e.g. McPherron and Lee, 1997; Grobet *et al*., 1997; Murphy *et al*., 2005). Genetic defects can be categorised in three groups, namely single gene or Mendelian disorders, multi-factorial and chromosome (Mahdieh and Rabbani, 2013). When one of the X-linked inheritance patterns are considered a mode of inheritance for a genetic disorder, it becomes more complex. An imbalance exists of the X chromosomes between males (XY) and females (XX) and the X chromosome would be over-expressed in females relative to males. However, this imbalance is resolved by the random inactivation of one of the X chromosomes in each of the somatic cell tissues (Lyon, 1961; Heard and Avner, 1994). This dosage compensation results in expression of only one allele of most genes in the non-homologous region of X in females (Belmont, 1996).

Generally, each maternal and paternal X chromosome contributes equally between tissues and causes females to exhibit an mosaic expression of both normal and mutant alleles when present (50:50) (Barr and Bertram, 1949; Lyon, 1961; Heard and Avner, 1994; Belmont, 1996; Clayton, 2009; Avery and Vrshek-Schallhorn, 2016). The location and size of these mosaic

expression patterns are different for all tissue types (Willemsen *et al*., 2002). However, occasionally an extreme deviation from the 50:50 expression ratio occurs. This is called X chromosome inactivation (XCI) skewing and it is found that one of the X chromosomes will contribute for example 90 % to most of the relevant tissue cells (Belmont, 1996; Migeon, 2008; Minks *et al*., 2008). Ørstavik (2009) described the severity of skewing by showing how some female carriers will display phenotypic recessive X-linked disorders. The skewing of the mosaic expression found in females is due to selection that favours the X chromosome carrying the mutant allele or *vise versa* (Belmont, 1996; Migeon, 2008; Deng *et al*., 2014). Furthermore, approximately 1000 X-linked genes escape XCI, thus resulting in the expression of both the inactive (Xi) and active (Xa) X chromosomes. Such genes are mostly located within or close to the end of the pseudoautosomal region (PAR) (Carrel *et al*., 1999).

Several methods exist that enable the analysis of XCI expression in females. Expression differs among different tissue material and should therefore be studied and reported accordingly (Ørstavik (2009). The two most general methods of XCI detection include methylation- and expression analysis (Ørstavik (2009). Amos-Landgraf *et al*. (2006) and Ørstavik (2009) proposed a methylation analysis of XCI using DNA to assess XCI ratios and -patterns. Methylation analysis generally makes use of the '*Humara*' and/or the *androgen receptor* (AR) genes due to their polymorphism and is amplified by the PCR process (Tommasini *et al*., 2002; Amos-Landgraf *et al*., 2006). Amplification products are sequenced and allele calling occurs by using software packages such as Genotyper (Applied Biosystems, https://www.thermofisher.com). XCI ratios are detected by using, for example, the calculated percentage of the predominant allele. When the predominant allele exceeds 75 %, it is considered as skewing.

These studies, using the *XIST* gene polymorphism, can also be used to determine XCI ratios (Wolff *et al*., 2000; Bolduc *et al*., 2008; Zhao *et al*., 2010). Synthesized cDNA from RNA is amplified by means of a PCR and thereafter digested with *Hinf*I. The *XIST* gene is known to only be expressed in the Xi chromosome and can therefore be used to determine XCI ratios (Rupert *et al*., 1995; Carrel and Willard, 1999).

2.5 AIMS AND OBJECTIVES

When the description and results of prior research are taken into account, no physiological, environmental or management system was conclusively identified as the causative agent of WCS. Previous research has also not considered a potential genetic basis for WCS or the potential for an interaction of genotype with the environment (stress). The tentative breed-specificity of the condition lends some credence to the potential for WCS to have a genetic basis and this is supported by phenotypically similar conditions that arise from single genes in other species. Therefore, the principal aim of this research was to attempt to identify a genetic basis that predisposes lambs to WCS. The following three null hypotheses provide the basis for these investigations:

1) Haplotypes and SNP in high LD with *RYR1* and RN^{-} are unrelated to the occurrence of WCS.

2) A genome-wide scan of SNP profiles of afflicted and unafflicted sheep fails to detect loci associated with WCS.

3) Runs of homozygosity in animals afflicted with WCS show no regions of genomic commonality.

CHAPTER 3

MATERIALS AND METHODS

3.1 DATA COLLECTION

3.1.1 Sample Collection

Samples were collected at abattoirs located in the geographical area where WCS had a relatively high incidence rate in the past. Abattoirs are located within or close to the towns of Groblershoop and Upington in the Northern Cape Province of South Africa (Figure 3.1). Additional samples were also collected in Johannesburg, Gauteng, South Africa, at a retail distribution centre. Sheep (Dorper, Figure 3.2) carcasses are regularly transported from Mariental in Namibia, to this location for further processing. A total of 84 samples were collected from Groblershoop (20 afflicted and 20 unafflicted), Upington (3 afflicted and 3 un-afflicted) and the retail centre (20 afflicted and 18 unafflicted). It was specified that all afflicted and unafflicted carcass samples should be selected as pairs from the same cohort. This would minimize the risk of false positive associations (Type I error) due to selection biases and population stratification (Cardon and Palmer, 2003; Hirschhorn and Daly, 2005; Turner *et al*., 2011). An apparent seasonal effect to WCS has been previously identified by Le Roux (2012). Late autumn (April to May) and winter (June to August) have been identified as the period with the highest occurrence rate of WCS. Therefore, sampling of WCS afflicted- and unafflicted carcasses were mainly collected during the autumn and winter months of 2014 – 2016. Thereby, an agreement with the abattoirs was established and stated that the collection of samples should occur during late autumn and winter.

Figure 3.1 Geographical distribution of samples collected from abattoirs in South Africa and Namibia.

Figure 3.2 Black head Dorper sheep (http://www.dorpersa.co.za).

3.1.2 Sample Collection Methods

Muscle samples were taken from only the hind leg (*biceps femoris* muscle) of lamb carcasses using biopsy punches (Figure 3.3). The biopsy punch was inserted into the carcass to obtain tissue (on an area with the least visible fat on the muscle) and placed into a 45 mL tube, sealed in an enclosed package, and stored at the lowest possible temperature at abattoirs until collection (Figure 3.4).

Figure 3.3 Biopsy punch used to collect muscle samples from sheep carcasses (http://www.cmecorp.com).

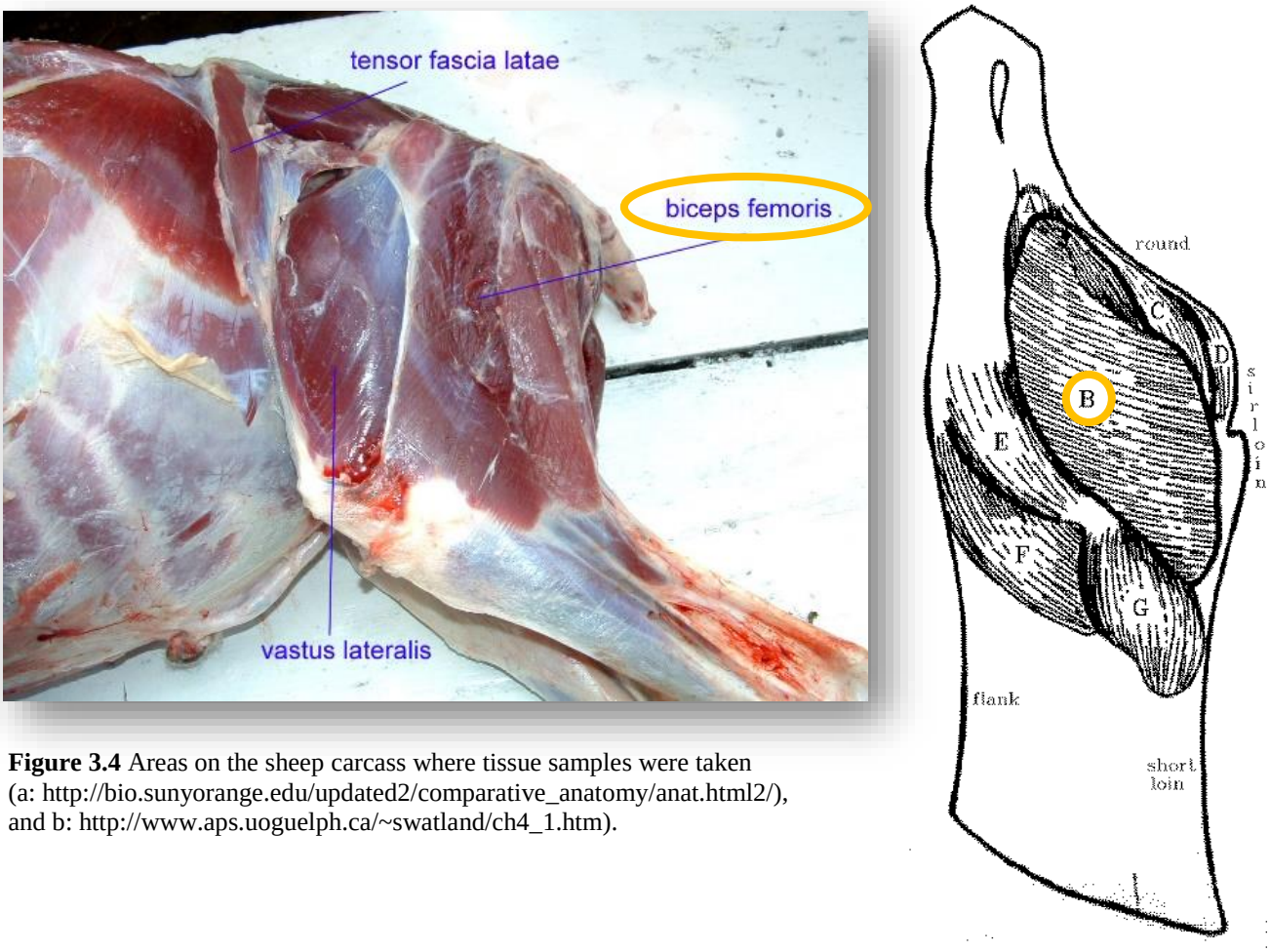


Figure 3.4 Areas on the sheep carcass where tissue samples were taken (a: http://bio.sunyorange.edu/updated2/comparative_anatomy/anat.html2/), and b: http://www.aps.uoguelph.ca/~swatland/ch4_1.htm).

3.2 SAMPLE PROCESSING

3.2.1 DNA Extraction

Extraction of genomic DNA from muscle samples was accomplished by using the Genial® First DNA All tissue extraction kit (Troisdorf, Germany) following the manufacturer's instructions, with minor modifications that best-suited muscle samples (http://www.genial.de/index.php/en/products/dna-extracionkits/all-tissue-dna-extraction-kit/).

Roughly chopped muscle was placed into a 1.5 milliliter (mL) reaction vessel with 500 microliters (µL) lysis buffer one, 50 µL lysis buffer two and 5 µL Enzyme (Proteinase K), and thereafter incubated for 90 min at 65 ºC. Centrifugation occurred for 10 min at 12 000 revolutions per minute (rpm) of which 500 µL supernatant was transferred to a fresh reaction vessel. A total of 375 µL of lysis buffer three was added, and thereafter vortexed for 20 seconds (sec). The samples were chilled in a freezer for 5 min and again centrifuged for 10 min at 13 000 rpm. A total of 800 µL supernatant were transferred into a fresh reaction vessel. A total of 640 µL isopropanol was added and carefully mixed. To obtain a DNA-pellet the samples were centrifuged for 15 min at 13 000 rpm. The supernatant was removed, and the pellet washed with 300 µL chilled 70 % ethanol (EtOH) and centrifuged for 5 min at 13 000 rpm. The pellet was finally air dried overnight and dissolved in 50 µL double distilled water (ddH_2O) and stored at -80 °C (http://www.gen-ial.de/index.php/en/products/dna-extracionkits/all-tissue-dna-extraction-kit/).

3.2.2 DNA Quality and Quantity Assessment

3.2.2.1 Nanodrop™

Nucleic acid (DNA) concentration was determined using a Thermo Scientific™ NanoDrop 2000 (https://www.thermofisher.com) spectrometer. For each DNA sample, the quantity and quality were tested three times to acquire an average for both measurements. A total of 1 µL of DNA was used for each measurement.

3.2.2.2 Gel Electrophoresis

DNA extraction products were also run on a gel electrophoresis system (Bio-Rad, California, USA, http://www.bio-rad.com/en-za/category/horizontal-electrophoresis-systems). A total of 3 µL DNA was added to 3 µL of loading dye, mixed and loaded into the gel (1 % 50x Tris/Acetic Acid/EDTA (TAE) and 0.003 % ethidium bromide (EtBr)). The gel ran for 20 min at 100 Volts (V), and was thereafter UV visualized with the Bio-Rad Molecular Imager® Gel Doc™ XR+ System controlled by Image Lab software.

3.2.2.3 Qubit™

The final DNA quantification step was completed with the use of the Qubit™ 3.0 fluorometer instrument (Invitrogen™, Thermo Fisher Scientific, Carlsbad, Calif, USA, https://www.thermofisher.com/za/), following manufacturer's instructions. The Qubit™ was used to test the purity of the DNA samples and provide the most accurate DNA quantity.

3.2.3 Sample Preparation and Beadchip Analysis

To ensure the correct concentration of genomic DNA was reached, sample normalization was applied. The DNA concentration for genotyping is generally approximately 50 nanograms/microliter (ng/µL). Therefore, after Qubit™ results were obtained, DNA samples with a concentration greater than 50 ng/µL were normalized to 50 ng/µL with elution buffer using a Hamilton robotic system, Microlab Star Plus. The DNA samples with concentrations less than 50 ng/µL were also used.

The Infinium® HD Assay Ultra Manual (experienced user card, https://emea.illumina.com) was used for genotyping of the samples during a three-day procedure. During the amplification process, the DNA was denatured and neutralized, whereas the incubation step amplified (or increases) the DNA. During the fragmentation process, the DNA was fragmented by means of an enzymatic process. The DNA was thereafter precipitated and re-suspended within a buffer. The hybridization process entailed the annealing of the DNA to locus-specific 50-mers and thereafter washed to remove all unhybridized DNA. Finally, the primers that were attached to the DNA were extended by adding labelled nucleotides, whereafter the primers and stained and

the BeadChips were coated for protection purposes. The latter processes were all completed according to manufacturer's recommendations (see Adler *et al*., 2013 and https://www.illumina.com). The Illumina iScan Control System on the Illumina HiScan system was used to read the BeadChip, and the raw data were transferred to Illumina GenomeStudio Genotyping Module™ Genotyping Module Software v2.0 for further analysis.

3.3 GENOTYPE QUALITY CONTROL

Genotyping of samples was performed at the Agricultural Research Council - Biotechnology Platform (ARC-BTP), Pretoria, South Africa using the Ovine Infinium® High -density (HD) SNP BeadChip of Illumina (Illumina, San Diego, California, USA, https://www.illumina.com). The BeadChip features 685 734 SNPs that are equally distributed across the domestic sheep (*Ovis aries*) genome on 26 autosomes and the X chromosome (Kijas *et al*., 2014; https://www.illumina.com). The Y-chromosome is not included in the Ovine Infinium® HD SNP BeadChip (https://www.illumina.com). The International Sheep Genomics Consortium (ISGC), in conjunction with FarmIQ (www.farmiq.co.nz), developed the Ovine Infinium® HD SNP BeadChip, but is not commercially available. After the manufacturer's quality control whereby a total of 79 728 non-functioning SNPs were removed, a total of 606 006 functional SNP genotypes were available for analysis.

3.3.1 Genotyping Quality Assessment

The Illumina GenomeStudio Genotyping Module™ Genotyping Module Software v2.0 was used to convert raw signal intensities into genotyping calls. Within the Genotyping module software, a project was created using the appropriate sample sheet containing all relevant sample identifications (sample ID). Along with the sample sheet, all raw signal intensities were converted into genotyping calls for each SNP and individual. The GenCall (GC) score cutoff was set at 0.15 (cutoff value recommended by manufacturer). The GC score was used to test the reliability of the genotype calls. The PLINK Input Report Plug-in v2.1.4 for GenomeStudio® Genotyping Module was used to create input files containing phenotype-, genotype- (PED file) and SNP information (MAP file).

3.3.2 Choice of Software Packages

Several packages have been developed for meta- and GWAS analysis. METAL (Willer *et al*., 2010), XWAS (Gao *et al*., 2015), GCTA (Yang *et al*., 2011), Golden Helix SNP Variation Suite (SVS) (http://goldenhelix.com/products/SNP_Variation/index.html) and several R packages including GWASTools (Gogarten *et al*., 2012) are amongst these. However, the majority of these are for the exclusive use in humans or are not publicly available for use.

PLINK is one of the most widely used, freely available and open-source toolsets for genomic association studies with large datasets containing both genotypes and phenotypes (Purcell *et al*., 2007). This software has the ability to integrate data management, quality control methodology, association analysis and annotation. Data manipulation includes the merging of datasets, the removal and update of SNPs and individuals simultaneously. Quality control (QC) methods include the call rate of individuals and SNPs, minor allele frequencies, inbreeding, IBD, identity-by-state (IBS), Hardy-Weinberg equilibrium (HWE), sex analysis and pedigree errors. Estimating population stratification, LD based SNP pruning and estimation of LD between SNPs, runs of homozygosity, association analysis based on case/control samples as well as the use of Fisher's exact test (assuming binary disease status which is the case for WCS and used by most GWAS software tools). All of the above can be done by using only one data set, thereby providing computational and time efficient analysis with relatively uncomplicated and a comprehensive output file, that is provided within a short space of time. Furthermore, other software packages such as Haploview (Barrett *et al*., 2005) as well as packages developed within R such as SNPRelate- (Zheng *et al*., 2012), qqman (Turner, 2014) and Manhattanly (http://sahirbhatnagar.com/manhattanly/) packages, use PLINK output files to create data visualization figures and diagrams. The latter allows for very few changes or new input files for other association analysis software.

Finally, for case-control study design, most GWAS software uses the Chi-Squared (χ^2) test instead of Fisher's Exact test. This is due to the former assuming large sample sizes (as with most GWAS studies) whereas the latter is more appropriate in the case of small sample sizes (Kim, 2017), as in the current study. PLINK have the ability to perform all the above-mentioned tests, making it the preferred software tools for the current study.

3.3.3 Individual and SNP Quality Assessment

The PLINK v1.07 (Purcell *et al*., 2007) software package was used for both individual- and SNP quality control (QC) measures. PLINK allows for the efficient storage, and analysis of high-throughput genetic data and can perform a wide range of analysis including quality control, association analyses, and haplotypic tests, to only mention a few. This ensures that no bias due to the sex of the animals influenced the analyses, the *--allow-no-sex* option within plink was also used within every command specified. Individual QC was based on three initial steps: i) missing rate per individual (*--mind*); ii) inbreeding coefficient (*--het*); and iii) pairwise IBD estimation (*--genome*).

One method of detecting poor quality DNA, is by observing the proportion of SNPs failing to genotype within a sample (Anderson *et al*., 2010; Turner *et al*., 2011). This allows the removal of individuals with a large set of failing SNPs. First, the missing rate per individual excluded individuals with excessive missing genotypes (call rate < 90 %; Zhang *et al*., 2013; Hao *et al*., 2017).

An excess of homozygosity is an indicator of population stratification and inbreeding (Anderson *et al*., 2010). Individuals with high inbreeding coefficients (calculated by using the *--het* command in PLINK) depicted observed heterozygosities (H_o) outside of the normal distribution of the given sample set. The average and standard deviation (SD) of H_o across the sampled individuals were therefore calculated. The outlier homozygous individuals with high inbreeding levels were identified by having H_o values greater or smaller than the mean or normal distribution (by using ± 3 SD).

These individuals were identified and removed by means of determining the observed heterozygosities for each individual, using the formula:

$$H_o=N(NM)-O(HOM)/N(NM)$$

The O(HOM) was observed homozygous SNPs, and N(NM) the total number of SNPs.

An excess of heterozygosity is generally due to sample contamination (a mixture of DNA of different samples) (Anderson *et al*., 2010). Finally, pairwise IBD estimation was used to identify individuals with shared ancestry as well as potential duplicate samples that were collected due to human error. To determine excessive heterozygotes and IBD, the same *--genome* command in PLINK was used. When a pair of individuals had a PI_HAT score of one,

they were regarded as duplicates, with 0.5 the animals were full-sibs, with 0.25 they are half-sibs (etc.) (Anderson *et al*., 2010; Turner *et al*., 2011; Alonso *et al*., 2015). One member of a pair of individuals was consequently removed from the dataset if their PI_HAT score was greater than 0.1875 (Anderson *et al*., 2010; Turner *et al*., 2011).

Throughout the slaughter and sampling process, information regarding the animal's sex was not recorded. Sex was therefore assigned to animals based on mean heterozygosity rates using the PLINK, *--check-sex*, command. This command uses the heterozygosity rates or the estimated inbreeding coefficient of the X chromosome. Except for the pseudo-autosomal region (PAR/homologous region; a region on the X chromosomes where homologous sequences exist between the X- and Y chromosomes), males cannot be heterozygous for any given marker on the X chromosome. Therefore, males will typically have homozygosity rates of one and females will have values less than 0.2, for the non-homologous region on the genome (Anderson *et al*., 2010). Therefore, a female call was made when the inbreeding coefficient (F) was less than 0.2 ($F \leq 0.2$) and a male call when F was more than 0.8 ($F \geq 0.8$) (Anderson *et al*., 2010; Turner *et al*., 2011; Alonso *et al*., 2015). If sex could not be established, the individual was excluded from the dataset.

To initiate the SNP marker QC, all SNPs without a known chromosomal position on the ovine genome (*Ovis aries* Oar_v4.0) were excluded. SNP QC was based on two steps: i) missing rate per SNP (call rate) (*--geno*), and ii) minor allele frequency (MAF) (*--maf*). To ensure that the maximum number of SNPs were included in the study, multiple combinations of the different quality control parameters had to be tested. Four different combinations (comb. 1-4) of SNP quality control parameters for 71 individuals (35 afflicted and 36 unafflicted) are presented in Table 3.1.

Table 3.1 Multiple combinations (Comb.) of the different SNP quality control parameters tested to determine the combination that fits the data appropriately.

	Comb. 1	**Comb. 2**	**Comb. 3**	**Comb. 4**
Total SNPs before QC				606 006
SNPs with no chromosomal position				1 291
Missing rate per SNP (*geno* ≥)	0.1	0.05	0.05	0.1
Minor allele frequency (*maf* ≤)	0.01	0.01	0.05	0.05

Generally, the SNP call rate was set between 98 and 99 %. Removing SNPs based on minor allele frequencies i.e. SNPs with a MAF of between 1 – 5 % (0.01 to 0.05) were excluded (Hao *et al*., 2017). Genotyping error, inbreeding, migration, non-random mating and population stratification are factors that influence deviations from Hardy-Weinberg equilibrium (HWE) (Wittke-Thompson *et al*., 2005; Yu *et al*., 2009; Ziegler *et al*., 2011a). Testing for HWE deviations has been the subject of wide interest. Some researchers believe it to be a necessity for GWAS studies (Xu *et al*., 2002; Thakkinstian *et al*., 2005) while others caution the use thereof (Nielsen *et al*., 1998; Wittke-Thompson *et al*., 2005). Deviations for HWE can be caused by a true association (Wittke-Thompson *et al*., 2005; Turner *et al*., 2011). As often mentioned, no prior genetic information, inheritance patterns or candidate genes prior to the current study have been identified for WCS. Testing for deviations from HWE was therefore not considered, since the removal of such SNPs may cause false-negative (Type 2 error) associations.

3.3.4 Linkage Disequilibrium

Linkage disequilibrium (LD) can be defined as the non-random association between two or more markers (Clarke *et al*., 2011). Several SNPs may be associated with the trait or phenotype under study and are therefore in linkage disequilibrium (LD) (Alonso *et al*., 2015). Furthermore, markers lying on the same chromosome that are in LD are inclined to be inherited together and causes shared ancestry within a population (Clarke *et al*., 2011). Within PLINK, the commands --*indep-pairwise 50 5 0.2* or --*indep-pairwise 50 5 0.5* (Kominakis *et al*., 2017) were tested. A window of 50 SNPs (***50*** *5 0.2*; ***50*** *5 0.5*) was used to calculate LD between each pair of SNPs. The number five (*50* ***5*** *0.2*; *50* ***5*** *0.5*) described the number of SNPs to shift the window i.e. sliding window, at each step. When a pair of SNPs had $r^2 > 0.2/0.5$ (*50 5* ***0.2***; 50 5 ***0.5***), one SNP per pair was removed (Purcell *et al*., 2007).

3.3.5 Population Stratification

Population stratification (PS) exists when the allele frequencies between afflicted and unafflicted individuals differ due to ancestral histories. Thereby, implying that the sampled individuals are from different populations or a hidden relatedness exists between the

individuals. This can cause false-positive (Type 1 error) associations (Pritchard *et al*., 2000; Cardon and Palmer, 2003; Price *et al*., 2006; Choi *et al*., 2009; Kang *et al*., 2010). In the present study, populations were not sampled *per se* but rather individuals from geographical regions. Finally, two methods, Principal Component Analysis (PCA) and quantile-quantile (Q-Q) plots (discussed in more detail under section 4.5) were used to detect within-breed PS. The principal aim of a PCA for this study was to identify those individuals causing underlying sub-structure within the sampled population and to remove them. A set of pruned SNPs were used to conduct the PCA, since SNPs in approximate linkage equilibrium (LE) (independent SNPs) with each other has proven to be valuable due to SNPs in approximate linkage disequilibrium (LD) having strong influences on a PCA (Anderson *et al*., 2010; Laurie *et al*., 2010). The SNPRelate and gdsfmt packages (Zheng *et al*., 2012) within R (R Core Team, 2013) were employed to perform a PCA. Individuals were labeled by their respective sampling areas i.e. Groblershoop, Mariental and Upington.

3.4 COMPARATIVE GENOMICS

Knowledge of phenotypically similar conditions such as PSE and the *Rendement Napole* phenotypes having a genetic basis indicated that it was essential to explore the possibility of their causal genes being positional and functional candidates for WCS. A direct candidate gene approach involving sequencing of polymerase chain reaction (PCR) products were not conducted. Sequences for each gene (*RYR1* and *PRKAG3*) were found from the swine genotype (*RYR1* (Fujii *et al*., 1991) and *PRKAG3* (Le Roy *et al*.,1990)), from where loci in the ovine genome with similar sequences were identified. By using Basic Local Alignment Search Tool (BLAST) within Ensembl (Zerbino *et al*., 2018), the nucleotide sequences and respective positions of the latter genes were identified on the ovine karyotype (Oar v3.1; https://www.ensembl.org/Ovis_aries/Location/Genome).

3.5 RUNS OF HOMOZYGOSITY

In an attempt to determine whether matching homozygous regions exist in afflicted males and females (autosomes) and afflicted females (X chromosome), the test ROH was used by PLINK (eg. Howrigan *et al*., 2011; Al-Mamun *et al*., 2015; Muchadeyi *et al*., 2015;

Mastrangelo *et al*., 2017; Purfield *et al*., 2017). By identifying such ROH, candidate loci also identified within these homozygous regions could harbor the causal mutation for a recessive genetic defect. Data from all 33 afflicted animals (19 males and 14 females) were extracted. Subsequently, 552 695 SNPs were available for analysis. For ROH, the --*homozyg* commands were used.

The following thresholds were combined under the --*homozyg* command:

--*homozyg-window-snp 50*	50 SNPs in sliding window
--*homozyg-window-het 1*	One heterozygous SNPs allowed in a sliding window
--*homozyg-window-threshold 0.05*	Proportion of overlapping windows (0.05) that must be called
--*homozyg-snp 50*	Minimum number of 50 SNPs in an ROH
--*homozyg-kb 1000*	Sliding window is a 1000 kilobase pairs (kbp)
--*homozyg-density 50*	Minimum density of one SNP in a 50 kbp)
--*homozyg-gap 1000*	1000 kbp in length is allowed between two SNPs that are in different segments
--*homozyg-window-missing 5*	Five missing calls allowed

3.6 ASSOCIATION ANALYSIS

To test for an association between the phenotypic condition (WCS) and genomic markers (SNPs), Fisher's Exact test within PLINK v1.07 (Purcell *et al*., 2007) software package was used. The phenotype of WCS was treated as a qualitative trait.

To conduct the basic case-control association test within PLINK, between WCS and the phenotype, the χ^2 test using the --*assoc* option was used. With Fisher's Exact test within PLINK, the --*fisher* option was used. Under the null hypothesis, which states that no association exists between a marker and the phenotype, the genotype and allele frequencies are predicted to be comparable in afflicted and unafflicted samples (Clarke *et al*., 2011).

To identify either the presence or absence of loci affecting WCS phenotype in sheep, a genome-wide association analysis (GWAS) using a case-control study design were conducted. This approach is designed to compare the allele frequencies between afflicted (case) and unafflicted (control) samples to find an association between the two given variables i.e. phenotype (WCS

carcass) and genotype. The genotype is represented by markers such as SNPs. The association analysis was performed using 69 individuals and 552 695 SNPs distributed across 26 autosomes and the X chromosome. The final step of the GWAS was conducted using the Fisher's Exact test, since it is more commonly used in small sample sizes (Kim, 2017).

Observed associations between the SNPs and the phenotype can generally be described in either of two ways. Firstly, the observed association can be caused by causal variants each with a small effect in high LD with the SNPs. Secondly, single or multiple causal variants each with large effects are, however, in low LD with the SNPs. When the allele frequencies of the SNPs and the unidentified causal variants are very different from each other, low LD will occur (Visscher *et al*., 2012).

The qqman- (Turner, 2014) and RColorBrewer packages within R (https://cran.r-project.org) were used to create and visualize the GWAS results by means of Manhattan- and quantile-quantile (Q-Q) plots. The Manhattan plot is a type of scatter plot where the physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values (representative of a significance level) are plotted on the y-axis. The strongest associations will have the smallest *P*-values accordingly; their negative logarithms will be the greatest. The blue line represents suggestive association ($-\log10e^{-5}$) whereas the red line depicts the genome-wide significance threshold of $-\log10e^{-8}$ after Bonferroni correction. The Q-Q plots are generally based on χ^2 statistics which tested for allelic association (Clarke *et al*., 2011). For the latter, the observed *P*-values (y-axis) are plotted against the expected *P*-values (x-axis). Each point represents an SNP and is plotted against the null hypothesis. In general, SNPs with a significant association will deviate from the expected *P*-values (Weir *et al*., 2004; Clarke *et al*., 2011). When the vast majority of SNPs follow the null distribution i.e. no association, and only a few deviates from the expected *P*-values at the tail of the distribution, it also provides evidence of the absence of population stratification (Clarke *et al*., 2011; Zhang *et al*., 2012; Alonso *et al*., 2015). The Q-Q plot can further demonstrate evidence of the appropriate use of QC methods during the current study and the quality of the data.

Since females carry two X chromosomes (can either be homozygous or heterozygous) and males carry one X chromosome, they will always be hemizygous for the X chromosome. To investigate the conceivable influence sex might have on WCS, individuals were grouped according to their sex (either male or females) subsequent to sex assignment. Analyses of the X chromosome were conducted using males and females separately. This would allow for the

investigation of sex ratios and the role of X-inactivation in females where it deem to be applicable. The allele frequencies were estimated for all SNPs, and by using the Fisher's Exact test (as described above), the SNP frequencies were compared between afflicted- and unafflicted males, and afflicted- and unafflicted females separately.

To adjust for multiple testing, the standard Bonferroni correction (Abdi, 2007) was used. The Bonferroni correction is commonly used to obtain an experimental *P*-value when multiple statistical tests, for example, hundreds and thousands of SNPs are performed simultaneously; and is calculated independently for each study. This newly estimated significance level will reduce the false positive rate (Abdi, 2007). The significance threshold was obtained by dividing the observed *p*-value (0.05) by the number of tests performed or loci tested (552 695):

$$P - \text{value} = \frac{0.05}{552\,695} = 9.05 \text{ x } 10^{-8}$$

3.7 SNP- AND GENE ANNOTATION

SNP and gene annotation were completed by using NCBI (*Ovis aries*_v4.0; https://www.ncbi.nlm.nih.gov/) and Ensembl (ovine genome assembly 3.1; https://www.ensembl.org). With NCBI, SNP names, -positions and positions of genes are provided. Gene function and -ontology (GO) were identified and described by using genomic databases including the latter and UniProt (http://www.uniprot.org/). Physiological functions and gene mechanisms of the genes involved were referenced from other species (mainly human, rat and mouse) using NCBI, Ensembl and UniProt databases and web pages, since very limited information from the genes on the ovine genome was available in the literature. By using the sheep QTL database (http://www.animalgenome.org), existing loci for both autosomes and sex chromosomes could be identified to associate candidate genes with the detected loci.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 QUALITY ASSESSMENT

Three animals were removed due to both the call rate and excess heterozygosity (one afflicted and two unafflicted). Analysis of pairwise IBD resulted in eight additional animals being removed. Thus, during the initial individual quality control assessment 11 individuals (seven afflicted and four unafflicted) were removed from the dataset. As a result, 36 afflicted and 37 unafflicted individuals were subjected to further SNP QC analysis. However, following sex assignment, two more individuals were removed since they failed --*check-sex* option whereby PLINK could not successfully assign their sex. As a result, a total of 13 individuals were excluded and 71 individuals (40 males and 31 females), representing 35 afflicted (21 males and 14 females) and 36 unafflicted (19 males and 17 females) carcasses remained in the data set for further analysis.

The first step in the SNP QC assessment excluded all SNPs (1 291 SNPs) without a known chromosomal position on the ovine genome (*Ovis aries* Oar_v4.0). Thereafter, a total of 604 715 SNPs were examined for low call rates, and low minor allele frequencies (MAF).

Testing multiple combinations of the different quality control parameters allowed for the creation of a comprehensive SNP dataset. Combining a SNP call rate of 0.1, MAF of 0.01 (Uemoto *et al*., 2015; Hao *et al*., 2017) (combination one from Table 3.1) allowed for the greatest number of SNPs to be used (Table 4.1). Turner *et al*. (2011) suggested the use of a lower call rate when small sample sizes are used within a GWAS study. All SNPs that were genotyped for less than 90 % of the samples were removed (Turner *et al*., 2011; Zhang *et al*., 2013; Hao *et al*., 2017). When the --*maf 0.05* parameter was used, at least 100 000 SNPs were removed, making it almost double compared to when --*maf 0.01* was used and approximately 56 000 SNPs were removed.

Table 4.1 Multiple combinations (comb 1-4) of marker quality control parameters using 71 individuals (35 afflicted and 36 unafflicted).

	Comb. 1	Comb. 2	Comb. 3	Comb. 4
Total SNPs before QC				606 006
No chromosomal position				1 291
Missing rate per SNP (*geno* ≥)	0.1	0.05	0.05	0.1
Minor allele frequency (*maf* ≤)	0.01	0.01	0.05	0.05
Total SNPs removed	52 020	55 932	110 437	106 818
Total SNPs available for analysis	552 695	548 783	494 278	497 897

For case-control studies, HWE is only assumed for control samples, therefore only SNPs deviating for HWE in the control samples would be removed (Balding, 2006; Ziegler *et al*., 2011b; Alonso *et al*., 2015). Deviations from HWE can occur either by chance, genotyping error, inbreeding or population stratification (Wittke-Thompson *et al*., 2005; Miyagawa *et al*., 2008). Subsequent to individual-, SNP QC and sex assignment the total number of individuals suitable for the final step of the statistical analysis were 71 animals, comprising of 35 afflicted- and 36 unafflicted carcasses and 552 695 SNPs.

4.2 LINKAGE DISEQUILIBRIUM

With the *--indep-pairwise 50 5 0.2* option, 487 442 SNPs were excluded and thus only 65 253 SNPs remained. For *--indep-pairwise 50 5 0.5*, 324 927 SNPs were excluded, thus 227 768 SNPs remaining. By using the latter LD estimation, an intermediate number of SNPs were removed, while with the former only around 13 % of the total number of SNPs would have been included.

Prior to the current study, no genetic information was available for WCS. Therefore, by not using an LD pruned dataset for the association analysis, all possible genomic regions of interest and available SNPs (after QC) could be explored to find any potential association with WCS. However, the LD pruned dataset was used to conduct the PCA analysis. SNPs in approximate linkage equilibrium (LE) have proven to be more valuable than SNPs in approximate linkage

disequilibrium (LD), due to the latter having a possible influence on the PCA (Anderson *et al*., 2010; Laurie *et al.*, 2010).

4.3 POPULATION STRATIFICATION

4.3.1 Principal Component Analysis

In principle, all the genomic data were used to calculate the relatedness between each possible pair of samples (Alonso *et al*., 2015). Each principal component was plotted against all others by using a scatter plot with each point representing an individual. As expected a relatively homogenous group of carcasses were sampled, implying very little population stratification (Figure 4.1). The proposed sampling of individuals from only one breed i.e. the Dorper within specified geographical regions may have allowed for a homogenous group. However, two animals, both afflicted individuals from the Groblershoop (GR) group (highlighted light blue) were identified as clear outliers and were removed for further analysis. Two additional individuals (highlighted in pink), both unafflicted and from Groblershoop, were also identified as being probable outliers. Results were similar whether data from the 67 animals were analysed or the analysis was conducted using these probable outliers as well. Thus, the two individuals were not removed. After individual- and marker quality control as

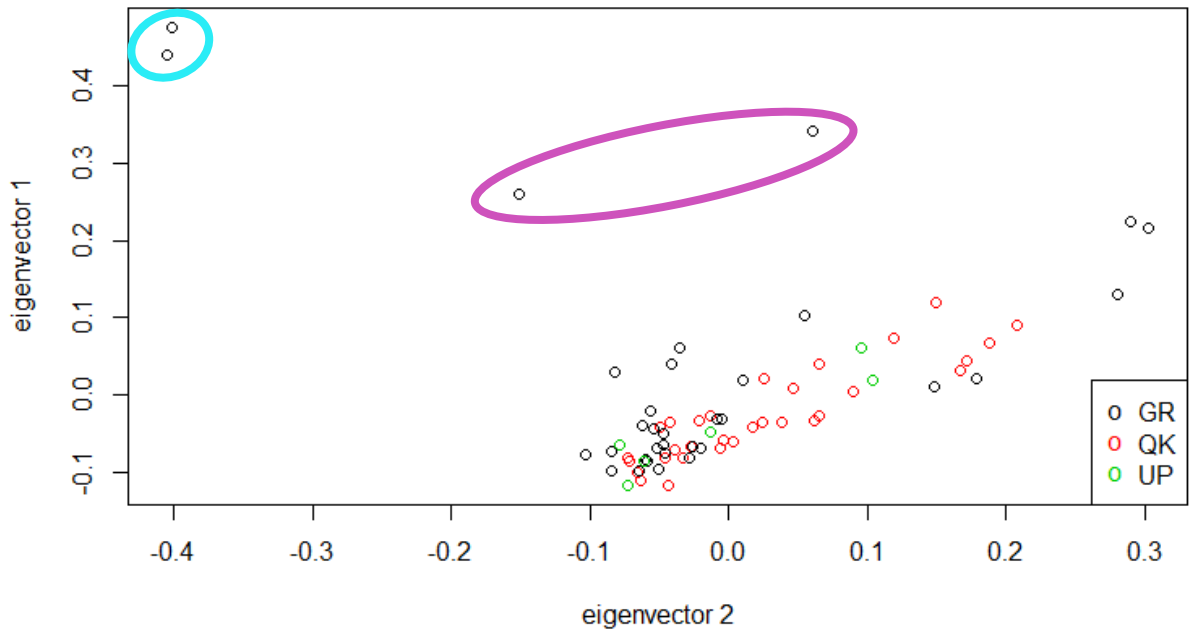


Figure 4.1 Principal component analysis after initial individual- and SNP quality control. Plot of the first two eigenvectors from the three sampled areas of Groblershoop (GR), Upington (UP), and Central distribution centre (QK).

well as PCA outlier removal, the total number of individuals suitable for the final steps of the statistical analysis were 69, comprising of 33 afflicted and 36 unafflicted samples.

4.4 COMPARATIVE GENOMICS

Since the current study is the first to investigate a genetic basis for WCS, candidate genes specific to WCS have not been identified previously. However, phenotypically similar conditions (PSE and the *Rendement Napole*) occured in pigs and result from mutations in *RYR1* and *PRKAG3*, respectively. Homologs of the porcine gene *RYR1* and *PRKAG3* are found at positions 473 243 80-474 469 18 on Chromosome 14 and 219 776 505-219 783 386 on Chromosome 2 in the *Ovis aries* (Oar_v4.0) reference genome (https://www.ncbi.nlm.nih.gov/), respectively. Synteny between the porcine *RYR1* and *PRKAG3* nucleotide sequences and the ovine genome confirmed their positions in the ovine genome (Oar v3.1) (Figure 4.2).

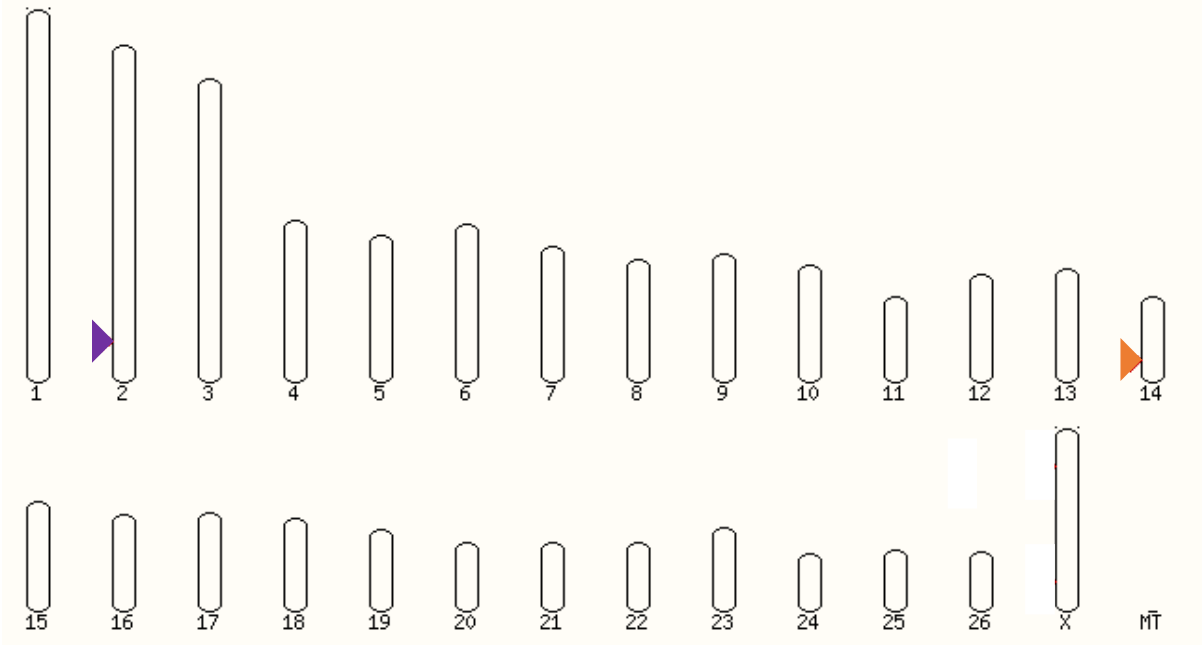


Figure 4.2 *PRKAG3* (purple) and *RYR1* (orange) genes as candidate loci for WCS based on synteny with the porcine *RYR1* and *PRKAG3* nucleotide sequence on *Ovis aries* (Oar v3.1) karyotype. Figure adapted from the Ensembl webpage (https://www.ensembl.org/Ovis_aries/Location/Genome).

As described in Chapter two, a single point mutation, at Nucleotide 1843 within the *RYR1* is responsible for the porcine stress syndrome (PSS) condition that results in PSE meat. The latter caused an alteration in the amino acid sequence where arginine is replaced with cysteine at Position 615. This mutation affects the SR Ca^{2+}-release channel and results in the channel opening, but closing is inhibited. Therefore, the mutation combined with pre-slaughter stress causes rapid glycolysis early post-slaughter where the pH of the carcass meat is lower than six (< 6) at 45 minutes' post-slaughter. Thus, causing PSE meat. Twenty-five SNPs, starting from oar3_OAR14_47433539 to oar3_OAR14_47543981, were located within the *RYR1* gene, however none of these was significantly related to the occurrence of the WCS phenotype.

The *PRKAG3* gene encodes the γ3 subunit of the enzyme 5′AMP-activated protein kinase (AMPK) (Cheung *et al*., 2000; Milan *et al*., 2000). This enzyme is primarily expressed in the skeletal muscles and is essential in the regulation of energy metabolism, helps with preservation of ATP in cells and is involved with the metabolism of fatty acids and carbohydrates in adipose tissue, skeletal muscle and other tissues (Winder and Hardie, 1999; Cheung *et al*., 2000; Milan *et al*., 2000). The causal mutation is a base pair (bp) substitution of R200Q between markers SW120 and SW936 on Chromosome 15 in the porcine genome. Post-slaughter, the glycogen is altered to lactic acid, thereby causes a lowered ultimate pH which results in reddish, soft and exudative meat. Three SNPs, oar3_OAR2_219781182, oar3_OAR2_219782879, and oar3_OAR2_219785153, were located within *PRKAG3*, and as with *RYR1*, no association was found between them and the WCS phenotype. It is concluded that neither *RYR1* nor *PRKAG3* were plausible candidate genes for the WCS phenotype.

4.5 RUNS OF HOMOZYGOSITY

If the causative mutation were an autosomal recessive inherited, homozygosity resulting from both the maternal and paternal lineages would be required for its expression in afflicted individuals. This homozygosity would presumably extend outward from the mutant locus due to LD (Broman and Weber, 1999; Gibson *et al*., 2006; Li *et al*., 2006). Thus, to determine whether matching homozygous regions existed in afflicted males and females (autosomes) and afflicted females (X chromosome), the test ROH was used by PLINK. By identifying ROH, candidate loci within these regions may harbor the causal mutation for a recessive genetic defect.

In sheep, several previous studies have assessed ROH (e.g. Al-Mamun *et al*., 2015; Muchadeyi *et al*., 2015; Mastrangelo *et al*., 2017; Purfield *et al*., 2017). Mastrangelo *et al*. (2017) and Purfield *et al*. (2017) specifically identified genomic regions of interest that may harbor candidate genes relating to traits involved in production.

The number of identified homozygous segments within individual females ranged from 36 to 129 with an average segment size ranging from 1588.8 kbp to 4734.85 kbp. For the afflicted females specifically, the number of homozygous segments ranged from 72 to 129 with an average segment size ranging from 2619.27 kbp to 3606.81 kbp. To search for ROH on the autosomes, all afflicted animals (N = 33 (19 males and 14 females)) were used. However, the results obtained did not show overlapping homozygous segments that were present within all the afflicted animals on any of the autosomes. The highest number of afflicted animals sharing an overlapping homozygous segment was 24 on Chromosome 3 (OAR3). Thus, the results of the test of ROH on the autosomes suggested the absence of a recessive mutation on any of the autosomes which harbors the causal locus for WCS.

To search for ROH on the X chromosome, only afflicted female animals (N = 14) were used. However, again no such overlapping homozygous regions on the X chromosome (OARX) were identified. The greatest number of afflicted females sharing an overlapping homozygous segment on OARX was 13. However, when the latter homozygous segment was tested against all unafflicted females, they also shared this specific segment. Therefore, the results yet again do not indicate a candidate homozygous region that could potentially harbor a recessive mutation causing WCS.

4.6 ASSOCIATION ANALYSIS

4.6.1 Association Analysis for the Autosomes

To test for an association between genomic markers and the WCS, the Fisher's Exact test using 69 animals were conducted. Approximately 13 SNPs distributed throughout the genome on five autosomes, Chromosome 1 (OAR1), Chromosome 4 (OAR4), Chromosome 8 (OAR8), Chromosome 11 (OAR11) and Chromosome 24 (OAR24) and eight SNPs on X Chromosome (OARX) were detected by demonstrating *P*-values ($P \leq 1 \cdot 10^{-5}$) below the suggestive significance threshold of WCS (Figure 4.3). These SNPs with the relevant information i.e. SNP

names, reference SNP ID, chromosomal positions, base pair (bp) position according to the most recent *Ovis aries* (Oar_v4.0) reference genome (https://www.ncbi.nlm.nih.gov/), unadjusted Fisher's exact test *P*-values, and their related genes are listed in Table 4.2. The related genes are described either as the gene of whom the relevant SNP lies within or an intergenic region i.e. a region in the genome where no genes have been annotated till yet (the gene within brackets next to the intergenic region is described as the gene in closest proximity to the relevant SNP).

Several loci positioned on the autosomes demonstrated *P*-values recognizable as suggestive associations. Given these SNPs, no other loci in approximate LD within their genomic regions showed association with WCS. Although the following singular SNPs, OAR1_245730132.1 (OAR1), oar3_OAR4_44743238 (OAR4), OAR8_45760589.1 (OAR8) and oar3_OAR24_9440581 (OAR24) showed suggestive associations with the WCS, it is possible that they are genotyping artefacts as a consequence of small sample size. However, it is possible that when a large number of animals are used, the frequency of these 'SNP artefacts' may decrease and minor peaks due to SNP association will become more apparent. The latter argument is especially applicable to the peak on OAR11 (oar3_OAR11_35812793), as it has the most significant *P*-value and the other loci in LD with the latter ensuing. All other genes with their respective influences on cellular components, molecular function and biological processes listed in Table 4.2 are described by and adapted from http://www.ensembl.org and http://www.uniprot.org, and recorded in Addendum A.

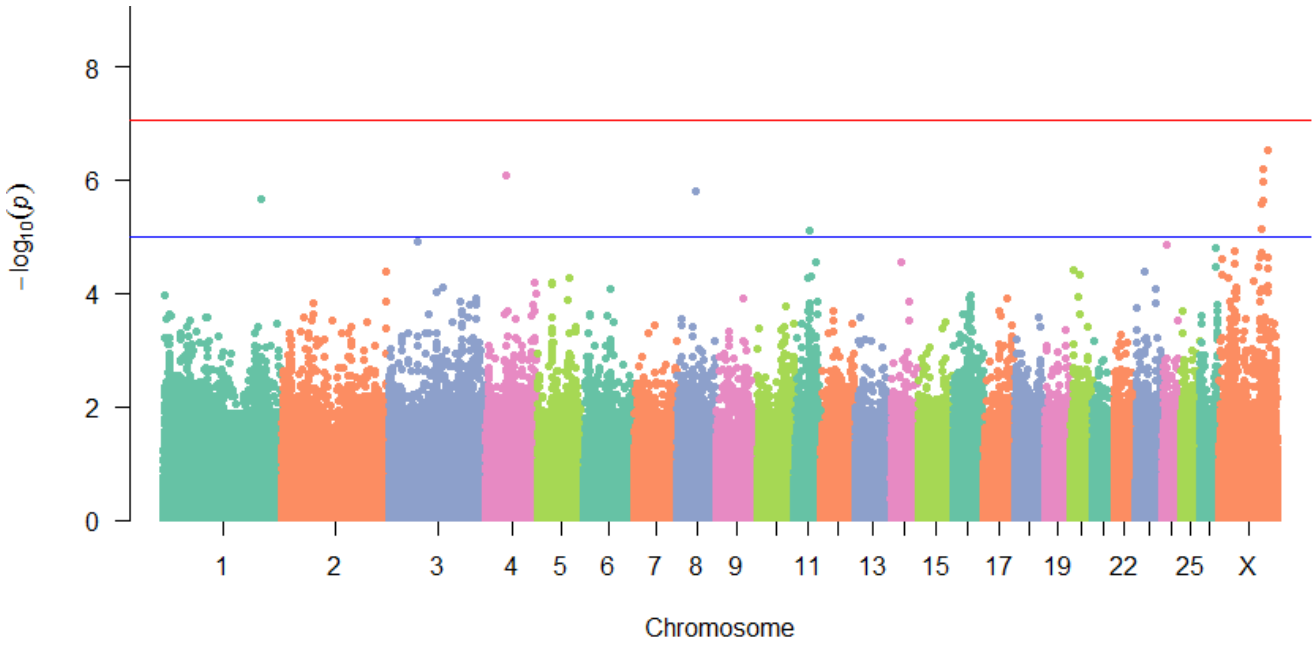


Figure 4.3 Manhattan plot displaying results of the Fisher's Exact test of 69 samples including both males and females. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values (representative of a significance level) are plotted on the y-axis. The strongest associations will have the smallest *P*-values accordingly; their negative logarithms will be the greatest. The blue line represents suggestive association (–log10($^{-5}$)) whereas the red line depicts the genome-wide significance threshold of –log10($9 \cdot 10^{-8}$) after Bonferroni correction.

The results from the current study: i) comparative genomics using nucleotide sequences causing phenotypically similar conditions to WCS in other species; ii) the lack of ROH in both afflicted males and –females on the autosomes; and iii) the lack of genome-wide significant SNPs from the GWAS provided conceivable evidence that previously identified candidate loci on some of the autosomes on the ovine genome are not supported as candidate loci for WCS. Compared to the autosomes, the apparent peak positioned on OARX motivated the analysis of males and females separately. Consequently, the following association analyses were conducted using males (afflicted and unafflicted) and females (afflicted and unafflicted) separately.

Table 4.2 Loci on the X chromosome and Autosomes showing suggestive association with WCS.

SNP	Chr	RefSNP ID	Position on Oar_v4.0 (bp)	Unadjusted *P*-value	Related genes
oar3_OARX_113973214	X	rs399985763	113751984	$4.67 \cdot 10^{-7}$	Intergenic (LOC101108550, HTR2C)
OAR1_245730132.1	1	rs414552741	227901188	$5.61 \cdot 10^{-7}$	intergenic (VEPH1)
oar3_OAR4_44743238	4	rs424871551	44720173	$2.09 \cdot 10^{-6}$	RELN
oar3_OARX_113988383	X	rs414187600	113767153	$2.43 \cdot 10^{-6}$	Intergenic (LOC101108550, HTR2C)
oar3_OARX_35953513	X	rs400747921	35932202	$3.59 \cdot 10^{-6}$	Intergenic (LOC101121576)
oar3_OARX_102834391	X	rs416468050	102667437	$4.24 \cdot 10^{-6}$	TENM1
oar3_OAR11_35812793	11	rs406416952	35763513	$4.56 \cdot 10^{-6}$	Intergenic (ACSF2)
oar3_OARX_102811671	X	rs413794928	102644717	$4.87 \cdot 10^{-6}$	TENM1
oar3_OARX_102817582	X	rs422039869	102650628	$4.87 \cdot 10^{-6}$	TENM1
oar3_OAR24_9440581	24	rs402465452	9442405	$5.40 \cdot 10^{-6}$	NUBP1
OAR8_45760589.1	8	rs403004733	42572637	$5.74 \cdot 10^{-6}$	Intergenic (LOC101107027)
oar3_OARX_98985496	X	rs416506282	98824617	$6.43 \cdot 10^{-6}$	TMEM255A
oar3_OARX_102819706	X	rs426965083	102652752	$9.40 \cdot 10^{-6}$	TENM1

4.6.2 Association Analysis for the Sex Chromosomes

Separate analyses for each sex were motivated by the loci on OARX that approached a significant association with the WCS phenotype. In the data there were 38 males (19 afflicted and 19 unafflicted) and 31 females (14 afflicted- and 17 unafflicted individuals).

4.6.2.1 Association Analysis using Male Only Individuals

Two loci were found on OARX to have significant ($P < 9.05 \cdot 10^{-8}$) associations with WCS. Both loci are within the non-homologous region of the X chromosome (Das *et al.*, 2009; Figures 4.4a and 4.5). Thus, SNPs and their associated genes in these regions are hemizygous in the males.

The four most significant SNPs (listed in order of significance) were oar3_OARX_29903534 ($P = 4.56 \cdot 10^{-8}$), oar3_OARX_113973214 ($P = 6.16 \cdot 10^{-8}$), oar3_OARX_102819706 ($P = 9.12 \cdot 10^{-8}$)) and oar3_OARX_102834391 ($P = 9.12 \cdot 10^{-8}$). The SNP oar3_OARX_29903534 and OARX_113973214 are separated by approximately 83 Mbp, OARX_113973214 is approximately 11 Mbp downstream of oar3_OARX_102819706 and oar3_OARX_102834391. The SNP oar3_OARX_29903534 is located within the *Duchenne muscular dystrophy* (*DMD*) gene (Position: 28400851 – 31063619; https://www.ncbi.nlm.nih.gov/genome/?term=sheep). The *DMD* gene is X-linked and encodes for the dystrophin protein (Hoffman *et al.*, 1987; Koenig *et al.*, 1988). The SNP oar3_OARX_113973214, was found to be in an intergenic region. However, this marker is positioned roughly 6 kbp to the left of the position of SNP oar3_OARX_113973214 (upstream) from the *5-hydroxytryptamine (serotonin) receptor 2C* (*LOC101108550/HTR2C*; Position: 113674155 – 113745911; https://www.ncbi.nlm.nih.gov/genome/?term=sheep) gene. SNPs oar3_OARX_102819706 and oar3_OARX_102834391 lies within the *Teneurin Transmembrane Protein 1* (*TENM1*) gene (Position: 102506451 – 103422241; https://www.ncbi.nlm.nih.gov/genome/?term=sheep). These genes and their functions are discussed in more detail in Section 4.7.

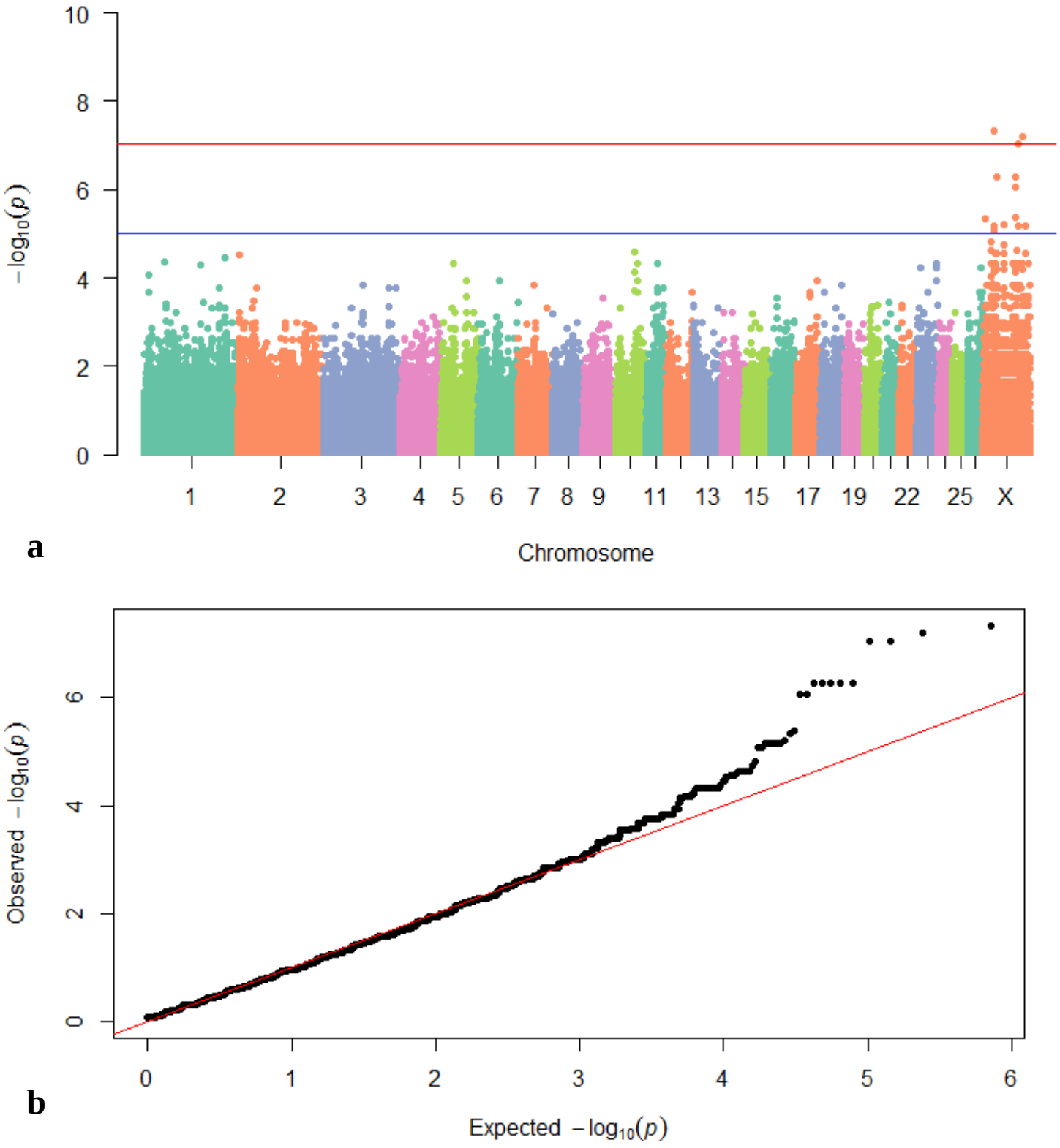


Figure 4.4 a) Manhattan plot displaying results of the Fisher's Exact test of only 38 male individuals. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values (representative of a significance level) are plotted on the y-axis. The strongest associations will have the smallest *P*-values accordingly; their negative logarithms will be the greatest. The blue line represents suggestive association (–log10($^{-5}$)) whereas the red line depicts the genome-wide significance threshold of –log10($9 \cdot 10^{-8}$) after Bonferroni correction; **b)** The quantile-quantile (Q-Q) plot displays both the distribution of results. The observed *P*-values (y-axis) is plotted against the expected *P*-values (x-axis). Each point represents a SNP and is plotted against the null hypothesis (red line).

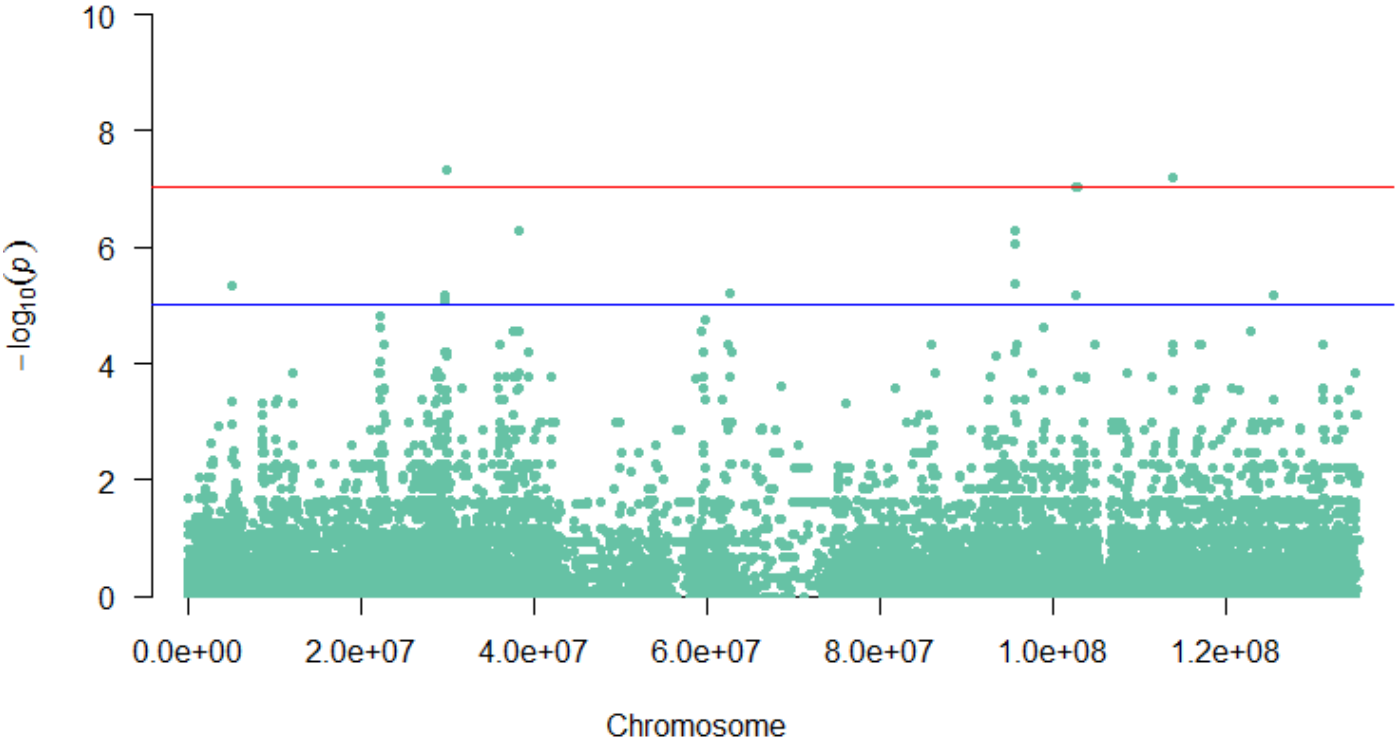


Figure 4.5 Manhattan plot displaying results of the Fisher's Exact test of 38 male individuals on the X chromosome only. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values (representative of a significance level) are plotted on the y-axis. The strongest associations will have the smallest *P*-values accordingly; their negative logarithms will be the greatest. The blue line represents suggestive association (–log10($^{-5}$)) whereas the red line depicts the genome-wide significance threshold of –log10($9 \cdot 10^{-8}$) after Bonferroni correction.

Table 4.3 Loci of the X chromosome and autosomes showing both suggestive- and genome-wide association using male only WCS individuals. Loci are ranked according to *P*-values.

SNP	Chr	RefSNP ID	Position on Oar_v4.0 (bp)	Unadjusted *P*-value	Related genes
oar3_OARX_29903534	X	rs403503557	29903897	$4.56 \cdot 10^{-8}$	DMD
oar3_OARX_113973214	X	rs399985763	113751984	$6.16 \cdot 10^{-8}$	Intergenic (LOC101108550, HTR2C)
oar3_OARX_102819706	X	rs426965083	102652752	$9.12 \cdot 10^{-8}$	TENM1
oar3_OARX_102834391	X	rs416468050	102667437	$9.12 \cdot 10^{-8}$	TENM1
oar3_OARX_38329627	X	rs415290122	38316347	$5.36 \cdot 10^{-7}$	USP9X
oar3_OARX_38320651	X	rs399727660	38307371	$5.36 \cdot 10^{-7}$	USP9X
oar3_OARX_95632854	X	rs399297015	95501768	$5.36 \cdot 10^{-7}$	Intergenic (MIR106A)
oar3_OARX_95618774	X	rs404382936	95487688	$5.36 \cdot 10^{-7}$	Intergenic (MIR106A)
oar3_OARX_95609744	X	rs401788046	95478658	$5.36 \cdot 10^{-7}$	Intergenic (MIR106A)
oar3_OARX_95695568	X	rs420523058	95563699	$8.62 \cdot 10^{-7}$	Intergenic (GPC3)
oar3_OARX_95694509	X	rs408024224	95562640	$8.62 \cdot 10^{-7}$	Intergenic (GPC3)
oar3_OARX_95670658	X	rs399200833	95539292	$4.11 \cdot 10^{-6}$	Intergenic (MIR106A/GPC3)
oar3_OARX_5074875	X	rs430050128	5080978	$4.47 \cdot 10^{-6}$	Intergenic (TRNAC-GCA)
oar3_OARX_62630133	X	rs404630971	62594195	$6.02 \cdot 10^{-6}$	Intergenic (LOC105605507)
oar3_OARX_29825132	X	rs427073791	29825543	$6.80 \cdot 10^{-6}$	DMD
oar3_OARX_29840625	X	rs418698048	29841036	$6.80 \cdot 10^{-6}$	DMD
oar3_OARX_125542461	X	rs412047967	125315529	$6.80 \cdot 10^{-6}$	TAF7L
oar3_OARX_102811671	X	rs413794928	102644717	$6.80 \cdot 10^{-6}$	TENM1
oar3_OARX_102817582	X	rs422039869	102650628	$6.80 \cdot 10^{-6}$	TENM1
oar3_OARX_29667826	X	rs413909812	29667017	$8.15 \cdot 10^{-6}$	DMD
oar3_OARX_29692647	X	rs408305408	29691838	$8.15 \cdot 10^{-6}$	DMD
OAR10_57796569.1	10	rs422191497	56593483	$2.55 \cdot 10^{-5}$	Intergenic (LOC101103736)
oar3_OAR2_3956155	2	rs417312952	3986051	$2.92 \cdot 10^{-5}$	Intergenic (BRINP1)
oar3_OAR1_236236258	1	rs421724992	236086685	$3.42 \cdot 10^{-5}$	Intergenic (PFN2)
oar3_OAR5_34267605	5	rs411401092	34223848	$4.58 \cdot 10^{-5}$	Intergenic (LOC105611207)
oar3_OAR11_35812793	11	rs406416952	35763513	$4.59 \cdot 10^{-5}$	Intergenic (ACSF2/LRRC59)
oar3_OAR23_58585201	23	rs423305155	58545189	$4.59 \cdot 10^{-5}$	Intergenic (CCBE1)
oar3_OAR26_35923697	26	rs426001544	35902344	$5.93 \cdot 10^{-5}$	Intergenic (CHRNB3)
oar3_OAR23_9724433	23	rs406979990	9673264	$5.93 \cdot 10^{-5}$	Intergenic (DSEL)

4.6.2.2 Association Analysis using Female Only Individuals

The female sample group included 31 animals (14 afflicted- and 17 unafflicted individuals). In contrast with the analysis of data from males, the association analysis using data from females found no loci that were associated with WCS at neither a suggestive- nor genome-wide level (Figure 4.6a).

The strong evidence of an association between hemizygous alleles and WCS in males and the lack of even suggestive evidence of a similar association in females may be explained by X-inactivation in the females. As already described in Chapter two, an imbalance of the X chromosomes between males (XY) and females (XX) and in the absence of X-inactivation genes on the X chromosome will be over-expressed in females relative to males.

Generally, each maternal and paternal X chromosome contributes equally between tissues and causes females to exhibit an mosaic expression of both normal and mutant alleles when present (50:50) (Barr and Bertram, 1949; Lyon, 1961; Heard and Avner, 1994; Belmont, 1996; Clayton, 2009; Avery and Vrshek-Schallhorn, 2016). This potentially explains the lack of association between SNP located on OARX and WCS in the females. One approach for analysing data from females in the presence of X-inactivation is to use only those individuals with homozygous haplotypes and search for evidence of association within these data sets. For the data that are currently available, this further reduced the number of observations and together with relative low minor allele frequencies, prevented testing of independent homozygous genetic classes and WCS in the females.

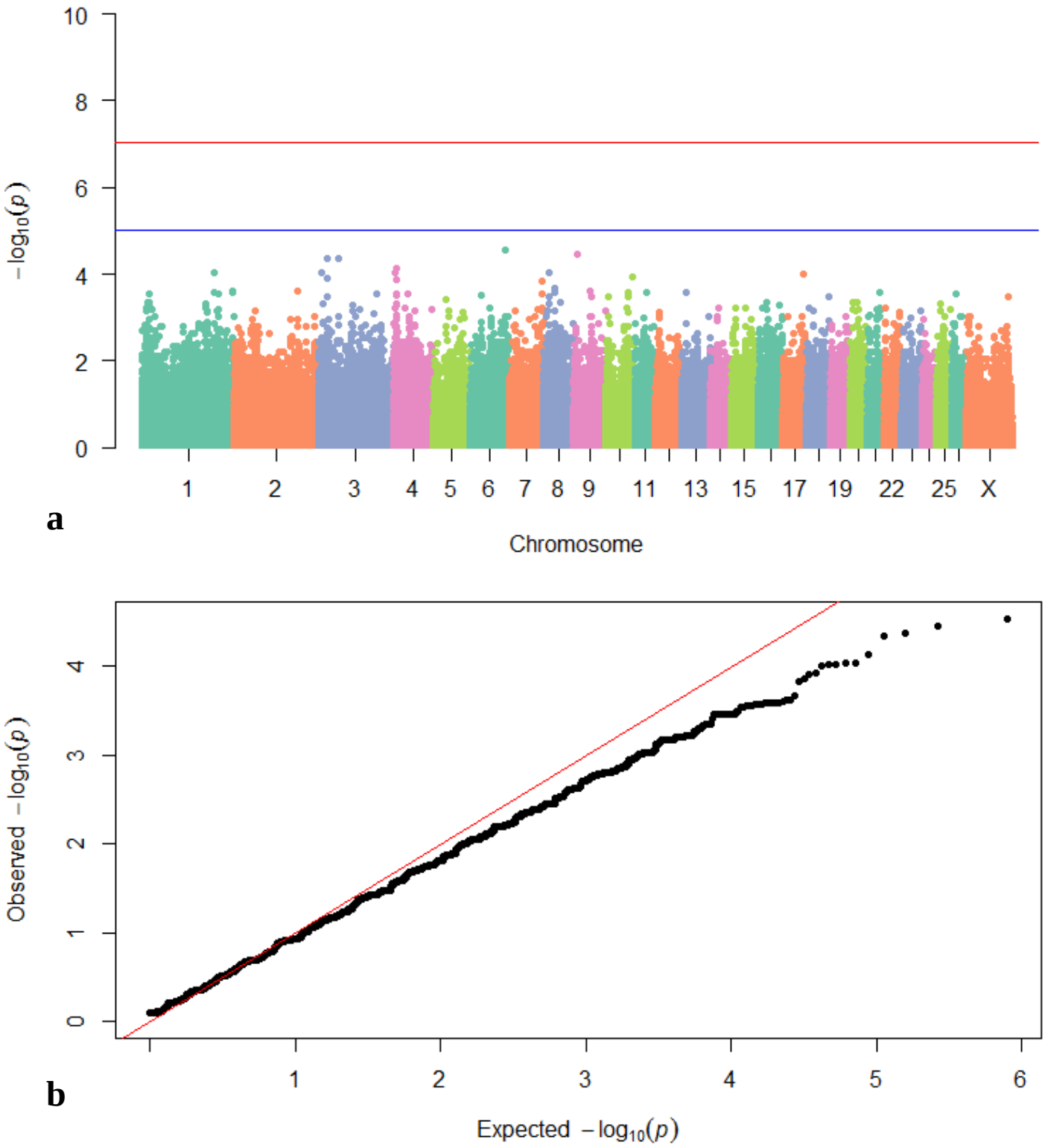


Figure 4.6 a) Manhattan plot displaying results of the Fisher's Exact test of only the 31 female individuals. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values (representative of a significance level) are plotted on the y-axis. The strongest associations will have the smallest *P*-values accordingly; their negative logarithms will be the greatest. The blue line represents suggestive association (–log10($^{-5}$)) whereas the red line depicts the genome-wide significance threshold of –log10($9 \cdot 10^{-8}$) after Bonferroni correction. **b)** The quantile-quantile (Q-Q) plot displays both the distribution of results. The observed *P*-values (y-axis) is plotted against the expected *P*-values (x-axis). Each point represents a SNP and is plotted against the null hypothesis (red line).

4.6.2.3 Genotype- and Allele Calls of Each Individual

To further investigate the separate relationship between males, females and WCS, the genotype calls for each individual at oar3_OARX_29903534 and oar3_OARX_113973214 were extracted from the raw data obtained from the Genotyping Module (https://emea.illumina.com). Since males are hemizygous for the X chromosome, they only have one allele whereas females have two alleles. Males therefore cannot be heterozygous for a particular genotype on the X chromosome. GenomeStudio® software called the allele(s) for each individual genotype for the two particular SNPs either A (DNA nucleotide = Adenine) or G (DNA nucleotide = Guanine). Since the genotype calls for each individual at both significant SNPs (oar3_OARX_29903534 and oar3_OARX_113973214) were investigated, the allele(s) for oar3_OARX_29903534 were depicted as A1 and G1, and for oar3_OARX_113973214, A2 and G2, to put it into more specified terms.

Tables 4.4 and 4.5 summarises the number of afflicted (males and females) and unafflicted (males and females) individuals carrying the different combinations of alleles possible for these loci. The males are presented with one only allele since they are hemizygous for the X chromosome, thereby requiring only the G2 allele from oar3_OARX_113973214 to manifest WCS phenotype.

For oar3_OARX_29903534 (Table 4.4), 16 afflicted males carried the G1 allele. A total of four unafflicted males also carried the G1 allele, while all others (a total of 15) carried the A1 allele. A total of three afflicted males therefore carried the A1 genotype. Thus, the ratio between afflicted males carrying the G1 allele and the unafflicted males carrying the associated A1 allele, was approximately 1:1 and *vise versa*.

For oar3_OARX_113973214 (Table 4.5), all afflicted males carried the same G2 allele. A total of nine unafflicted males also carried this G2 allele, while the other 10 were carriers of the A2 allele. Compared to the unafflicted males, almost double the number of afflicted males carried the G2 allele. Therefore, according to the allele frequencies of the afflicted males, the G2 allele presented with the strongest association with WCS, whereas the A2 allele represented the normal phenotype.

Table 4.4 Summarising the presence of oar3_OARX_29903534 (males carry either G1 or A1, whereas females carry either A1/G1, G1/G1 or A1/A1) on the X chromosome in both unafflicted and afflicted, males and females.

Phenotype	Number of animals	Number of animals in which one copy of the afflicted G1 allele is present (males only)	Number of animals in which one copy of the unafflicted A1 allele is present (males only)	Number of animals in which one copy of the G1- and one copy of the A1 allele are present (females only)	Number of animals in which both afflicted G1 alleles are present (females only)	Number of animals in which both unafflicted A1 alleles are present (females only)
Unafflicted Males	19	4	15	N/A	N/A	N/A
Afflicted Males	19	16	3	N/A	N/A	N/A
Unafflicted Females	17	N/A	N/A	9	7	1
Afflicted Females	14	N/A	N/A	6	6	2

Table 4.5 Summarising the presence of oar3_OARX_113973214 (males carry either G2 or A2, whereas females carry either A2/G2, G2/G2 or A2/A2) on the X chromosome in both unafflicted and afflicted, males and females.

Phenotype	Number of animals	Number of animals in which one copy of the afflicted G2 allele is present (males only)	Number of animals in which one copy of the unafflicted A2 allele is present (males only)	Number of animals in which one copy of the G2- and one copy of the A2 allele are present (females only)	Number of animals in which both afflicted G2 alleles are present (females only)	Number of animals in which both unafflicted A2 alleles are present (females only)
Unafflicted Males	19	9	10	N/A	N/A	N/A
Afflicted Males	19	19	0	N/A	N/A	N/A
Unafflicted Females	17	N/A	N/A	6	11	0
Afflicted Females	14	N/A	N/A	2	12	0

With regard to the females on the oar3_OARX_29903534 (Table 4.4), six afflicted females carried the G1/G1 genotype, while seven unafflicted females also carried the same G1/G1 genotype. Three females (one unafflicted and two afflicted) carried the A1/A1 genotype. As a result, approximately 50 % (a total of nine) of the females were heterozygous (G1/A1) for the oar3_OARX_29903534 SNP. The afflicted females (a total of six) and unafflicted females (a total of seven) carrying the 'associated' genotype (G1/G1) were in approximately equal numbers, thus providing the reasoning behind no apparent association being found within the female association analysis (Figure 4.6). Females carrying the heterozygous genotype for oar3_OARX_29903534, included six afflicted- and nine unafflicted for afflicted- and unafflicted individuals, respectively, accounted for approximately 50 % of the total number of females in the sample set.

For the oar3_OARX_113973214 SNP, nearly all afflicted (a total of 12) and 60 % of the unafflicted (a total of 11) female individuals (Table 4.5) carried the G2/G2 genotype. As with the former SNP, the ratio of afflicted females to unafflicted females carrying the G2/G2 genotype again resulting in the apparent lack of found association with the WCS phenotype (Figure 4.6). A total of six unafflicted- and two afflicted females were heterozygous (A2/G2) for oar3_OARX_113973214. Neither afflicted- nor unafflicted females carried the A2/A2 associated normal genotype.

A total of 28 of the 33 afflicted animals (19 males and 14 females) carried at least one G1 allele for oar3_OARX_29903534. However, for oar3_OARX_113973214, all afflicted animals carried at least one G2 allele. Consequently, results implicated that a strong association in both males and females exists with the oar3_OARX_113973214 SNP.

Table 4.6 summarises the number of males carrying allele combinations of oar3_OARX_29903534 (G1 and A1) and oar3_OARX_113973214 (G2 and A2) while, Table 4.7 produce similar results based only on the female individuals. Given the information provided in Table 4.4, two afflicted females carried the A1/A1 genotype, though, when combined with their genotypes of oar3_OARX_113973214 i.e. A1/A1-G2/G2 and A1/A1-A2/G2, they carried at least one G2 allele. The female with the A1/A1-G2/G2, presented strong genotypic and phenotypic association with WCS, therefore most likely independent of XCI, due to the individual carrying the homozygous genotype (G2/G2) at oar3_OARX_113973214. The female with A1/A1-A2/G2 genotype, also presented with both a genotypic and phenotypic association with WCS. This female was heterozygous (A2/G2) at oar3_OARX_113973214,

and therefore the association was likely to be dependent on XCI skewing, selecting against the normal A2 allele.

It is also worth mentioning that from Table 4.5 and 4.6, 11 unafflicted females (G2/G2) and nine unafflicted males (G2) presented with a strong genotypic but not a phenotypic association with WCS. Though given the apparent influence of stress on WCS which is continuously discussed throughout this study, these males and females, in all likelihood did not experience adequate levels of stress to manifest the WCS condition post-slaughter.

Proc FREQ of SAS/STAT software (SAS, 2015) was used to perform a Chi-Square (χ^2) test of dependence, for males, between the two variables: WCS condition (afflicted and unafflicted) and SNP G2 (presence of G2 and absence of G2) for oar3_OARX_113973214. The data from the two by two contingency table, Table 4.8, was analysed yielding significant dependence between WCS condition and SNP G2 with $\chi^2 = 13.57$ ($P = 0.0002$). Furthermore, residual analysis of the results showed that unafflicted males were significantly associated with the absence of G2 ($\chi^2 = 5$, $P<0.05$), thus, a strong relationship exists between unafflicted males and not carrying the G2 of oar3_OARX_113973214. To the contrary, a strong positive relationship exists between afflicted males and the presence of G2 oar3_OARX_113973214 ($\chi^2 = 5$, $P<0.05$).

Table 4.6 Summarising the number of males carrying the different allele combinations of oar3_OARX_29903534 (G1 and A1) and oar3_OARX_113973214 (G2 and A2).

	G1/G2	**A1/A2**	**G2/A1**	**A2/G1**
Unafflicted males (19)	3	9	6	1
Afflicted males (19)	16	0	3	0

Table 4.7 Summarising the number of females carrying the different allele combinations of oar3_OARX_29903534 (G1 and A1) and oar3_OARX_113973214 (G2 and A2).

	G1G1/G2G2	**G1A1/G2G2**	**A1A1/G2G2**	**A1A1/A2G2**	**G1A1/G2A2**	**A1A1/A2A2**	**G1G1/G2A2**	**A2A2/G1G1**
Unafflicted Females (17)	5	5	1	0	4	0	2	0
Afflicted females (14)	6	5	1	1	1	0	0	0

Table 4.8 Number of afflicted- and unafflicted males that are either carriers- or non-carriers of allele G2 from SNP oar3_OARX_113973214.

	Afflicted	**Unafflicted**
Carrier of G2	19	9
Non-carrier of G2	0	10

4.7 DISCUSSION ON CONCEPTS AND OBSERVATIONS

4.7.1 Phenotypic and Genotypic Observations

The genotypic profiles for some of the sampled individuals indicated the presence of genetically susceptible animals (unafflicted animals carrying the same genotype as the afflicted animal G/G or G genotype) not expressing the WCS phenotype. A sensible explanation could be the lack of sufficient stress the animals experienced prior to slaughter. This phenomenon is known as incomplete penetrance. Accordingly, some individuals carry the mutation but do not express the phenotype (Zlotogora, 2003; Shawky, 2014). The latter has previously been observed in PSS pigs (http://omia.org/OMIA000621/9823/). A relationship between WCS and incomplete penetrance may perhaps exist when the true causal variant is identified as being one of the two most significant SNPs i.e. oar3_OARX_29903534 or oar3_OARX_113973214. Therefore, it can be speculated that the phenotype is expressed through the causal mutation and linked to one of the proposed candidate genes. However, it is very rare that a causal variant is one of the actual genotyped SNPs (Pearson and Manolio, 2008; Manolio, 2010). It is therefore most improbable, but not impossible that one of these highly significant SNPs are the actual causal variant for WCS.

Given the concepts and deliberations described above, a rare X-linked dominant pattern of inheritance seem to fit in with a scenario regarding WCS, given only female individuals is taken into account, since males are hemizygous. Moreover, considering the sample size and distinctive individuals with unusual genotype-phenotype ratios regarding the relevant significant SNPs, the role of incomplete penetrance should also be carefully considered. However, it should only be regarded as a theoretical statement since all discussions were solely based on loci that demonstrated a strong association with the phenotype, but is most likely not the direct causal variant of WCS.

4.7.2 Concepts Behind Possible Inheritance Patterns for WCS

To determine whether overlapping homozygous segments exist in all afflicted animals (both males and females), the current study used ROH. No such regions were identified. Therefore, the lack of any overlapping ROH provided plausible evidence to support the notion of WCS not being a recessive inherited genetic condition. The results obtained from the current study including the significant association between genomic markers on the X chromosome

and WCS as well as the frequency of afflicted males *vs.* afflicted females and frequency of the number of heterozygous afflicted- and unafflicted females led to the careful consideration of theoretical concept relating to the potential mode of inheritance i.e. X-linked inheritance, for WCS.

4.7.2.1 X-Linked Inheritance

To recapture the discussion from Chapter two, PSS is an autosomal recessive inherited condition. Therefore, the latter statement combined with the phenotypic similarities between PSS and WCS, led to the consideration of WCS also potentially being a recessive inherited condition. However, the observed association between WCS and SNPs on the X chromosome would suggest an X-linked condition instead of an autosomal recessive inherited condition. Nevertheless, this assumption is wholly based on the causal mutation essentially being one of the actual genotyped SNPs, with this rarely being the case as well as very low occurrence rate of WCS (0.2 to 1.5 % annually) that is similar to what is found in PSS cases.

Manifestation of a X-linked recessive condition within the homologous region of X has generally higher frequencies in males than females since males need only carry one copy of the mutated allele while females must have two copies of it to express the phenotype. Data from the current study consists of a sample set of approximately equal numbers of afflicted males and females. Furthermore, evidence of several WCS afflicted females carrying heterozygous genotypic profiles has been observed for some of the most significant SNPs. The latter two statements, combined with the absence of homozygous segments in afflicted females, provided credible evidence that the causal genetic variant for WCS is rare, X-linked dominant inherited and located within the non-homologous region on the X chromosome of the *Ovis aries* genome.

With an X-linked dominant inheritance, females only need one copy of the mutant allele (observed heterozygous genotypes from several WCS afflicted females) to exhibit the condition or phenotype (https://ghr.nlm.nih.gov/primer/inheritance/inheritancepatterns). Genotypic presentation of X-linked dominance is typically represented by $X^{D}X$, while the normal phenotype is represented by XX (Dudek, 2009). These principles can be applied to WCS afflicted males and -females. All offspring, both males and females, from an ewe that is a carrier of mutant allele causing WCS will have a 50 % chance to inherit the condition. It is

expected that the female offspring will either be a heterozygous afflicted, homozygous afflicted (provided the mutant allele is inherited from both ewe and ram) or homozygous unafflicted. All female offspring from a carrier ram will always be afflicted since the X chromosome is inherited whereas all male offspring will be unafflicted. When a female is a carrier and the male not, all offspring will present with a 50 % chance to inherit the condition. When both the male and female are carriers, all female offspring will inherit the condition while the male offspring will still have a 50 % chance to inherit the condition.

4.7.3 Concepts Behind X-Inactivation in WCS Afflicted Females

The concept behind X-inactivation, mosaic expression and WCS can be explained by females carrying the heterozygous genotype for the causal variant. These heterozygous females either: 1) exhibit the WCS phenotype i.e. afflicted carcass or; 2) exhibit normal carcass characteristics i.e. unafflicted carcass. Thus, in both cases the mutant- and the normal allele is present but only one is expressed. It is possible that with WCS afflicted female carcasses, skewing from the normal 50:50 (mutant:normal alleles) expression ratio may occur and the active X chromosome (carrying either mutant or normal allele) contributes the largest proportion to most tissue cells. As previously stated, the latter has either been discussed or observed by Belmont (1996), Migeon (2008), Minks *et al*. (2008) and Ørstavik (2009). If this condition can be linked to performance, as is the case with PSE in pigs, the skewing of the mosaic expression found in WCS heterozygous females can possibly be due to selection that favours the X chromosome carrying the mutant allele, thereby heterozygous afflicted females exhibit the phenotype. Conversely, selection can also possibly favour the X chromosome carrying the normal allele, thereby heterozygous unafflicted females that do not exhibit the phenotype. However, stress- i.e. transport and/or pre-slaughter stress, environmental conditions i.e. nutritional stress, individual variability, and even hormonal control (Castagné *et al*., 2011) as contributors to the phenotypic expression in WCS females should not be neglected.

4.7.4 Candidate Genes

Given the significant associations between WCS and SNPs on the X-chromosome greater attention is given to candidate genes in close proximity to oar3_OARX_113973214 and oar3_OARX_29903534. The positions of the candidate loci, *HTR2C* and *DMD*, are also illustrated by using the ovine karyotype (Oar v3.1) that was generated by Ensembl (https://www.ensembl.org/Ovis_aries/Location/Genome) (illustrated in Figure 4.7).

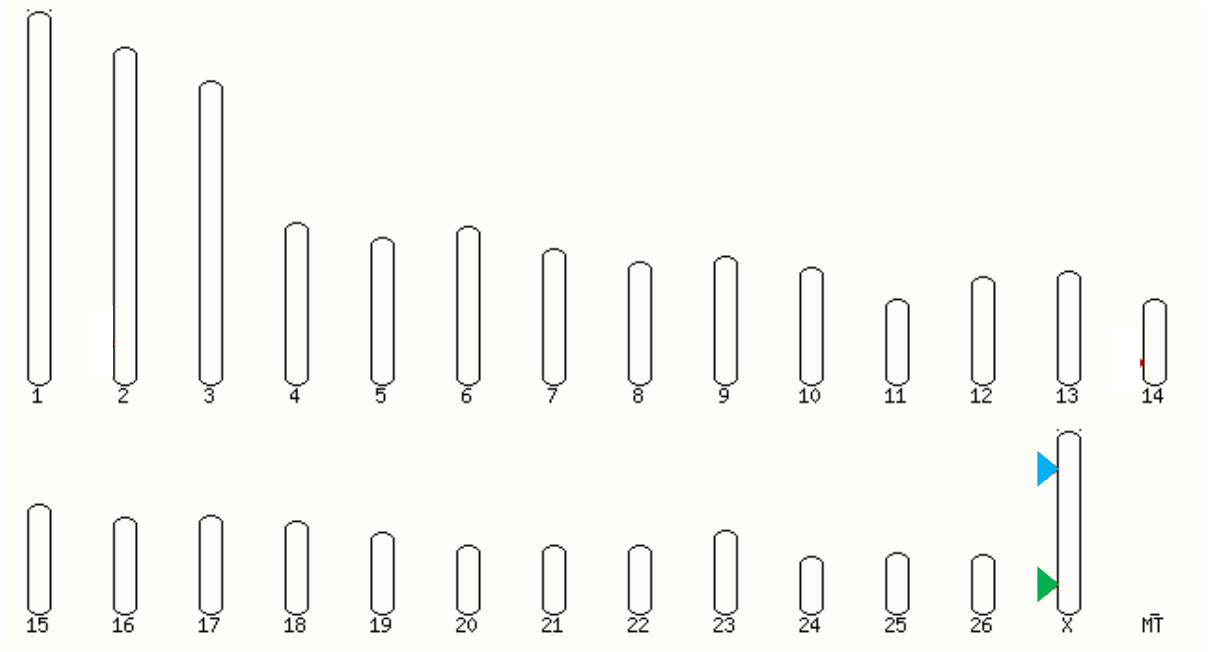


Figure 4.7 *DMD* (blue) and *HTR2C* (green) and genes as candidate loci for WCS positioned on the X chromosome of the *Ovis aries* (Oar v3.1) karyotype. Figure adapted from the Ensembl webpage (https://www.ensembl.org/Ovis_aries/Location/Genome).

4.7.4.1 HTR2C Gene

The SNP oar3_OARX_113973214 ($P = 6.16 \cdot 10^{-8}$), in the analysis of the samples from males) is closely linked to and downstream of the *5-hydroxytryptamine (5-HT, serotonin) receptor 2C* (*LOC101108550*/*HTR2C)* gene which maps to 113,815,511-114,086,474 bp on OARXq. This gene encodes the 5-HT_{2C} receptor.

Many functions of the *HTR2C* gene and its receptor exists, however, given the physiological characteristics of WCS, two of these functions specifically stand out that may show some

conceivable relevance to WCS (Addendum A). These functions included stress responsiveness and calcium ion homeostasis. It has been well speculated that stress (Jansen and Pretorius, 1986; Jansen, 1991) and the relationship between WCS (PSS in pigs and its relationship with stress (Hall *et al*., 1980; MacLennan and Phillips, 1992)) has a major influence on the manifestation of WCS in lambs. Mutations in the *RYR1* gene cause the calcium release channel to function improperly in PSS afflicted pigs, and under acute stress conditions PSS pigs result in PSE meat. It has also been well established that a phenotypic relationship exists between PSS and WCS. Therefore, the potential is recognised in that the *HTR2C* gene and gene product i.e. 5-HT_{2C} receptor, may disrupt the calcium ion homeostasis (described below) of WCS carcasses, and therefore result in a phenotypically similar carcass to PSE carcasses. Thus, these two genes *RYR1* (in pigs) and *HTR2C* (in sheep) may act either independently from each other or may as well be part of the same gene network that is functionally related to cause a similar phenotype in different species. Consequently, the likely role that calcium ion homeostasis and stress play in WCS can be established from the latter argument.

The 5-HT_{2C} receptors are involved in stress response (Miller and O'Callaghan, 2002; Donovan and Tecott, 2013) and anxiety (Griebel, 1995). Heisler *et al*. (2007) indicated the importance of 5-HT_{2C} receptors in the expression of anxiety-like behaviour while Avery and Vrshek-Schallhorn (2016) proposed that the increased activity of this receptor is related to an increased stress response in humans.

To use PSS afflicted pigs as an example, stress disrupts an animals' state of homeostasis (Miller and O'Callaghan, 2002). To counteract this effect, the hypothalamic-pituitary-adrenal (HPA) axis is activated in these pigs (Miller and O'Callaghan, 2002). The response by the HPA is mediated through the hypothalamus by the formation of corticotropin-releasing factor (CRF) (also known as corticotropin-releasing hormone (CRH); Denver, 2009). Finally, CRF/CRH in return regulates the processing of hormones such as the adrenocorticotropic hormone (ACTH) (Wurtman, 2002; Heisler *et al*., 2007a) that will assist an animal to effectively respond to stress. From all the different types of serotonin receptors, the 5-HT_{2C} receptor is predominantly expressed in the hypothalamus (Heisler *et al*., 2007b; Yadav *et al*., 2009) and can act as a neurotransmitter while assisting in the regulation of ACTH (Frazer and Hensler 1999). Heisler *et al*. (1998, 2003) described the interaction between HPA, serotonin and the role both play in anxiety, while the review of Lanfumey *et al*. (2008) described the interaction between the 5-HT_{2C} receptor and hypothalamic-pituitary-adrenal (HPA).

Serotonin receptors are grouped into seven families, 5-HT_1 to 5-HT_7 (Schaerlinger *et al*., 2003), of which most are G-protein-coupled receptors. These receptors are mostly associated with psychiatric and neurological disorders in humans (Villalón *et al*., 1997). The 5-HT_2 receptors are for example divided into three subtypes, 5-HT_{2A}, 5-HT_{2B}, and 5-HT_{2C} (Pompeiano *et al*., 1994; Villalón *et al*., 1997; Clemett *et al*., 2000). The latter receptor is involved in calcium ion homeostasis. The latter interacts with proteins such as the G_{q11}, Gi3 and G13 and increases both the inositol triphosphate hydrolysis and the intracellular Ca^{2+} concentration (Roth *et al*., 1984; Bonhaus *et al*., 1995; Cussac *et al*., 2002; Pytliak *et al*., 2011). More, specifically, 5-HT_{2C} receptors are also involved in processes such as the coordination of the intracellular responses to serotonin, specifically in central nervous system (CNS) of mammalian species, homeostatic and behavioural responses, stress response, sexual behaviour, control of appetite, obesity and feeding behaviour in mice (Tecott *et al*., 1995; Nonogaki *et al*., 2003; Quilter *et al*., 2012). The binding of 5-HT_2 receptors by agonists (extracellular messengers such as hormones), stimulates phospholipase C (PLC) by attaching to the G-protein (Roth *et al*., 1984; Chang *et al*., 2000; Campbell and Farrell, 2007). Phospholipase C is an enzyme which hydrolyses *phosphatidylinositol 4,5*-bis*phosphate* (PIP_2), which in return causes the formation of intracellular *inositol 1,4,5-triphosphate* (IP_3) and diacylglycerol (DAG) (Roth *et al*., 1984; Chang *et al*., 2000; Campbell and Farrell, 2007). Elevated levels of IP_3 then acts as a second messenger through the cytosol to mobilize the release of Ca^{2+} from the sarcoplasmic reticulum (SR) (Porter *et al*., 1999; Jerman *et al*., 2001; Campbell and Farrell, 2007; Knauer *et al*., 2009). As Ca^{2+} forms a complex with another protein called calmodulin within the cytosol, it also activates a protein kinase to phosphorylase target enzymes (Campbell and Farrell, 2007). This final activated product is the foundation for the normal desired cellular response such as smooth muscle contraction (Cohen *et al*., 1985; Campbell and Farrell, 2007). Knauer *et al*. (2009) demonstrated how 5-HT_2 receptors that are expressed in non-neuronal cells can link to phosphoinositide hydrolysis, Ca^{2+} utilization and the activation of mitogen-activated protein kinases (MAPK) via the utilization of protein kinase C (PKC) or Ca^{2+}.

Summarized here are two wholly hypothetical mechanisms that may result in the manifestation of WCS. 1) Genetic inactivation or malfunctioning of the 5-HT_{2C} receptor disrupts the regulation of the HPA axis through serotonin (Heisler *et al*., 2007b). This disruption causes the live animal to respond ineffectively to stress, whether it be long-term on the farm or acute pre-slaughter or handling and as a final consequence to manifest WCS post-slaughter. 2) The normal biochemical process of muscle contraction that is initialized by serotonin binding to the

receptor and ending with the continuing release of Ca^{2+} from the SR to the cytoplasm of the cells is disrupted with the end result being the reduced water holding capacity of the meat.

Finally, under the condition that the causal variant exists within the *HTR2C* gene of afflicted WCS carcasses, the above-mentioned pathways provided reputable theoretical evidence in what way the biochemical mechanism of WCS potentially works. Nevertheless, it is important to realize that this discussion is only from a hypothetical point of view and the exact mechanisms of the above-mentioned pathways and their interactions with the WCS phenotype are unknown since nearly all research regarding the *HTR2C* gene has been conducted using human and mouse trials. However, it serves as evidence that the *HTR2C* gene can be highlighted as a prospective candidate gene for WCS.

4.7.4.2 DMD Gene

From the male-only group, one specific SNP, oar3_OARX_29903534, also on OARX, displayed the highest genome-wide significance with WCS with a *P*-value of $4.56 \cdot 10^{-8}$. SNP oar3_OARX_29903534 is located within the dystrophin (*DMD*) gene.

The *DMD* gene is also X-linked and encodes the dystrophin protein (Hoffman *et al*., 1987; Koenig *et al*., 1988). The latter is a cytoskeletal protein that forms a linkage between dystrophin-associated protein complex (DAPC) intracellular and *y*–actin (Campbell, 1995; Allen and Whitehead, 2011). A further link is formed between DAPC and the extracellular matrix via laminin (Campbell, 1995; Allen and Whitehead, 2011). In humans, mutations in the *DMD* gene are the leading cause of Duchenne Muscular Dystrophy (DMD) in young boys, Becker Muscular Dystrophy (BMD) as well as X-linked dilated cardiomyopathy (XLCM) (Muntoni *et al*., 2003). In humans, dystrophin is absent in DMD patients and will manifest itself with symptoms such as progressive muscle weakness (Carlson and Makiejus, 1990; Cox *et al*., 1993; Blake *et al*., 2002) early in life. BMD has a later onset in life. However, with both diseases, increased plasma creatine phosphokinase (CPK) levels and dilated cardiomyopathy are observed (Kirchmann *et al*., 2005; Magri *et al*., 2011). Costamere disorganization, sarcolemmal instability and necrosis are also affected by the absence of dystrophin (Torres and Duchen, 1987; Weller *et al*., 1990; Matsuda *et al*., 1995; Williams and Bloch, 1999). Allen and Whitehead (2011) proposed increased CPK levels are indicative of an increase in permeability of the muscle surface membrane, which allows for calcium ions moving into the intracellular

region and enzymes to move out. However, the exact cause of the muscle permeability is unclear. Nonneman *et al*. (2012) identified the *DMD* gene as a causal gene for a novel porcine stress syndrome. Animals affected by the gene showed reduced dystrophin protein in both the skeletal and heart muscle, as well as increased CPK levels (Nonneman *et al*., 2012). With WCS carcasses, if a mutation truly exists in the *DMD* gene, the phenotype could be due to an increase in permeability of the cell membranes of muscles causing the typical shiny wet appearance of WCS. However, the possibility of strong LD with another close-by gene to *DMD*, which may harbor the causal mutation cannot be ignored. Within a 10 Mb region surrounding the *DMD* gene, approximately 62 genes have been annotated to date. However, few of these genes have not been characterized and or functionally annotated.

4.7.4.3 TENM1 Gene

Another group of SNPs including oar3_OARX_102819706 and oar3_OARX_102834391 ($P = 9.12 \cdot 10^{-8}$), oar3_OARX_102811671 and oar3_OARX_102817582 ($P = 6.80 \cdot 10^{-6}$) are located within the *TENM1* gene. *TENM1* encode for type II transmembrane glycoproteins (Tucker *et al*., 2007). This gene together with *TENM2*, have been mapped to loci associated with mental retardation in humans (Tucker and Chiquet-Ehrismann, 2006). A group of large transmembrane proteins called the Teneurins is highly conserved in most organisms including *Drosophila* and humans (Tucker and Chiquet-Ehrismann, 2006; Young and Leamey, 2009). Conversely, all Teneurins have only been discovered in recent years, consequently their precise functions are unclear (Rubin *et al*., 1999; Tucker and Chiquet-Ehrismann, 2006; Young and Leamey, 2009). It is, however, known to be expressed in the nervous system and some in non-neuronal tissues associated with pattern formation and morphogenesis as well as limb development (Tucker *et al*., 2007; Young and Leamey, 2009). Teneurins consists of a minimum of 2500 amino acids with a molecular weight of at least 300kDa (Tucker and Chiquet-Ehrismann, 2006; Tucker *et al*., 2007). A total of four Teneurin genes have been identified including *TENM1* (Levine *et al*., 1994). The first research studying Teneurins did establish its presence in both neuronal- and non-neuronal tissues, but primarily in subpopulations of neurons (Otaki and Firestein, 1999; Rubin *et al*., 1999).

Very little research was done on the biological functions of the *TENM1* (type II transmembrane glycoproteins), *TMEM255A* (transmembrane protein 255A), *ACSF2* (Acyl-CoA synthetase family member 2) candidate genes and the pathways they are involved in. It is, however,

established that their functions are much more complex than initially thought (Watkins *et al*., 2007; Young and Leamey, 2009; Schöler *et al*., 2015). Furthermore, their possible involvement or roles in WCS is also unclear since no interaction or relationship could be established, compared to the plausible relationships that have been established between *HTR2C* and *DMD*, and the WCS phenotype. However, the latter is stated with absolute caution since a greater number of loci within the *TENM1* demonstrated strong signals of suggestive significance with WCS and should be included as a possible candidate gene for WCS.

4.8 DETECTION OF LOCI

For the current study, at least two possible major loci have been identified as having a possible effect on WCS. Both loci have been mapped to the X chromosome of the ovine genome and is perhaps one to two centiMorgan (cM, approximately 1 000 000 bp) each in length. The position of locus1 most likely contains several genes that may include *DMD*, *LOC105611207, ACSF2, CHRNB3*, and *USP9X*, with the *DMD* gene possibly having a major effect. Locus2 also contains several genes that may include *HTR2C* and *TENM1* with the *HTR2C* gene possibly having a major effect. To date, a total of 22 loci's have been mapped to OARX. These loci are related to traits such as tail fat deposition, teat number, fecal oocyst count, *Haemonchus contortus* fecal egg count (FEC), total number of lambs born, and Maedi-Visna virus susceptibility (https://www.animalgenome.org).

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

By using a candidate gene approach, it was possible to map the genetic loci, *RYR1* and *PRKAG3* (*RN*$^{-}$), causative of phenotypically similar conditions such as PSS and RSE to the ovine genome. The positions of these loci (*RYR1* on OAR14 and *PRKAG3* on OAR2) mapped to the ovine genome were not in accordance with the loci showing significant association with the WCS phenotype. The visual representation of the GWAS results by means of Manhattan plots, also confirmed that no valuable relationship exists between SNPs located within these genes on the autosomes and the WCS phenotype. The results obtained during the present study therefore, do not support either *RYR1* or *PRKAG3* as positional candidate genes for WCS. Furthermore, to determine whether matching homozygous regions existed in afflicted males and females (autosomes) and afflicted females (X chromosome), the test ROH was used. By identifying ROH, candidate loci within these regions that are either on the autosomes or X chromosome may harbor the causal mutation for a recessive genetic defect. However, the results obtained did not show overlapping homozygous segments that are present within all the afflicted animals on any of the autosomes or within all the females on the X chromosome. Therefore, results from the GWAS, assessment of candidate genes and ROH, suggest the causal variant for WCS may be unlikely to be on one of the autosomes. Results obtained from the GWAS and presented within the Manhattan plots, attested to stronger evidence of an association between at least two loci, oar3_OARX_29903534 and oar3_OARX_113973214 positioned within the non-homologous region on the X chromosome.

The Fisher's Exact Test which compares the frequency of this SNP in cases (afflicted) to the frequency of this SNP in controls (unafflicted), led to the investigation of raw genotype- and allele call data. The results obtained demonstrated the reasoning behind why the lack of association was found. The allele frequencies of oar3_OARX_29903534 and oar3_OARX_113973214 from afflicted- and unafflicted females were in approximately equal numbers. Therefore, the Manhattan plot did not show the association. The allele frequencies of

the latter SNPs from afflicted and -unafflicted males were not in equal numbers, and therefore showed the association within the Manhattan plot.

The investigation of the raw genotype- and allele call data also led to the discovery that all the afflicted animals (both males and females) carry at least one G2 allele from oar3_OARX_113973214, which showed, according to its frequencies, to have a significant influence on the WCS phenotype. On the contrary, 20 unafflicted animals also carried this G2 allele. Given the apparent influence of stress on WCS which is continuously discussed throughout this study, these unafflicted males and females in all likelihood did not experience adequate levels of stress to manifest the WCS condition post-slaughter.

Given the physiological description of the WCS phenotype, two major genes, *HTR2C* and *DMD*, positioned on the non-homologous region of the X chromosome have been identified as positional and functional candidate genes for WCS in sheep. Assuming the genetic predisposition of WCS exists as a causal mutation within the *HTR2C* gene, a disruption in cell homeostasis will occur, either during the pre-slaughter period of lambs by means of stress and anxiety; or during the post-slaughter period, through the calcium ion homeostasis mechanism within the cells of affected muscles. Similarly, assuming the genetic predisposition of WCS exists as a causal mutation within the *DMD* gene, an increase in cell membrane permeability, by means of influencing the water holding capacity (WHC) of muscle, may play a major role.

It should also be considered that instead of describing the influence of these genes separately, a robust relationship or epistasis effect existing between them is a very likely and plausible explanation. Consequently, two possible major regions surrounding the two candidate genes as well as other genes in close proximity (also showing some suggestive association with the phenotype) have been identified and are perhaps one to two cM each in length. The position of the locus1 would specifically contain the *DMD* gene and others (e.g. *LOC105611207*, *ACSF2*, *CHRNB3*, and *USP9X*) surrounding the latter; while locus2 most certainly contain *HTR2C* and *TENM1*.

The second concept that needs special consideration and discussion is the mode of inheritance for WCS. The results of the current study indicate a possible rare X-linked dominant inherited condition, provided only female individuals are taken into account. The following evidence supports this statement: i) a sample set of approximately equal numbers of afflicted males and -females, ii) evidence of females carrying heterozygous genotypes for the significant SNPs,

and manifests the WCS phenotype and iii) evidence of absent homozygous segments in afflicted females on both the autosomes and the X chromosome.

The roles X-inactivation and mosaic expression play in the manifestation of WCS can be explained by females carrying the heterozygous genotype for the causal variant. These heterozygous females either: 1) exhibit the WCS phenotype i.e. afflicted carcass or; 2) exhibit normal carcass characteristics i.e. unafflicted carcass. Thus, in both cases the mutant- and the normal allele is present but only one is expressed. It is possible that with WCS afflicted female carcasses, skewing from the normal 50:50 (mutant:normal alleles) expression ratio may occur and the active X chromosome (carrying either mutant or normal allele) contributes the largest proportion to most tissue cells.

By carefully examining the genotype- and allele calls of the most significant SNPs, of both afflicted and unafflicted, and males and females, it was possible to identify the existence of an association between these SNPs and the actual causal variant. Thereby also identifying a GxE interaction. The genotype (G) was identified by means of the latter approach, whereas the environment (E) interaction is theorised as being a stress effect. On condition that both the afflicted genotypes and stress as the environmental trigger are present, the animal will exhibit the WCS phenotype post-slaughter. Thereby, albeit a limited number of approaches were used to analyse the SNP data, the most strategic and fundamental analyses was used to successfully achieve the principal aim of the study.

The precise biological mechanism involved in the manifestation of WCS is presently unknown. Future studies (discussed below) can either confirm or reject the hypotheses' set by the current research.

5.2 LIMITATIONS OF THE CURRENT STUDY

Having limited and inaccurate knowledge of the environmental- and nutritional factors, and the biochemical- and physiological characteristics of WCS played a major role in the current study. As described in Chapters one and two, previous research has considered these factors; however, no results could be validated by similar independent studies. Furthermore, information regarding the animals' histories, including the phenotypic status of previous generations (given these animals have been slaughtered) were not available. The latter was

driven by breeders within the specified geographical regions having a relatively large number of animals within a cohort and thus tend to avoid keeping pedigree records.

This study was challenging to due limitations in financial resources and availability of afflicted animals. The limitations precluded assessment of quantitative trait loci with the consequence of WCS being viewed as a Mendelian (qualitative) trait. However, this also allowed the current study to make use of the Ovine Infinium® HD SNP BeadChip (600K) instead of the OvineSNP50 assay (50K), thus allowing a 12-fold increase in coverage on the sheep genome. Moreover, with the funding being a major limiting factor, it precluded the current study to explore other research avenues such as sequencing and NGS (as discussed in Chapter two) of both afflicted and unafflicted individuals, as well as the theorised candidate- (from phenotypically similar conditions) and newly proposed candidate regions by the current study.

Given the results obtained from the current study and since the fact that the WCS phenotype only manifest itself post-slaughter, an on-farm family study using afflicted animals and using experimental matings seems to be an impractical next step.

5.3 RECOMMENDATIONS

1. In this study, WCS was regarded as a qualitative trait. However, development of an improved description of the phenotype would be beneficial for future research.

2. Sequence X of both afflicted and unafflicted MALES to potentially identify the exact position of the causal variant for WCS. As a consequence, the development of a genetic test that is commercially available to identify carrier or genetically predisposed animals to WCS will be the ultimate and final step to dramatically reduce the prevalence of WCS.

3. Validation of the association found between the SNPs, their respective genes and the WCS phenotype, could be established by a follow-up study. At a minimum, 100 to 200 (presumably the Dorper breed) animals that carry the G2 SNP should be randomly selected (both males and females). These animals should then be subjected to the various stressors described by the authors that investigated the stress on WCS. The animals must then be slaughtered and the incidence of WCS among the various treatment groups investigated. Provided the animals carrying the G1 and G2 SNPs manifest the WCS

carcasses post-slaughter, the significant association between the loci and WCS can be validated. Depending on the outcome of such a study the association with the G1 SNPs can also be investigated.

4. Since there is a lack of information regarding accurate pedigree records, a pedigree analysis to identify the mode of inheritance using historical data is impractical. If the causal variant for WCS has been identified, a comprehensive study examining the suggested inheritance patterns would be possible. An on-farm family study using carrier animals and experimental matings would provide a direct hands-on approach identify the mode of inheritance.

5. OMIC technologies include a widespread and to some degree the simultaneous detection of genes, mRNA, proteins and metabolites using the methods of genomics, transcriptomics, proteomics and metabolomics (Westerhoff and Palsson, 2004; Te Pas *et al*., 2006; Kell, 2007). These disciplines together will provide a full description of the biological mechanisms influencing a trait (Kadonaga, 1998; Te Pas *et al*., 2006; Horgan and Kenny, 2011; Woelders *et al*., 2011). With OMICS, the choice of the biological sample to investigate is of high importance since the latter will be dependent on which trait and the biological mechanisms underlying the trait is investigated. The end product of such a large combination of biological disciplines coming together is biomarkers, the different technologies may yield different biomarkers (Te Pas *et al*., 2017). Biomarkers are often referred to as the levels or concentrations of molecules from different biological mechanism found in the cell that show differential expression of a given phenotypic trait (Horgan and Kenny, 2011; Te Pas *et al*., 2017). One often shortcoming using OMICS technologies is the need to use the biological samples from the same animals within in given timeframe, thus requiring high financial- and technical resource inputs (Te Pas *et al*., 2017). By using transcriptomics, biological differences between PSS- and normal high-quality carcasses have been investigated. Differences in PSS carcasses (characterized by high drip loss, meat with pale colour and high rate of pH decline) and normal carcasses (exhibiting low drip loss, meat with a non-pale colour and slow rate of pH decline) were compared (Te Pas *et al*., 2010). *Longissimus dorsi* samples and microarray (SNP) technology was used. The differentially expressed genes between the two groups indicated dissimilarities in the physiological processes such as muscle contraction and contractile fibre type, intracellular processes and oxygen transport (Te

Pas *et al*., 2010; Te Pas *et al*., 2017). Pierzchala *et al*. (2014) validated the genes through PCR technology influencing traits such as meat colour, drip loss, pH decline and other carcass traits that were found by Te Pas *et al*. (2010). Picard *et al*. (2010) reviewed the study of proteomics of skeletal muscle from livestock species including porcines.

The use of OMICs technology to discover the biological mechanism underlying WCS would grant the ideal opportunity since it is a relatively undiscovered carcass condition in Southern Africa. The current study provided the first step in this discovery process of the genomic background of WCS by using microarray technology. The validation of such study have already been proposed whereas transcriptomics, proteomics and metabolomics will follow by using normal and afflicted sheep carcasses. It is necessary to apply these technologies to determine which genes and proteins are highly expressed in sheep carcasses manifesting the WCS phenotype under normal and stressed conditions. The first biomarker from OMIC technology that will be developed for WCS is a genetic test (as previously discussed). Altogether and with the discovery of the environmental factors that directly affect sheep and their carcass quality, will provide a meaningful and biological explanation of a relatively unknown trait such as wet carcass syndrome.

SUMMARY

Wet carcass syndrome (WCS) is a condition predominantly found in sheep, which negatively affects the quality of their carcasses. The Northern Cape Province in South Africa and the southern part of Namibia have been identified as geographic regions with a high occurrence rate of WCS. During the pre-slaughter period, the animal appears to be clinically normal, showing no symptoms of an abnormality. However, after the removal of the skin during the slaughter process the carcass appears to be "wet". The condition is phenotypically described as a subcutaneous accumulation of watery fluid. The areas on the carcass most affected are the brisket, flanks, hindquarters, sides, and back. The watery fluid is also found in the intramuscular connective tissue layers of both the flank and subscapular area. Unfortunately, WCS results in sheep carcasses that are deemed to be unacceptable by the end consumer from both an appearance point of view and an apparent reduced shelf life. When the description and results of previous research are considered, no physiological-, environmental- or management system was conclusively identified as a causative agent of WCS. However, stress experienced by the animals during the pre-slaughter period was identified as a possible cause and is continuously discussed throughout the current research to date. The tentative breed-specificity, i.e. Dorper sheep breed, of the condition lends some credence to a potential genetic basis for it. However, previous research has not considered a genetic variant as a causal agent of WCS. The current study employed the Ovine Infinium® HD SNP BeadChip and a genome-wide association analysis (GWAS) approach to scan the genomes of both afflicted- and unafflicted sheep in search of putative quantitative trait loci associated with the WCS phenotype. This study was not only one of the first in Southern Africa to make use of this specific BeadChip but also the first to investigate the role of genetics as a causative factor of WCS. Muscle samples from sheep carcasses (33 afflicted and 36 unafflicted) were collected from three different abattoirs in the Northern Cape Province of South Africa and Southern Namibia.

By using a candidate gene approach it was possible to map genetic loci, *RYR1* and *PRKAG3* (*RN*$^{-}$) causative of phenotypically similar conditions such as porcine stress syndrome and red, soft and exudative meat to the ovine genome. The positions of these loci (*RYR1* on Chromosome 14 and *PRKAG3* on Chromosome 2) mapped to the ovine genome were not in accordance with the loci showing significant association with the WCS phenotype; and no relationship was found between single nucleotide polymorphisms located within these genes

and WCS. Furthermore, along with the latter approach the test of runs of homozygosity presented similar results as well as providing plausible evidence that WCS is not a recessive inherited condition. To test for an association between the phenotype (WCS) and an autosomal genetic marker(s) i.e. SNPs, a case-control study design was implemented. Given the relatively small sample size of the current study, the results obtained from the GWAS attested strong evidence of at least two loci, oar3_OARX_29903534 and oar3_OARX_113973214 positioned within the non-homologous region of the X chromosome for WCS carcasses. All afflicted animals, both males and females, carried at least one G2 allele for marker oar3_OARX_113973214, which was shown to be related to the WCS phenotype. Given the apparent influence of stress on WCS, these unafflicted males and females in all likelihood did not experience adequate levels of stress to manifest the WCS condition post-slaughter. The results of the current study also indicated that WCS may possibly be a rare X-linked inherited condition, provided only female individuals are considered. Finally, two possible major loci involving two major genes, *HTR2C* and *DMD*, positioned on the non-homologous region of the X chromosome have been identified as novel positional and functional candidate genes for WCS in sheep.

OPSOMMING

Natkarkassindroom (NKS) is ‘n toestand wat hoofsaaklik onder skape voorkom en wat die gehalte van hul karkasse negatief beïnvloed. Die Noord-Kaap-provinsie in Suid-Afrika asook die suidelike deel van Namibië is geïdentifiseer as geografiese gebiede met ’n hoë voorkoms van NKS. Gedurende die tydperk voor die skape geslag word, kom die dier klinies normaal voor en toon geen simptome van abnormaliteit nie. Nadat die vel egter gedurende die slagproses verwyder word, kom die karkas as “nat” voor. Die toestand word fenotipies beskryf as die onderhuidse aansameling van ‘n waterige vloeistof. Die dele van die karkas wat die meeste geaffekteer word is die borsstuk, lies, agterkwart, sye en rug. Die waterige vloeistof word ook aangetref in die binnespierse bindweefsel-lae van beide die lies- en die bladbeen-area. Ongelukkig is NKS skaapkarkasse nie aanvaarbaar vir die eindverbruiker, nie slegs omdat dit ’n onaanvaarbare voorkoms het nie, maar ook omdat dit ’n korter rakleeftyd het. Wanneer die beskrywings en resultate van vorige navorsing in ag geneem word, kon geen fisiologiese-, omgewings-, of bestuurstelsel oortuigend geïdentifiseer wat NKS veroorsaak nie. Die stres wat die diere gedurende die tydperk voor die slagproses ervaar, is egter geïdentifiseer as ’n moontlike oorsaak en word deurlopend in die navorsing tot nou toe bespreek. Die tentatiewe ras-spesifieke geneigdheid dat die toestand meer onder Dorperskape voorkom dui daarop dat daar ’n moontlike genetiese basis daarvoor is. Vorige navorsing het egter nie ’n genetiese variant as ’n veroorsakende faktor van NKS ondersoek nie. Die huidige studie het gebruik gemaak van die ‘Ovine Infinium® HD SNP’ skyfie en ’n genoomwye assossiasie-analise (GWA) om die genome van beide aangetaste- en onaangetaste skape te skandeer in die soeke na vermeende kwantitatiewe-eienskap-loki (KEL) wat geassosieer kan word met die NKS-fenotipe.

Hierdie studie is nie net slegs een van die enigste studies in Suider-Afrika om hierdie spesifieke skyfie te gebruik nie, maar ook die eerste studie wat die rol van genetika as ’n veroorsakende faktor van NKS ondersoek. Spiermonsters van 33 aangetaste- en 36 onaangetaste skaapkarkasse is versamel vanaf drie verskillende abattoirs in die Noord-Kaap-provinsie van Suid-Afrika, asook die suidelike dele van Namibië.

Deur ’n kandidaatgeenbenadering te gebruik, was dit moontlik om genetiese loki, *RYR1* en *PRKAG3* (*RN*$^{-}$), wat fenotipies-soortgelyke toestande soos varkstressindroom, en rooi, sagte

en afskeidingsvleis veroorsaak, in die skaapgenoom te beskryf. Liggings van hierdie loki (*RYR1* op Chromosoom 14 (OAR14) en *PRKAG3* op Chromosoom 2 (OAR2)) wat op die skaapgenoom afgebeeld is, was nie in ooreenstemming met die loki wat ’n beduidende assosiasie met die NKS-fenotipe getoon het nie; en geen verhouding kon gevind word tussen enkel-nukleotied-polimorfismes (ENPs) binne hierdie gene en NKS nie. Voorts, tesame met die laasgenoemde benadering, het die genomiese soektog vir gebiede van homosigositeit (GVH) soortgelyke resultate gelewer, asook aanvaarbare bewyse dat NKS nie ’n resessiewe oorerflike toestand is nie. Om te toets vir ’n assosiasie tussen die fenotipe (NKS) en ’n outosomale genetiese merker(s), m.a.w. ENP, is daar van ’n geval-kontrolestudie gebruik gemaak. Gegewe die relatiewe klein steekproef van die huidige studie, het die resultate van die GWA-analise sterk bewyse getoon van ten minste twee loki, oar3_OARX_29903534 en oar3_OARX_113973214, in die nie-homogene area van die X-chromosoom vir NKS-karkasse. Alle aangetaste diere, beide ramme en ooie, het ten minste een G2-alleel vir merker oar3_OARX_113973214 gedra, wat duidelik ’n beduidende invloed op die NKS-fenotipe gehad het. Gegewe die oënskynlike invloed van stres op NKS, het onaangetaste ramme en ooie wat ook hierdie G2 allele dra, in alle waarskynlikheid nie genoegsame vlakke van stres ervaar om die NKS-toestand ná die slagproses te toon nie. Die resultate van die huidige studie het ook daarop gedui dat NKS moontlik ’n skaars, X-gekoppelde oorerflike toestand kan wees, gegewe slegs vroulike diere word in ag geneem. Laastens, twee moontlike KEL-areas wat twee hoofgene, *HTR2C* en *DMD*, betrek, wat geleë is op die nie-homogene area van die X-chromosoom, is geïdentifiseer as moontlike posisionele en funksionele kandidaatgene vir NKS in skape.

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ADDENDUM A

Possible candidate genes with their respective influences on cellular components, molecular functions and biological processes as described by and adapted from http://www.ensembl.org and http://www.uniprot.org/.

Gene name	Cellular components	Molecular functions	Biological processes
TENM1	Plasma membrane	Protein homodimerization activity	Signal transduction
	Membrane	Protein heterodimerization activity	
TMEM255A	Membrane	Unknown	Unknown
VEPH1	Unknown	Unknown	Unknown
ACSF2	Mitochondrion	Catalytic activity	Metabolic process
HTR2C	Plasma membrane	Gq/11-coupled serotonin receptor activity	Behavioral fear response
	Membrane	Signal transducer activity	Cgmp biosynthetic process
		G-protein coupled receptor activity	Cellular calcium ion homeostasis
		G-protein coupled serotonin receptor activity	Signal transduction
		Drug binding	G-protein coupled receptor signaling pathway
		Serotonin binding	Phospholipase C-activating G-protein coupled receptor signaling pathway
		1-(4-iodo-2,5-dimethoxyphenyl)propan-2-amine binding	Phospholipase C-activating serotonin receptor signaling pathway
			Serotonin receptor signaling pathway
			Locomotory behavior
			Feeding behavior
			Positive regulation of phosphatidylinositol biosynthetic process
			Regulation of neurological system process
			Regulation of appetite
			Response to drug
			Regulation of corticotropin-releasing hormone secretion
			Positive regulation of fat cell differentiation
			Release of sequestered calcium ion into cytosol
			Positive regulation of cytosolic calcium ion concentration involved in phospholipase C-activating G-protein coupled signaling pathway
			Positive regulation of ERK1 and ERK2 cascade

RELN	Proteinaceous extracellular matrix Extracellular space Cytoplasm Plasma membrane Membrane Dendrite	Very-low-density lipoprotein particle receptor binding Lipoprotein particle receptor binding	Cell morphogenesis involved in differentiation Neuron migration Axon guidance Central nervous system development Brain development Learning Long-term memory Associative learning Glial cell differentiation Regulation of gene expression Positive regulation of neuron projection development Positive regulation of phosphatidylinositol 3-kinase signaling Dendrite development Cell migration Peptidyl-tyrosine phosphorylation Spinal cord patterning Ventral spinal cord development Hippocampus development Cerebral cortex tangential migration Layer formation in cerebral cortex Cerebral cortex development Forebrain development Positive regulation of TOR signaling Positive regulation of CREB transcription factor activity Protein localization to synapse Reelin-mediated signaling pathway Positive regulation of protein kinase activity Response to pain Positive regulation of peptidyl-tyrosine phosphorylation Regulation of behavior Modulation of synaptic transmission Positive regulation of small gtpase mediated signal transduction Positive regulation of synaptic transmission, glutamatergic

			Long-term synaptic potentiation Positive regulation of dendritic spine morphogenesis Positive regulation of protein tyrosine kinase activity Positive regulation of synapse maturation NMDA glutamate receptor clustering Postsynaptic density protein 95 clustering Receptor localization to synapse Lateral motor column neuron migration Positive regulation of long-term synaptic potentiation Positive regulation of lateral motor column neuron migration Regulation of NMDA receptor activity Positive regulation of excitatory postsynaptic potential Positive regulation of AMPA receptor activity
USP9X	Membrane	Thiol-dependent ubiquitinyl hydrolase activity	Ubiquitin-dependent protein catabolic process
	Growth cone Cytoplasm	Co-SMAD binding	Transforming growth factor beta receptor signaling pathway Protein deubiquitination Neuron migration BMP signaling pathway Axon extension
MIR106A	Unknown	Unknown	Unknown
NUBP1	Plasma membrane Extracellular exosome Cytosol Cytoplasm	Nucleotide binding Metal ion binding Iron-sulfur cluster binding ATP binding 4 iron, 4 sulfur cluster binding	Protein localization to cell cortex Negative regulation of centrosome duplication Iron-sulfur cluster assembly Centrosome localization Cellular iron ion homeostasis Cell growth
SLC9A1	Plasma membrane	Sodium:proton antiporter activity	Transmembrane transport