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Identification of genomic regions that contribute to wet carcass syndrome in sheep

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Abstract

Wet carcass syndrome (WCS), which is observed predominantly in sheep, affects carcass quality negatively. After slaughter the carcass appears to be 'wet' with a subcutaneous accumulation of watery fluid. Not all animals in a contemporary group are afflicted, and experimental attempts to induce WCS have been unsuccessful. The reported prevalence of WCS in Dorper and Dorper crosses gave rise to the hypothesis that it may have a genetic basis. Therefore, the primary objective of this investigation was to test this hypothesis using a high-density SNP assay to search loci that may predispose sheep to WCS. Muscle samples from 43 afflicted and 41 unafflicted sheep were collected from slaughterhouses in the province of Northern Cape, South Africa, and in southern Namibia. Tests against candidate genes proved uninformative, as did runs of homozygosity. Potential associations between WCS and an autosomal genetic marker were investigated further in a case-control genome-wide association study. Separate analyses for each sex were motivated because single nucleotide polymorphisms (SNPs) on the X chromosome suggested quantitative trait loci. These analyses revealed significant associations between SNP and WCS in males, but not in females. Three SNPs that reached genome-wide significance in males are in strong linkage disequilibrium with the Duchenne muscular dystrophy, 5-hydroxytryptamine receptor 2C, and Teneurin transmembrane protein 1 genes. These genes are identified as positional candidate genes, and the Duchenne muscular dystrophy, 5-hydroxytryptamine receptor 2C genes have biological effects that have been documented in other species, making them plausible functional candidate genes for WCS in sheep.

Keywords: association analysis, Dorper, sheep carcass, single nucleotide polymorphisms, X chromosome
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Introduction

Wet carcass syndrome (WCS) is a condition that is found predominantly in sheep, which affects the quality of their carcasses negatively. Although WCS has been the subject of research since 1981 (Brock *et al*., 1983; Hattingh *et al*., 1983), its aetiology remains undetermined. The first incidence of WCS was recorded in January of 1981 at Chambor abattoir in Krugersdorp, Gauteng, South Africa (Jansen, 1991). It is most frequently observed in Dorper and crosses of Dorper with indigenous and locally developed breeds of South Africa and Namibia (Brock *et al*., 1983; Webb & Van Niekerk, 2011). During the pre-slaughter period the animal appears to be clinically normal. However, after slaughter and removal of the skin the carcass appears to be 'wet' (Hattingh *et al*., 1983). As shown in Figure 1, the condition can be described as a subcutaneous accumulation of watery fluid (Brock *et al*., 1983). The areas of the carcass that are 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 the flank and subscapular area. Afflicted carcasses do not dry off with overnight cooling (Joubert *et al*., 1985), pose difficulties during meat processing and the meat has a diminished shelf life (Joubert *et al*., 1985). Therefore, these carcasses are deemed unacceptable, and are condemned by the meat inspectors. In South Africa, economic losses that stem from WCS were approximately R27 million in 2010 (Webb & Van Niekerk, 2011; Le Roux, 2012).

Not all animals in a flock that are sent to slaughter at one time are afflicted, and inducing the condition

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experimentally has proved unsuccessful. To date, many physiological and nutritional factors, as well as environmental agents and management systems, have been postulated and evaluated without a cause for the condition having been identified (reviewed by Van der Westhuizen, 2018). Despite apparent over-representation of WCS in Dorper, which may indicate genetic causation, there have been no prior investigations of a potential genetic predisposition to WCS (Van der Westhuizen, 2018). Furthermore, pedigree data are currently unavailable with which to perform analyses that would assist in estimating heritability or the mode of transmission of WCS.

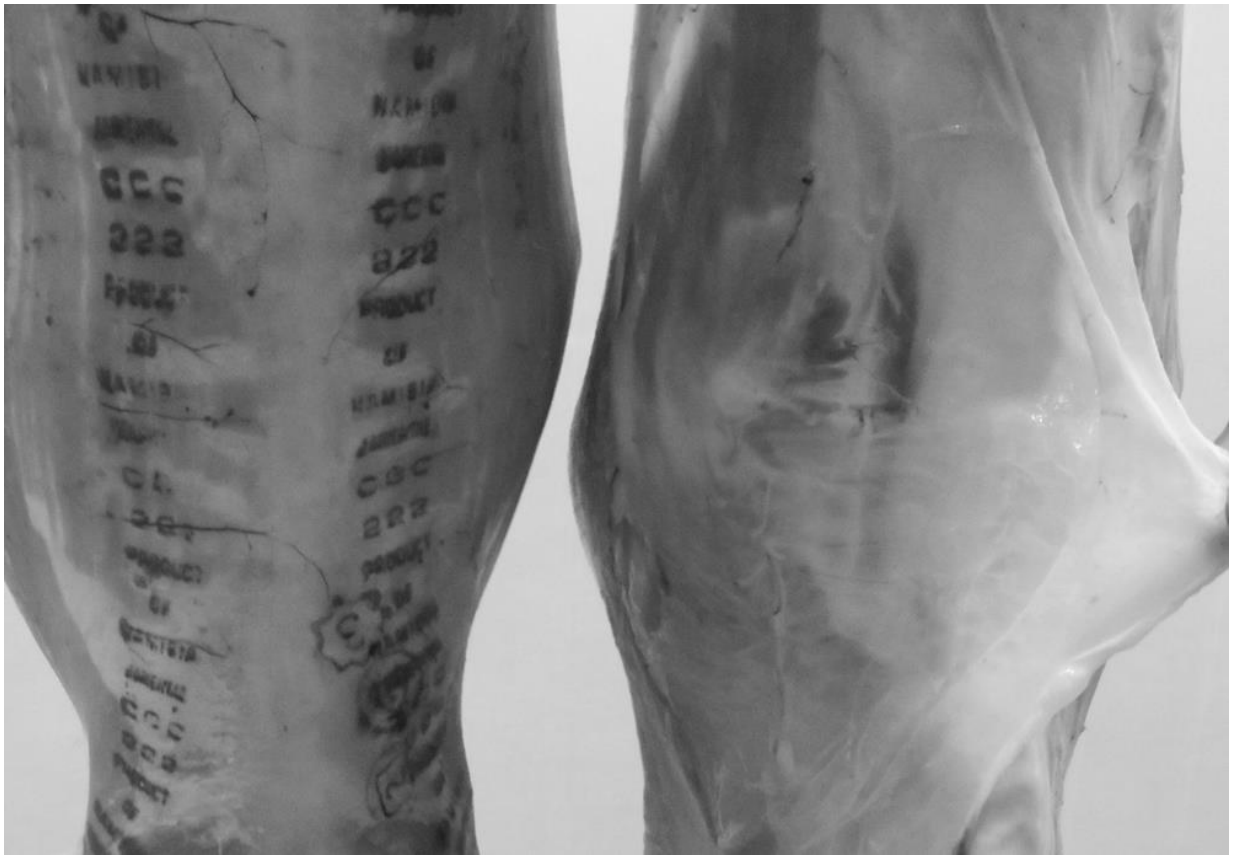

Figure 1 Carcass from a sheep that was not afflicted with wet carcass syndrome (left) compared to the carcass from an afflicted sheep.

The study of genetic diseases falls into two categories, that is, population-based studies and family-based studies. Population-based studies generally use a case-control study design to identify candidate gene regions (Miyagawa *et al*., 2008). In early forms of population-based studies, candidate gene studies relied on the similarity of phenotypes across species and previous identification of a causative locus in a species other than the one that was currently under investigation (Harhay & Keele, 2003). From the early 2000s to date, genome-wide association studies (GWAS) using high-density SNPs have become the conventional method of identifying statistically significant loci that underlie common and complex diseases (Hirschhorn & Daly, 2005; Miyagawa *et al*., 2008; Clarke *et al*., 2011).

The GWAS approach has been used successfully to identify quantitative trait loci (QTL) and putative candidate genes in wool (Wang *et al*., 2014), meat (Zhang *et al*., 2013), milk production (García-Gámez *et al*., 2012), bodyweight (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), and chondrodysplasia (Zhao *et al*., 2012) in sheep. For a GWAS, populations consisting of afflicted and unafflicted individuals are compared using the frequencies of alleles or genotypes, that is, a case and control study. If a higher frequency of a SNP variant exists in the afflicted group (afflicted individuals with a specific phenotype), or is statistically more common in the afflicted group, the alleles or genotypes are regarded as being associated with the disease (Hirschhorn *et al*., 2002). SNPs, which are spaced approximately evenly across the genome, may be tested independently for an association with a specific phenotype (Balding,

2006; Kemper *et al*., 2012).

Two conditions that are phenotypically similar to WCS are porcine stress syndrome (PSS), which results in pale, soft, and exudative (PSE) meat (Ludvigsen, 1957; Wismer-Pedersen, 1959), and reddish, soft, and exudative meat (RSE) (Le Roy *et al.,* 1990). Both PSE and RSE become apparent after slaughter and result from mutations at single loci. Porcine stress syndrome, which results in PSE pork, is caused by a single autosomal recessive mutation in the ryanodine receptor 1 (*RYR1*) gene (Hall *et al*., 1980; Fujii *et al*. 1991; MacLennan & Phillips, 1992). In the porcine species, this gene is located on chromosome 6 (SSC6) (Harbitz *et al*., 1990) (map position on *Sus scrofa* Sscrofa11.1 is 47,339,759-47,458,457 (Sayers *et al*., 2019a,b). This mutation affects the calcium ion release channel of the sarcoplasmic reticulum. The channel opens properly, but closing is inhibited (Endo *et al*., 1983; O'Brien, 1986; Fill *et al*., 1990). 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 (Wismer-Pedersen, 1959; Aalhus *et al*., 1998; Schaefer *et al*., 2001), causing PSE meat. There have been reports of 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 & Nurul (2011) stated that PSE meat can be found in all species, with its manifestation being dependent on pre-slaughter handling.

The RSE condition was proposed by Le Roy *et al*. (1990) and Warner *et al*. (1997) to result from a mutation in the Rendement Napole (RN^{-} or *PRKAG3*) gene. Warner *et al*. (1997) also proposed that the low pH and high glycogen content in the muscle results in low processing yields, which are typical characteristics in the presence of the RN^{-} genotype. The RN^{-} gene has an autosomal dominant inheritance pattern (Le Roy *et al*., 1990) and is located between markers SW120 and SW936 on chromosome 15 (SSC15) in the porcine genome (Milan *et al*., 1995; Mariani *et al*., 1996). A total of five causal substitution mutations 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). The *PRKAG3* gene mechanism encodes for a muscle isoform of the regulatory γ-subunit, which forms part of the adenosine monophosphate (AMP) activated protein kinase (AMPK). The enzyme AMPK is essential to the regulation of energy metabolism. During the post-slaughter period, glycogen is altered to lactic acid. The presence of lactic acid (Lundström *et al*., 1996; Enfält *et al*., 1997) causes a low ultimate pH (Miller *et al*., 2000; Lindahl *et al*., 2004; Škrlep *et al*., 2010). Therefore, RSE or acid meat exhibits a high drip loss and has low water-holding capacity (Le Roy *et al*., 1996; Le Roy *et al*., 2000; Škrlep *et al*., 2010).

Given this background, the overarching goal of this research was to determine whether there is a genetic predisposition for WCS. Thus, the specific objectives were i) to map sequences for porcine *RYR1* and *PRKAG3* onto the ovine genome and test co-located SNP variants for association with WCS; ii) to detect autosomal loci that showed consistent homozygosity in all afflicted sheep; iii) to identify genomic regions that harboured genetic variants that predispose sheep to WCS; and iv) to postulate candidate genes in any identified regions based on functional similarities that have been identified in the annotation of genomes of other species.

Materials and Methods

Muscle samples from 43 afflicted and 41 unafflicted sheep carcasses were collected from three abattoirs in the province of Northern Cape, South Africa, and in southern Namibia. Abattoirs in South Africa were selected from those in the geographical areas in which WCS had had a relatively high prevalence in the past. At each abattoir, personnel were instructed to select afflicted and unafflicted carcasses 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 & Palmer, 2003; Hirschhorn & Daly, 2005; Turner *et al*., 2011). Sex of the animals was not recorded at the abattoirs. Sex was therefore assigned to animals based on mean heterozygosity rates using the --check-sex command of PLINK (Purcell *et al*., 2007). In examining the genotypes from the X chromosome, an animal was assumed to be a female when the inbreeding coefficient (F) was less than 0.2 ($F \leq 0.2$) and a male when F was greater than 0.8 ($F \geq 0.8$) (Anderson *et al*., 2010; Turner *et al*., 2011; Alonso *et al*., 2015).

Genomic DNA was extracted from muscle samples using the First-DNA all tissue extraction kit (GEN-IAL GmbH, Troisdorf, Germany) following the manufacturer's instructions, with minor modifications that best suited muscle samples. Roughly chopped muscle was placed in a 1.5 millilitre (mL) reaction vessel with 500 microlitres (µL) lysis buffer 1, 50 µL lysis buffer 2 ,and 5 µL Proteinase K, and incubated for 90 min at 65 °C. Centrifugation occurred for 10 min at 12 000 revolutions per minute (rpm) from which 500 µL supernatant was transferred to a fresh reaction vessel. A total of 375 µL of lysis buffer 3 was added, and then vortexed for 20 seconds. 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 was transferred into a fresh reaction vessel. Then 640 µL isopropanol was added and mixed carefully.

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 air dried overnight, dissolved in 50 µL double distilled water (ddH_2O), and stored at -80 °C. Nucleic acid (DNA) concentration was determined using a NanoDrop 2000 spectrometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA). A total of 1 µL of DNA was used for each measurement. DNA extraction products were also run on a gel electrophoresis system (Bio-Rad, Hercules, California, USA). 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 then UV visualized with the Bio-Rad Molecular Imager® Gel DocTM XR+ System controlled by Image Lab software. The final quantity of DNA and its purity was determined using the Qubit 3.0 fluorometer (Invitrogen, Thermo Fisher Scientific, Carlsbad, California, USA) following the manufacturer's instructions.

All DNA samples were genotyped at the Agricultural Research Council Biotechnology Platform, using the Ovine Infinium® HD SNP BeadChip (Illumina Inc., San Diego, California, USA). The BeadChip features 685 734 SNPs that are approximately equally distributed throughout the domestic sheep *(Ovis aries)* genome on 26 autosomes and the X chromosome (Kijas *et al*., 2014). Illumina GenomeStudio Genotyping Module Software v2.0 (Illumina Inc., San Diego, California, USA) was used to convert raw signal intensities into genotype calls.

PLINK v1.07 (Purcell *et al*., 2007) was used for individual and SNP quality control (QC) measures. The missing genotype rate per individual was calculated using the *--mind* command and individuals with call rate < 90 % were excluded following Zhang *et al*. (2013) and Hao *et al*. (2017). Individuals with observed homozygosity (calculated using *--het*) outside the range µ ± 3SD were deleted from the data. Pairwise identity-by-descent (IBD) estimation (*--genome,* IBD > 0.1875) was used to identify individuals with shared ancestry and potential duplicate samples. Markers without a known chromosomal position on the ovine genome sequence (*Ovis aries* Oar_v4.0) were also excluded from further analyses, as were markers with call rates (*--geno*) < 0.90 and minor allele frequency (MAF) (*--maf*) < 0.01. Sex was assigned to animals based on the presence (F ≤ 0.2 = female) or absence (F ≥ 0.8 = male) of heterozygosity of the X chromosome. A pruned SNP dataset (227 768 SNPs) by means of linkage disequilibrium (*--indep-pairwise* 50 5 0.5) was used to conduct principal component analysis using the SNPRelate and gdsfmt packages (Zheng *et al*., 2012) in R (R Core Team, 2013). Only two samples were removed because of population stratification.

After editing, 69 individuals (33 afflicted and 36 unafflicted) and 552 490 SNPs remained for analyses. To identify loci that were associated with the WCS phenotype, a GWAS applicable to a case-control study design was implemented along with Fisher's exact test (*--fisher*) in PLINK v1.07 (Purcell *et al*., 2007). Fisher's exact test avoids problems of approximation that are exacerbated by small sample sizes (Kim, 2017). This test compares genotype frequencies between afflicted (case) and unafflicted (control) samples to associate the WCS phenotype with SNP genotype. The classical Bonferroni correction of the *P*-values was used to provide a critical value for significance testing at $P = 0.05$.

The qqman- (Turner, 2014) and RColorBrewer packages in R (R Core Team, 2013) were used to create and visualize the GWAS results by means of Manhattan- and quantile-quantile (Q-Q) plots. Gene and SNP annotation were completed by using NCBI *Ovis aries*_v4.0 (Sayer et al., 2019a,b) and Ensembl (Aken *et al*., 2016) using ovine genome assembly 3.1.

Results and Discussion

Using BLAST (Altschul *et al.*, 1997), homologs of the functional candidate genes *PRKAG3* and *RYR1* were positioned at 219,781,028-219,787,070 on chromosome 2 (OAR2) and 47,426,122-47,552,121 on chromosome 14 (OAR14) within the *Ovis aries* genome, respectively (Figure 2). Three SNPs on the Ovine Infinium® HD SNP BeadChip were located in the *PRKAG3* gene on OAR2, and 25 SNP were located in the *RYR1* gene on OAR14 (Van der Westhuizen, 2018). Two additional SNP near the upstream promoter regions were also identified. Logistic regression of the presence or absence of WCS on each SNP genotype indicated that for *PRKAG3*, oar3_OAR2_219782879 had the smallest *P*-value (0.19) and for *RYR1*, oar3_OAR14_47532583 had the smallest *P*-value (0.01). However, no other SNP within *RYR1* approached significance in affecting the occurrence of WCS and it was concluded that although PSS and RSE are phenotypically similar to WCS, the current results do not support the hypothesis that mutations in *RYR1* or *PRKAG3* cause WCS.

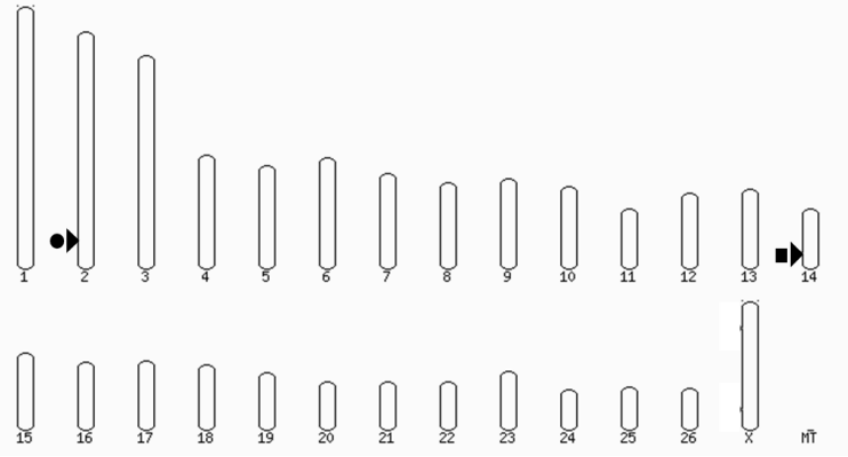

Figure 2 Position of *PRKAG3* on chrosome 2 and *RYR1* on chromosome 14 in the ovine genome

To determine whether there is an autosomal region that harbours a causative mutation(s) that affect WCS, the genomes of all afflicted animals (n = 33) were scanned for common runs of homozygosity. A total of 527 694 SNP loci on 26 autosomes were used in this evaluation. The number of identified homozygous segments in all afflicted animals ranged from 47 to 140, with a mean of 88.06 ± 3.97. The segment sizes ranged from 2344.03 kb to 3625.414 kb with a mean of 2856.54 ± 238.71 kb. No homozygous segments were identified as overlapping on any of the autosomes in all of the 33 afflicted animals. The greatest number of afflicted animals that shared an overlapping homozygous segment was 24. This consensus region was initiated with oar3_OAR3_106048097 and was terminated with oar3_OAR3_107139271. Thus, these results are interpreted to indicate the absence of a recessive mutation on any of the autosomes that harbour the causal locus for WCS and support the interpretation that *RYR1* and *PRKAG3* are not causal genes.

A similar analysis was conducted with the SNP genotypes of afflicted females (n = 14) to search for runs of homozygosity on OARX. A total of 25 001 SNPs were available for this analysis. The number of identified homozygous segments in all afflicted animals ranged from 4 to 13 with a mean of 8.14 ± 0.78 and the average segment sizes ranged from 1968.27 kb to 3757.46 kb, with a mean of 2538.51 ± 123.45 kb. Two loci were found to have overlapping homozygous segments in 13 of the 14 afflicted females. The first consensus region was initiated with oar3_OARX_73198255 and was terminated with oar3_OARX_73994257. This region was also found to be homozygous in 13 unafflicted females. The second consensus region was initiated with oar3_OARX_56609654 and was terminated with oar3_OARX_56624555. This region was also found to be homozygous in 12 of the unafflicted females. The lack of any overlapping runs of homozygosity provided plausible evidence to support the notion that WCS is not inherited as a simple recessive condition, unless there had been an error in genotyping or an individual had been mistakenly identified as being afflicted.

The initial GWAS included data from 19 afflicted and 19 unafflicted males and 14 afflicted and 17 unafflicted females (Figure 3). It detected 14 SNPs that were suggestively associated ($P \leq 1 \cdot 10^{-5}$) with WCS (Table 1). Five of the SNPs were located on chromosome 1 (OAR1), chromosome 4 (OAR4), chromosome 8 (OAR8), chromosome 11 (OAR11), and chromosome 24 (OAR24), and nine SNPs were located on the X chromosome (OARX). For the autosomal loci that were associated with WCS, no other nearby loci were similarly associated and these associations could result from genotyping artefacts or chance as a consequence of small sample size. The X chromosome was clearly distinguished from the autosomes by the presence of several SNPs that formed an apparent QTL peak. Because X-linked variants may have different effects between males and females (Dobyns *et al*., 2004) there was adequate incentive to conduct a separate GWAS analysis for each sex. Unfortunately, the power of these separate analyses was compromised owing to reduced allelic sample size (Gottipati *et al*., 2011).

In the data from males, two SNPs on OARX were found to reach the genome-wide significance threshold relating to WCS (Figure 4). The loci oar3_OARX_29903534 ($P = 4.56 \cdot 10^{-8}$) and oar3_OARX_113973214 ($P = 6.16 \cdot 10^{-8}$) are located in the region of OARX, which is not pseudo-autosomal (Das *et al*., 2009). These loci are separated by approximately 83 megabase pairs (Mbp), where OARX_113973214 is approximately 11 Mbp downstream from oar3_OARX_102819706 and oar3_OARX_102834391. However, the analysis of the data from females failed to detect suggestive associations with WCS.

Table 1 Loci on the X chromosome and autosomes showing suggestive association with wet carcass syndrome when both sexes were used in the genome-wide association analysis

SNP	Chr	RefSNP ID	Locus	*P*-value	Related genes[1]
OAR1_245730132.1	1	*rs414552741*	227901188	$5.61 \cdot 10^{-7}$	intergenic
OAR3_OAR4_44743238	4	*rs424871551*	44720173	$2.09 \cdot 10^{-6}$	*RELN*
OAR8_45760589.1	8	*rs403004733*	42572637	$5.74 \cdot 10^{-6}$	Intergenic
OAR3_OAR11_35812793	11	*rs406416952*	35763513	$4.56 \cdot 10^{-6}$	Intergenic
OAR3_OAR24_9440581	24	*rs402465452*	9442405	$5.40 \cdot 10^{-6}$	*NUBP1*
OAR3_OARX_29903534	X	*rs403503557*	29903034	$4.56 \cdot 10^{-8}$	Intergenic
OAR3_OARX_35953513	X	*rs400747921*	35932202	$3.59 \cdot 10^{-6}$	Intergenic
OAR3_OARX_98985496	X	*rs416506282*	98824617	$6.43 \cdot 10^{-6}$	*TMEM255A*
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_OARX_102819706	X	*rs426965083*	102652752	$9.40 \cdot 10^{-6}$	*TENM1*
OAR3_OARX_102834391	X	*rs416468050*	102667437	$4.24 \cdot 10^{-6}$	*TENM1*
OAR3_OARX_113973214	X	*rs399985763*	113751984	$4.67 \cdot 10^{-7}$	Intergenic
OAR3_OARX_113988383	X	*rs414187600*	113767153	$2.43 \cdot 10^{-6}$	Intergenic

SNP: name of the single nucleotide polymorhism; Chr: chromosome; refSNP ID: SNP identification (Oar_v4.0); Locus: base pair position on Opr_v4.0; *P*-value: *P*-values from Fisher's exact test

[1] RELN: Reelin; NUBP1: nucleotide binding protein 1; TMEM255A: transmembrane protein 255A; TENM1: Teneurin transmembrane protein 1; Intergenic: regions in which no genes have been annotated

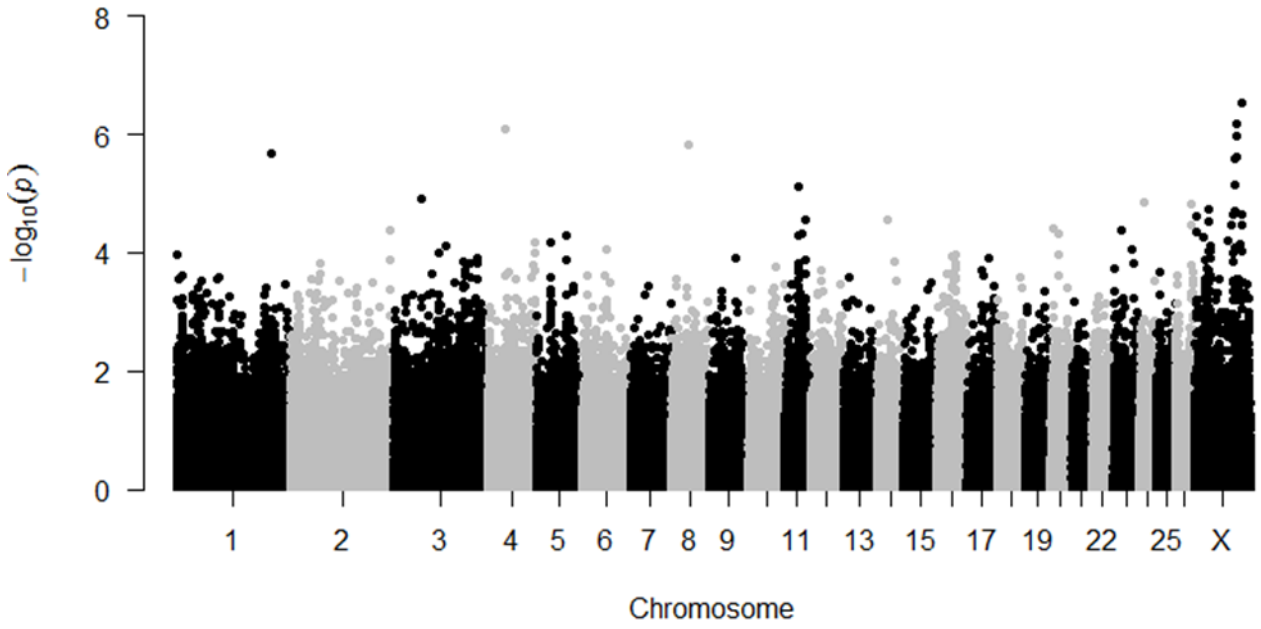

Figure 3 Manhattan plot of results from Fisher's exact for association of SNP genotype with wet carcass syndrome based on 69 samples from both males and females. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values are plotted on the y-axis

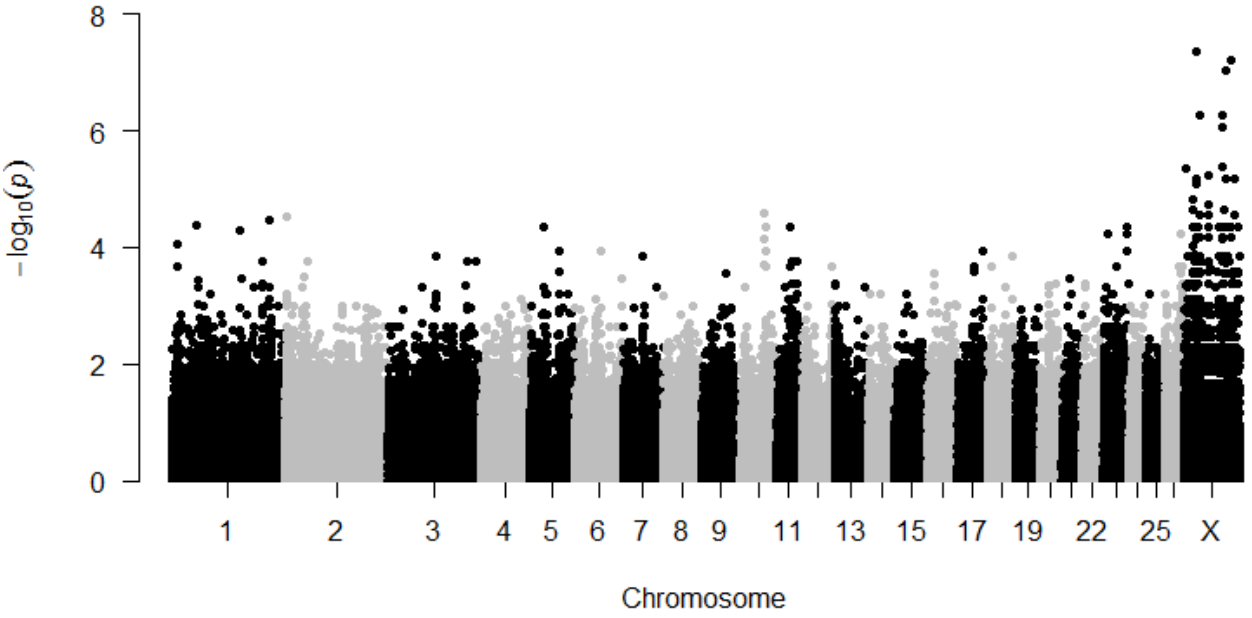

Figure 4 Manhattan plot of results from Fisher's exact test for association of SNP genotype with wet carcass syndrome based on samples from only the 38 males. The physical position of the SNP according to chromosome number is plotted on the x-axis, while the –log10*P*-values are plotted on the y-axis

Without discounting possible sex dimorphism in the effects of variants (Dobyns *et al*., 2004), the strong evidence for 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 the possible co-expression of heterozygous genotypes at autosomal loci, because random X-inactivation results in a mixture of cells in different tissues (Taylor, 2014). An imbalance of the X chromosomes between males (XY) and females (XX) and in the absence of X-inactivation, genes on OARX would be over-expressed in females relative to males. However, this imbalance is resolved by random inactivation of one of the X chromosomes in each of the somatic cells (Lyon, 2001). This dosage compensation results in the expression of only one allele of most genes in the non-homologous region of X in females.

Findings from this study that are potentially related the differences observed between males and females include i) afflicted and unafflicted females in approximately equal numbers carried the 'associated' genotypes at oar3_OARX_29903534 and oar3_OARX_113973214, thus providing a plausible reason for the lack of apparent association found in the GWAS analysis of females; ii) all afflicted animals had a 'G' allele for marker oar3_OARX_113973214, which had a significant influence on the WCS phenotype; iii) 20 unafflicted animals also carried the 'G' allele of oar3_OARX_113973214; and iv) some females, both afflicted and unafflicted, were heterozygous for oar3_OARX_113973214. Given the apparent influence of stress on phenotypically similar conditions in other species, it was speculated that these unafflicted males and females that carried this specific allele might not have experienced adequate levels of stress to manifest the WCS condition post slaughter. This ambiguity may be the result of incomplete penetrance (Zlotogora, 2003; Shawky, 2014). Incomplete penetrance affecting the expression of PSS has previously been observed in pigs (Ollivier et al., 1975; Smith & Bampton, 1978). In humans, penetrance in expression of the afflicted condition resulting from mutations in *RYR1* was significantly greater in males than in females (50% versus 30%; *P* =0.002), despite similar levels of exposure to trigger anaesthetics (Ibarra Moreno *et al*., 2019).

Marker oar3_OARX_29903534 is located within the *DMD* gene. The *DMD* gene is X-linked, encodes the dystrophin protein (Hoffman *et al*., 1987; Koenig *et al*., 1988), and is the leading cause of DMD in young boys (Moser *et al*., 1984; Allen & Whitehead, 2011). Increased plasma creatine phospho kinase (CPK) levels and dilated cardiomyopathy have been observed in DMD patients (Kirchmann *et al*., 2005; Magri *et al*., 2011). Allen & Whitehead (2011) proposed that increased CPK levels are indicative of an increase in the permeability of the muscle surface membrane, which allows for calcium ions to move into the intracellular region and enzymes to move out. However, the exact cause of the muscle permeability is unclear. Nonneman *et al*. (2012) found that a variant of the *DMD* gene caused a novel porcine stress syndrome. Nonneman *et al*. (2012) then showed that this condition could be induced by handling, transport and isoflurane anaesthesia.

Non-ambulatory response and fatigue, coupled with the classical symptoms of acute stress and sensitivity to halothane anaesthesia, have been observed in pigs and are similar to the symptoms exhibited by animals that carry a mutated ryanodine receptor 1 (*RYR1*) gene (Fujii *et al*., 1991; Allison *et al*., 2005). Moreover, animals that are affected by mutations in the *DMD* gene showed reduced dystrophin protein in the skeletal and heart muscles, and in increased CPK levels (Nonneman *et al*., 2012). With WCS carcasses, if there is a causal mutation in the *DMD* gene, the phenotype could be because of an increase in permeability of the cell membranes of muscles, which causes the typical shiny wet appearance of WCS. Similarly, if one assumes that the genetic predisposition of WCS exists as a causal mutation in the *DMD* gene, a hypothetical mechanism by which the *DMD* gene may cause WCS is through an increase in cell membrane permeability. An increase in cell membrane permeability by influencing the water-holding capacity of muscles may play a major role.

Marker oar3_OARX_113973214 was found to be in an intergenic region, positioned roughly 6 kbp downstream of the position the 5-hydroxytryptamine (serotonin) receptor 2C (*LOC101108550* or *HTR2C*) gene. Two functions of the *HTR2C* gene are potentially relevant to WCS given its physiological characteristics. These functions included stress responsiveness and calcium ion homeostasis. Stress may influence the manifestation of WCS in sheep (Jansen & Pretorius, 1986; Jansen, 1991) similar to the relationship between stress and PSS in pigs (Hall *et al*., 1980; MacLennan & Phillips, 1992). Mutations in the *RYR1* gene cause the calcium release channel to function improperly in afflicted pigs and poultry, resulting in the production of PSE meat under stressful conditions (Paião *et al*., 2013; Fisher *et al*., 2000). There is little doubt that there is a phenotypic similarity between PSS and WCS (Van der Westhuizen, 2018). Therefore, the *HTR2C* gene and its product the 5-HT2C receptor may disrupt the calcium ion homeostasis of WCS carcasses, and therefore result in a phenotypically similar carcass to PSE. Thus, these two genes *RYR1* (in pigs) and *HTR2C* (in sheep) may either act independently of each other or may be part of the same gene network that is functionally related to cause a similar phenotype in different species. It is well established that the 5-HT2C receptors are involved in stress response in humans (Miller & O'Callaghan, 2002; Donovan & Tecott, 2013; Avery & Vrshek-Schallhorn, 2016) and in anxiety (Griebel, 1995; Heisler *et al*., 2007a). Additionally, 5-HT2C receptors are involved in processes such as the coordination of the intracellular responses to serotonin, specifically in the central nervous system of mammalian species, homeostatic and behavioural responses, stress response, sexual behaviour, appetite control, obesity, and feeding behaviour in mice (Tecott *et al*., 1995; Nonogaki *et al*., 2003). Two wholly hypothetical mechanisms that may result in the manifestation of WCS (Quilter *et al*., 2012) are summarized here.. First, genetic inactivation or malfunctioning of the 5-HT2C receptor disrupts the regulation of the hypothalamic-pituitary-adrenal axis through serotonin (Heisler *et al*., 2007b). This disruption causes the live animal to respond ineffectively to stress, whether it is long term on the farm or acute pre-slaughter, and as a final consequence to manifest WCS post slaughter. Second, the normal biochemical process of muscle contraction that is initialized by serotonin binding to the receptor and ending with the continuing release of calcium ions from the sarcoplasmic reticulum to the cytoplasm of the cells is disrupted with the end result being the reduced water-holding capacity of the meat. Assuming the genetic predisposition of WCS exists as a causal mutation within the *HTR2C* gene, a disruption in cell homeostasis may occur, either during the pre-slaughter period of sheep by means of stress and anxiety or during the post-slaughter period through the calcium ion homeostasis mechanism within the cells of affected muscles.

Conclusions

The results attest to a potential genetic basis for WCS and its putative association with oar3_OARX_29903534 and oar3_OARX_113973214. Given the physical proximity of these loci with the *DMD* and *HTR2C* genes and the effects of these genes in other species, they are suggested as positional and functional candidate genes for WCS in sheep. The numbers for the current study would have been adequate, based on the original hypothesis that the genetic basis for WCS was caused by a simple autosomal recessive allele. Since this is probably not the case, and the mechanism seems to be more complex, involving the X chromosome, more samples are needed. The inconclusiveness of the genetic findings here leads to the suggestion that future studies should conclusively establish a genetic basis for WCS. Progression toward developing a genetic test to identify animals that are genetically predisposed to WCS is warranted only once this genetic basis is verified.

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Author's Contributions

LW carried out the research and developed the initial manuscript. MDM and MMS formulated the original hypothesis that lead to the investigation. MDM provided research direction, assisted in the interpretation of the results, and assisted in finalizing the manuscript. MMS obtained funding to support the research, supervised LW in its conduct, and assisted in finalizing the manuscript. FWCN provided academic supervision for LW, and assisted in interpreting the results and in finalizing the manuscript.

Conflict of Interest Declaration

The authors declare that there are no conflicts of interest.

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