Genomic Scars of the CRISPR/Cas9 System and the Pleiotropic Effects of *Socs2(+/-)* Genotype on Lamb Production Traits

By

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THESIS

Submitted in partial satisfaction of the requirements for the degree of

MASTER OF SCIENCE

in

Animal Biology

in the

OFFICE OF GRADUATE STUDIES

of the

UNIVERSITY OF CALIFORNIA

DAVIS

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2022

Acknowledgments

Firstly, I want to thank my parents, for the opportunities you've given me to learn and grow as a person. I can't express enough gratitude for the sacrifices you've made that have allowed me to pursue my education and current career. You've shown me the value of an education in your own lives and I hope to honor that by learning about the world and trying to make it a better and more interesting place!

Next, I want to thank Marcela Vilariño, for taking the time to teach me about your research project and for introducing me to the Ross lab while I was a Freshman undergraduate. You were the one that ignited my interest in gene editing and reproductive biotechnology by sharing your project with me and giving me a framework for understanding the relevance of what I was learning throughout my undergraduate classes.

Pablo Ross, you gave the opportunity to intern in your lab, see how science was conducted, practice techniques, support myself as a junior specialist, and pursue my own research project. I'm very thankful for the time I was able to spend in the Ross lab and it has advanced my scientific career.

Ahmed Mahdi, you mentored me, taught me many skills, and gave me opportunities to teach, which helped me develop my ability to formulate ideas, conduct research, teach others, and prepare me for graduate school and life.

Alison Van Eenennaam, you took me in as a graduate student on short notice and made me feel at home. Also, your continual effort to improve agriculture through the use of genome editing technology and your fight against the nonsensical regulations that hold back science and genetic improvement in livestock is aspirational.

Trish Berger, Lee Allen Pettey, Anna Denicol, Danika Bannasch, Christine Diepenbrock, Alex Nord, and Steven Roderick, I want to thank you for being exceptional teachers that have mentored, helped, and inspired me along the way!

Abstract

The *Suppressor of cytokine signaling 2* (*Socs2*) gene encodes the SOCS2 protein, a potent negative regulator of growth hormone receptor signaling. In mice, loss of SOCS2 function results in 30-50% increases in post-weaning growth without increases in birth or pre-weaning weight and without excessive adiposity or leanness. However, *Socs2* KO also reduces fertility, increases mortality rate, and prolongs inflammation in response to infection in mice. In sheep, a naturally occurring point mutation in *Socs2*, that is predicted to eliminate SOCS2 function, is associated with increases in size, weight, and milk production, at the expense of susceptibility to mastitis. We aimed to test whether *Socs2* knockout (KO) in a meat breed of sheep had detrimental effects on reproduction, and evaluate the growth, feed efficiency, survival, and carcass characteristics of *Socs2* heterozygous KO lambs. Additionally, we aimed to characterize the genomic alterations created by electroporation of sheep zygotes with Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) ribonucleoprotein complexed with dual guide RNAs targeting an 85 base pair span of *Socs2*. Whole genome sequencing (WGS) of six genome edited sheep, revealed that three healthy lambs carried large deletion alleles that evaded detection by initial PCR and Sanger sequencing. All six genome edited lambs were compound heterozygotes for *Socs2* mutations that were predicted to functionally ablate SOCS2 function and all the offspring of the two healthy genome edited rams were carriers for a *Socs2* KO allele. The pre-weaning (including prenatal) mortality rate of *Socs2(+/-)* lambs was 57% compared to 20% for *Socs2(+/+)* lambs, which was a large effect that trended towards statistical significance ($p = .052$). *Socs2(+/-)* rams were 17% taller at birth and *Socs2(+/-)* genotype was associated with a 22% increase in male growth rate during the preweaning period and a 28% increase in female growth rate in the post-weaning period.

Socs2(+/-) lambs did not differ significantly in feed efficiency and minor differences in carcass characteristics were observed. The modestly improved growth of *Socs2* heterozygous KO sheep is unlikely to benefit commercial lamb production unless the detrimental reproductive and survival phenotypes observed in *Socs2(+/-)* sheep are addressed.

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Chapter 1: Literature Review

Discovery of the Effects of *Socs2* on Mouse Growth

Research on the effects of the suppressor of cytokine signaling 2 (*Socs2*) gene on the growth of animals began in 1984 with the identification of a subset of mice that spontaneously arose in the 25th generation of a mouse breeding experiment whose post-weaning growth was 3 standard deviations above the mean (Bradford & Famula, 1984). The high growth subset of mice were born at the same weight as their littermates and grew indistinguishably from them during the pre-weaning period, but grew 30–50% more than controls without developing obesity in the post-weaning period (Bradford & Famula, 1984; Greenhalgh et al., 2002; Metcalf et al., 2000). The exceptional growth rate of these mice, was caused by a single partially recessive mutation termed *high growth (hg)*, with heterozygous (HG/*hg*) male mice growing to a weight slightly below the mid-parental average and female mice not growing to a significantly different weight (Bradford & Famula, 1984; Horvat & Medrano, 1996b; Metcalf et al., 2000). The *high growth* locus was later mapped to a 460kb deletion that encompassed the second exon of *Socs2,* the *Raidd/Cradd* gene, and the first two exons of the *Plexin C1* gene resulting in a fusion transcript of *Socs2* and *Plexin C1* (Horvat & Medrano, 1996a; Wong et al., 2002). Ablation of SOCS2 signaling was identified as the cause of the high growth phenotype after the generation of *Socs2(-/-)* mice was found to result in the same 30-50% increase in post-weaning growth (Metcalf et al., 2000).

The mechanisms underlying the phenotypes observed in *Socs2(-/-)* mice are still not completely elucidated, but a substantial amount of research in the last four decades has provided a reasonable understanding of the basic interactions between SOCS2 and the growth hormone receptor. When growth hormone (GH) binds to its receptor, Janus Kinase 2 (JAK2) dimerizes

and phosphorylates tyrosine residues on the intracellular domain of the GHR which serve as docking sites for signal transducer and activator of transcription 5a (STAT5a) and STAT5b; JAK2 then phosphorylates STAT5a and STAT5B as well as STATs 1 and 3 directly (Brooks et al., 2008). Additionally, there is JAK2-independent GHR signaling mediated by Src family kinase (SFK) signaling and activation of MEK, Ras and ERK1/2 through PLC γ (Rowlinson et al., 2008). Collectively, GHR signaling results in the activation of a variety of transcription factors involved in growth, metabolism, and physiological processes related to the hepatobiliary, cardiovascular, renal, gastrointestinal, and reproductive systems (Dehkhoda et al., 2018). In the liver, GH stimulation results in the expression of IGF-1 and other IGF-binding proteins that enter the circulatory system and exert effects on growth that are independent of GH and downregulate growth hormone secretion at the hypothalamic and pituitary levels (Dehkhoda et al., 2018). On the cellular level, GHR signaling is regulated by cytokine-induced members of the suppressor of cytokine signaling (SOCS) protein family. SOCS2 is one of eight members of the SOCS protein family, and is a particularly potent regulator of growth hormone receptor signaling (Postel-Vinay & Kelly, 1996). SOCS2 interacts with phosphorylated tyrosine residue 595 (pY595) and to a lesser degree phosphorylated pY487 of the GHR, via its Src homology 2 (SH2) domain and blocks further recruitment and activation of STAT5b (Greenhalgh et al., 2005). Additionally, via the SOCS-box domain, SOCS2 recruits Elongins B and C to form an E3 Ubiquitin Ligase complex that tags the GHR for proteasomal degradation (Bullock et al., 2006) (Figure 1.1).

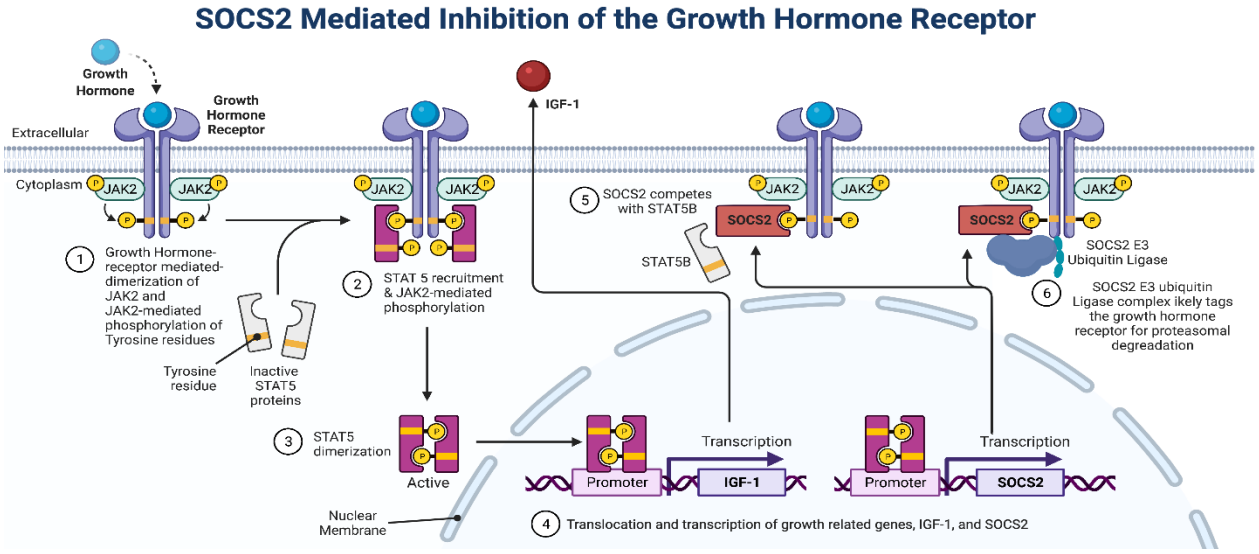


Figure 1.1: Predicted mechanism of Socs2 inhibition of growth hormone receptor signaling and the expression of growth-related genes. Figure generated with Biorender.

Socs2(-/-) mouse primary hepatocytes were shown to have prolonged growth hormone receptor activation and phosphorylation of STAT5b *in vitro.* Additionally, GH administration in *Socs2(-/-)* mice *in vivo* resulted in prolonged GH-regulated gene expression in the liver (Greenhalgh et al., 2002; Greenhalgh et al., 2005). Finally, the ablation of *Socs2* in mice already lacking GH-releasing hormone receptor (that causes a nearly complete deficiency in pituitary-derived circulating GH and induces dwarfism) or STAT5b resulted in minimal effects on growth suggesting that the increased post-weaning growth phenotype of *Socs2(-/-)* mice was dependent on GH mediated activation of STAT5b (Greenhalgh et al., 2002). While the growth effects of *Socs2* KO are known to be dependent on growth hormone and STAT5b signaling, the modulatory roles of secondary messengers like insulin-like growth factor-1 (IGF-1) that are transcriptionally activated by STAT5b remain poorly understood (Chia et al., 2006).

Interestingly, GH levels in *high growth* mice are consistently 20-30% lower and IGF-1 levels vary from normal to slightly elevated, depending on genetic background in *Socs2 (-/-)* mice (Medrano, 1991). This pattern is attributable to the negative feedback of IGF-1 on the secretion of GH by the anterior pituitary, with inhibition occurring at the hypothalamic and pituitary levels (Smith et al., 1996). Despite low levels of systemic GH, *Socs2(-/-)* mice have elevated local levels of IGF-1 in many tissues indicating increased GH signaling (Metcalf et al., 2000). High local levels of IGF-1 affect the deposition of collagen primarily in the skin of males, but also in lung bronchioles and occasionally in ductal tissues (Metcalf et al., 2000). While increased local IGF-1 levels were originally proposed as the mechanism of increased bone growth, recent evidence suggests that GHR signaling may be directly responsible and that both systemic and local IGF-1 levels have little effect on bone growth and size (Dobie et al., 2015). The increased bone growth of *Socs2(-/-)* metatarsal bones *in vitro* is independent of IGF-1, but depends on the IGF receptor suggesting a role for IGF-2 or IGF binding protein 3, which are both upregulated in growth hormone challenged *Socs2(-/-)* bones (Dobie et al., 2015). Evidence for the direct role of growth hormone receptor signaling on long bone growth comes from *Socs2(-/-)* 6-week-old mouse chondrocytes that have increased phosphorylation of STAT5, and greater longitudinal bone length and width (Pass et al., 2012). Thus, it is proposed that *Socs2* deficiency mediates bone growth directly through relaxing inhibition of greater phosphorylation of STAT5 and downstream signaling of non-IGF-1 secondary messengers with high local IGF-1 levels in some tissues mediating some phenotypic effects (Dobie et al., 2015).

In addition to dramatically increased post-weaning growth, homozygous *high growth* (*hg/hg*) mice with a C57BL/6J background do not experience major changes in feed efficiency or body composition. The amount of feed consumed by C57BL/6J-*hg/hg* mice is higher, but

proportional to the 51% increase in mature weight at 12 weeks of age (Corva & Medrano, 2000). The percentage of fat observed in *hg/hg* mice also does not differ from controls when both were fed a control diet. When fed a high energy diet, the fat percentage of the control mice increases while the fat percentage of the *high growth* mice does not and a heavier mature weight is achieved compared to diets that are not high energy (Corva & Medrano, 2000).

Prior to the identification of genetic markers for the rapid post-weaning growth of *high growth* mice, the trait was presumed to be completely recessive (Bradford & Famula, 1984). However, identification of genetic markers for the *high growth* locus, enabled the discovery that heterozygotes also expressed some of the high post-weaning growth phenotype, but that the increase was less than half the increase observed in homozygotes and that the increase was independent of sex (Horvat & Medrano, 1996b). However, *Socs2(+/-)* male mice grew 14% heavier during the post-weaning period and *Socs2(+/-)* female mice did not grow significantly larger than controls (an increase in post-weaning growth of 35% was observed in *Socs2(-/-)* males in this study) (Metcalf et al., 2000). Additionally, the increase in growth of *Socs2(+/-)* males was delayed relative to *Socs2(-/-)* males, with significant differences occurring at 11 and 6 weeks of age for *Socs2(+/-) and Socs2(-/-)* mice, respectively (Metcalf et al., 2000). The effects of *Socs2* deficiency on growth also interestingly has disproportionately large effects on longitudinal bone growth relative to flat bone growth that forms through intramembranous oddification instead of endochondral ossification (Li et al., 2022; Macrae et al., 2009; Rupp et al., 2015).

The Roles of *Socs2* Beyond Growth

The *high growth* mutation was first identified in a line of mice bred for high postweaning growth and prior to the identification of *high growth* mice in this line, low fertility was observed which suggested a natural tradeoff between postweaning growth and fertility (Bradford & Famula, 1984). Of the lines that were crossbred with *hg/hg* mice, some were infertile, and others had a range of normal to 40% fewer offspring than controls (Cargill et al., 1999; Lathan, 2012). Additionally, *hg/hg* mice had reduced lifespans with 50% of *Socs2(-/-)* mice dying before 450 days of age compared to 750 days for controls, which is also consistent with a tradeoff between growth and longevity (J. Casellas & J. F. Medrano, 2008). Another side effect of increased growth hormone receptor signaling is increased neurogenin production and the inhibition of neuronal differentiation; overexpression of *Socs2* in mouse neurospheres results in greater neuronal growth while *Socs2(-/-)* neurospheres exhibit decreased growth, which corroborates *in vivo* evidence of a 30% reduction in cortical neuron number and increased greater grey matter percentage in *Socs2(-/-)* mice (Turnley, 2002). While *Socs2* was originally identified through a screen for high post-weaning growth and its role in growth hormone receptor signaling is its most well-studied, SOCS2 has pleiotropic effects, regulates other SOCS proteins, and interacts with other cytokine signaling receptors including the erythropoietin, leptin, prolactin, estrogen, IGF-1, and inflammatory cytokine signaling receptors (Kung et al., 2019). One important function of *Socs2* is that the lipoxin A_4 mediated anti-inflammatory response is dependent on SOCS2 (Machado et al., 2006). A consequence of this is that upon infection with toxoplasma gondii, *Socs2(-/-)* mouse dendritic cells launched a pro-inflammatory response that initially decreased microbial numbers, but resulted in aberrant leukocyte infiltration and elevated mortality (Machado et.al., 2006). SOCS2 has been shown to reduce inflammation in a variety of disease models, but the cellular mechanism identified

differs substantially between models (Sobah et al., 2021). In experimental autoimmune encephalomyelitis infected mice, a decreased Th1 and Th2 response is observed initially, but it is followed by a prolonged increase in Th1 cells and a decrease in Th2 and Treg cells (Cramer et.al., 2019).

The Effects of *Socs2* Mutations in Sheep and Other Large Livestock

Many of the effects of *Socs2* KO in mice are also observed in livestock including sheep. In a French study investigating genetic susceptibility to mastitis, a naturally occurring nonsynonymous point mutation in *Socs2* (p.R96C) was identified that disrupts the tyrosine binding pocket of the SH2 domain of *Socs2* (Rupp et al., 2015). *In vitro* experiments showed a nearly complete lack of interaction between SOCS2 and its highest affinity phosphorylated residue on the GHR. Furthermore, homozygous p.R96C SOCS2 sheep were 24% taller, 18% heavier, and produced 4.4% more milk. However, there was also a strong positive correlation between this mutation and lifetime somatic cell count score (a proxy for genetic susceptibility to mastitis) (Rupp et al., 2015). Despite the apparently detrimental effects on mastitis susceptibility, the point mutation responsible for the p.R96C SOCS2 mutant protein was present at an allele frequency of 21.7% suggesting balancing selection and the potential for heterozygote advantage or incomplete penetrance of the detrimental mastitis phenotype. Heterozygous p.R96C SOCS2 ewes were significantly taller at the elbow, heavier, and produced more milk with a lower fat percentage than controls. However, the differences in weight from the controls were only significant starting at 530 days of age for the homozygotes and 3 years of age for the heterozygotes. Additionally, this comparison of growth was performed between two sets of triplet ewes per genotype over 3 years.

Thus, the increase in growth may not occur fast enough to be relevant to lamb producers and the effects of the mutation on male growth in sheep are unknown.

Evidence for a selective sweep in primarily Northern French breeds of meat sheep, with complete absence in Southern breeds, indicates a potential advantage of *Socs2* loss of function in meat breeds of sheep (Rochus et al., 2018). Additionally, *Socs2* was identified as having strong selective signatures in Tibetan sheep and is proposed to provide an adaptive advantage to the sheep living in the hypoxic Tibetan plateau environment through its regulation of the Erythropoietin receptor (Yang et al., 2016). However, it's important to note that *Socs2* and *STAT5B* have also been identified in selective sweeps for resistance to *H. contortus* infection and growth hormone transgenic overexpression sheep have elevated fecal egg counts (Adams et al., 2002; Benavides et al., 2015; Estrada-Reyes et al., 2019). While further functional analyses of the *Socs2* mutations in these studies is necessary to determine if *Socs2* deficiency is associated with parasite resistance, it's unlikely that loss of SOCS2 function would be selected for in environments with high rates of parasitism or infection based on the phenotypes *Socs2(-/-)* mice with toxoplasmosis and the negative association between postweaning weight and fecal egg count (Machado et al., 2006; Safari et al., 2005). In pigs, some SNPs in *Socs2* are associated with increased fat deposition, faster growth, and improved feed conversion efficiency in the post-weaning period (Chen et al., 2011; Ramos et al., 2009). Additionally, a nonsynonymous SNP in the *Socs2* gene is associated with size in some large breeds of dog (Rincón et al., 2007).

Generation of *Socs2* KO Sheep

Given the beneficial growth characteristics of *Socs2(-/-)* and *hg/hg* mice and the association of naturally occurring *Socs2* deficiency with increased growth in sheep and other large mammals,

Socs2 knockout (KO) or *Socs2* heterozygous KO sheep may offer a production advantage, particularly as terminal sires. The increase in post-weaning growth could decrease the time to market weight, the amount of feed consumed, the amount of labor required, and the greenhouse gas emissions. However, more data is necessary to determine how *Socs2* deficiency may negatively influence other production traits.

Recently, sheep with the same p.R96C SOCS2 mutant protein described by Rupp et al. (2015) were recreated by programmable base editing (Zhou et al., 2019). A cytosine base editor was used, which consists of a fusion protein between dead Cas9 protein and a cytidine deaminase that is directed to a target sequence by a guide RNA (gRNA)/Cas9 protein complex that localizes a cytosine deaminase that converts cytosines to uracils that are later converted to T•A complementary base pairs following replication or DNA repair (Huang et al., 2021). In the previously mentioned study, 53 zygotes were microinjected and 4 lambs were born; one lamb was unedited, two had *Socs2* indel mutations and the third had primarily the intended single base pair substitution. However, bystander editing of nearby cytosine nucleotides occurred in all lambs and the total rate of intended substitution was below 45% for all animals making the inheritance of the intended mutation inefficient. The two lambs with indels in *Socs2* did not have any observable health issues, and the phenotype of the three genome edited founder animals showed increased pre-weaning weight gain, but observations of more animals with more homogeneous genotypes over a longer period of time is necessary to draw any conclusive results from these SOCS2 pR96C sheep (Zhou et al., 2019).

Additionally, a second group used a dual gRNA approach to editing *Socs2* at the zygote stage (Mahdi, 2021). The use of dual gRNAs, is an approach that can increase the likelihood of a gene knockout by creating double-strand breaks at two target sites that creates either protein

disrupting indel mutations at both target cut sites, or deletions that span the sequence between the two cut sites (Binda et al., 2020). Two gRNAs were designed to target an 85 bp region of exon 1 of *Socs2* for deletion and functional knockout that encompassed part of the extended SH2 domain of the protein (Bullock et al., 2006). 80ng/μL of Cas9 ribonucleoprotein was complexed with dual guide RNAs at 40ng/μL and electroporated into zygotes to produce one stillborn, two lambs that died pre-weaning, and three healthy sheep. All six sheep were high percentage *Socs2* knockouts (Chapter 2 of this thesis is the genomic analysis of these sheep). The three healthy genome edited founders grew at a rate consistent with a 30-50% increase in growth, and this growth was observed in the pre-weaning period which is consistent with the base editing group, but different from what is observed in *Socs2* KO mice. There are still many caveats to analysis of growth from this study of founder animals including highly limited sample size, known epigenetic effects of *in vitro* fertilization on birth weight, differences in sex between edited and control lambs, and litter size.

The extent to which the *Socs2* KO mouse phenotype translates to *Socs2* KO sheep remains poorly studied and the improved growth of *Socs2* KO mice, p.R96C SOCS2 sheep, and genome edited founder sheep suggests that the introduction of *Socs2* mutations that decrease or eliminate SOCS2 function may be a powerful tool to rapidly improve the growth of sheep. However, it's critical that the tools used to create genome edited sheep are well understood so that scientists and regulators can understand their capabilities, specificity, and potential dangers. A broader picture of the effects of *Socs2* on production characteristics beyond growth is necessary to understand whether there are any detrimental effects of *Socs2* KO on other production characteristics such as fertility, survival, feed efficiency, and carcass characteristics that potentially outweigh any improvements in growth. The degree to which heterozygous *Socs2* KO balances the potential harms of *Socs2* KO with it potential benefits is a major aim of chapter 3 of this thesis. In the next

chapter of this thesis, the genomic alterations created by the CRISPR/Cas9 system will be assessed in an effort to better understand the effectiveness of a dual gRNA approach to gene KO, the accuracy of mutagenesis, and the repair pathways used by the cell to repair CRISPR/Cas9 induced double strand breaks in sheep zygotes.

Chapter 2: Genomic Analysis of *Socs2* Knockout Sheep

Abstract

The standard method of genotyping Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) genome edited mammalian embryos with PCR primers located close to the target site and Sanger sequencing is limited to the identification of short indel mutations. Whole genome sequencing (WGS) allows for the characterization of large deletions, insertions, transversions, and translocations that can also occur following CRISPR/Cas9 mediated double-strand breaks. Generation of six genome edited sheep was performed via electroporation of Cas9 protein complexed with dual guide RNAs (dgRNA) targeting an 85 base pair span of the *suppressor of cytokine signaling 2* (*Socs2)* gene. Initial genotyping of three healthy genome edited lambs by PCR and Sanger sequencing of a 479 base pair amplicon suggested that all three were homozygous *Socs2* KOs. However, 14/21 of the offspring sired by the two healthy rams were initially genotyped as wild type and forced a reevaluation of initial genotypes. Whole genome sequencing (WGS) of the genome edited lambs revealed that all three healthy offspring carried an additional large deletion allele, up to 2127 base pairs, that eliminated PCR primer binding sites for those alleles. Subsequent genotyping of the offspring with redesigned primers resolved *Socs2* genotypes as heterozygous for one of the father's knockout alleles. Additionally, all six of the genome edited lambs were compound heterozygotes for *Socs2* mutations that deleted the start site, resulted in frameshifts, or deleted 82bp or more of exon 1 and were predicted to functionally ablate SOCS2 function. Trace levels of mosaicism were detected in the WGS data of some of the edited lambs, but none of the 21 offspring of the two genome edited rams inherited any of these low-frequency variants. Analysis of the breakpoints of some alleles suggested that microhomology-mediated end joining (MMEJ) was used to repair double strand breaks . Genotyping of live offspring by WGS revealed that 3 of

the 6 genome edited sheep carried alleles that evaded detection by PCR and Sanger sequencing of a standard-length amplicon.

Introduction

Single-step microinjection or electroporation of CRISPR/Cas9 reagents into Metaphase II (MII) oocytes or early embryos can have mutational efficiencies upwards of 95% (Mahdi et al., 2022). A standard method for optimizing the electroporation conditions and genome editing reagent concentrations for the desired editing efficiency or mutation is conventionally performed by genotyping blastocysts through PCR, Sanger sequencing, and TIDE analysis of the target region (Sakurai et al., 2014). While this approach is effective at detecting the presence of unedited alleles and short indels, other classes of mutations known to occur from CRISPR/Cas9-mediated double-strand DNA breaks can evade detection. Large deletions of up to 20,000 base pairs, large insertions that exceed the required PCR extension time, chromosomal rearrangements, loss of heterozygosity, and inversions are all types of mutations that have been observed following CRISPR/Cas9 genome editing and that are often undetectable using standard genotyping approaches (Kosicki et al., 2018; Simkin et al., 2022). Detection of these mutations can be performed by whole genome sequencing, but this approach is not feasible for genotyping preimplantation embryos because it requires a greater quantity of DNA than what is present in the embryo. Thus, analysis of genome editing outcomes in live offspring is critical to establishing the efficiency of editing, and characterizing the mutations created following CRISPR/Cas9 mediated strand break. There is currently a paucity of comprehensive genomic

evidence on the effects of CRISPR/Cas9 genome editing in livestock MII oocytes and embryos, which limits applications of the technology in agricultural and biomedical fields.

One of the major limitations of CRISPR/Cas9 genome edited livestock generation is mosaicism caused by post-zygotic editing. Editing beyond the one-cell stage results in different alleles in different cells of an organism that complicates the phenotypic analysis of genome edited animals and necessitates multiple generations of breeding to achieve animals that are confirmed to be biallelic for a desired mutation. In genome edited livestock production, this is a major time and economic drain due to the long generation interval and often costly phenotypic assessments. The variety of genome editing outcomes is primarily mediated by the unpredictable indels generated by nonhomologous end joining of two strands of DNA with little or no homology (Song et al., 2021). One method used to reduce variation in editing outcomes is to bias the cell towards the microhomology mediated end joining (MMEJ) pathway by designing a single gRNA or dgRNAs that create two arms with microhomology (1-16bp) on either side of the breakpoint (Ata et al., 2018). The MMEJ repair pathway identifies homologous sequences and deletes the sequence in between as well as one of the homology arms (Mateos-Gomez et al., 2015). Additionally, synthesis-dependent MMEJ can result in the insertion of nearby sequences between two flanking homologous sequences (Yu & McVey, 2010). The design of sgRNAs that create short regions of homology is an approach used in genome editing to increase the likelihood of specific mutations occurring and limit the variation in mutations (Ata et al., 2018). However, this approach was not intentionally used in this study, but may have incidentally biased MMEJ by the design of dgRNAs located close to two sequences of homology.

The *suppressor of cytokine signaling 2* (*Socs2*) gene is two exons long and encodes a single transcript. SOCS2 interacts with cytokine receptors via an SH2 domain, primarily coded

for in exon 1 and a SOCS box domain that interacts with Elongins B/C to form an E3 ubiquitin ligase complex that tags cytokine receptors for degradation (Bullock et al., 2006; Kung et al., 2019). There is also an extended SH2 subdomain that bridges the interface between the SOCS box and the SH2 domains and links E3 ubiquitin ligase activity to substrate capture and inhibits Jak2 phosphorylation of the receptor (Bullock et al., 2006; Yasukawa et al., 1999). Mutagenesis of all three domains of SOCS2 suggests that all are critical to the inhibition of its primary target, the growth hormone receptor.

To characterize the on-target effects of CRISPR/Cas9 genome editing in six sheep, WGS and PCR informed by WGS data were performed. Amino acid sequence, functional effect, and mechanism of repair was analyzed for each major allele. Additionally, the potential presence of mosaicism and off-target mutations was assessed. Finally, the genotypes of 21 offspring of two genome edited rams were established by PCR.

Materials and Methods

Animals

All experiments involving adult genome-edited lambs and their offspring were approved and performed in accordance with the University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol #18343).

Synthetic Guide RNAs

The CHOPCHOP and CasOFFinder web tools were used to select single guide RNAs (sgRNA) 1 and 2 (target sequence in Figure 2.1) (Bae et al., 2014; Labun et al., 2016). Guide RNAs 1 and 2 were chosen because of their low potential for off-target mutations and because

both had 3 mismatches or more between the spacer sequence and the target sequence, and at least one mismatch in the 10bp seed region adjacent to the protospacer adjacent motif (PAM).

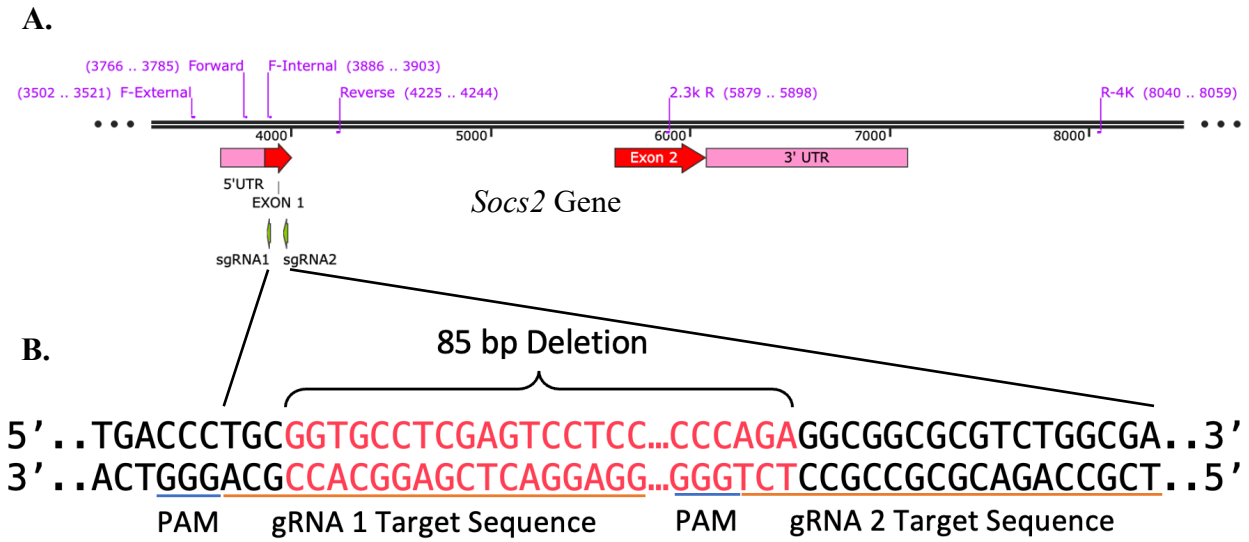


Figure 2.1: Socs2 gene map and target loci. (A) Locations of single guide RNAs (sgRNA) and primer binding sites on the Socs2 gene. Primer binding sites and locations on DNA are indicated with purple, Untranslated regions (UTR) are indicated with pink, exons are indicated with red, and gRNA target sites are indicated in green. (B) Guide RNA target and PAM sequences are underlined in orange and red, respectively, and the bases predicted to be deleted are colored in red.

Additionally, the guides were selected to be 85 bp apart in exon 1 of *Socs2* to increase the likelihood of creating a frameshift early in translation of the gene or if an in-frame deletion was created that it would eliminate a critical part of the protein, part of the extended SH2 domain including residues necessary for the kinase inhibitory region (Bullock et al., 2006). The list of potential off-target sites with up to 4bp mismatches from the crRNA sequence was assessed with the online tool CasOFFinder. 3 potential off-targets with 3 mismatches or fewer were identified

for sgRNA 1, 1 was identified for sgRNA 2 and 90 targets with 4 bp mismatches were identified for sgRNA 1 and 39 were identified for sgRNA 2 (Appendix Table 2.6).

Generation of Genome Edited Lambs

Generation of genome edited lambs was performed as previously described (Mahdi, 2021). Briefly, oocytes were aspirated from ovaries collected from ewes processed at the Superior Farms abattoir. Oocytes were matured in 400 µL of maturation medium (BO-IVM, IVF BIOSCIENCE) and incubated for 22-24 hours. Fresh sperm was collected from two whiteface primarily Dorset background rams (Ram 1 and Ram 2) at the UC Davis sheep barn and used for *in vitro* fertilization. Electroporation was performed 6 hours post fertilization with sgRNAs 1 and 2 at 40ng/µL each (Synthego Corporation,Redwood City, CA, USA) and Cas9 protein (PNA Bio, Thousand Oaks, CA, USA) at a concentration of 160ng/µL (Target sequence in Figure 2.1B). Electroporation of zygotes 6 hours post fertilization was performed with the NEPA21 Super Electroporator and Nepa 1 mm gap Electroporation Cuvettes (EC-001) (Nepagene, Ichikawa-City, Japan) with 4 unipolar pulses of 40 volts for 3.5 milliseconds, and 5 transfer pulses of 5 volts for 50 milliseconds each. 52 blastocysts were transferred to 13 recipient ewes and 6 lambs were born: one healthy ewe tag # 7181, two healthy rams #7182, and #7183 were born, along with one stillborn lamb Stillbir, and two lambs that died within the first week of life Male and Female. Rams 7182 and 7183 were mated to 11 unedited ewes, resulting in 21 fetuses (alive and dead). A wildtype ram #7068 was also mated to 6 ewes resulting in 10 fetuses (pedigree of relationships in Figure 3.1 ; and the identification of all animals in this experiment is in Table 3.6).

Whole Genome Sequencing

Genomic DNA from the six genome edited lambs (7181, 7182, 7183, Male, Female, and Stillbir) and their two sires (Ram 1 and Ram 2) were used for WGS with the Illumina NovaSeq platform following manufacturer instructions. Insert sizes were approximately 300 bp with 2x150 bp paired-end reads. Qualified reads were mapped to the ARS-UI_Ramb_V2.0 and Oar_v4.0 reference genomes using Burrows-Wheeler Aligner-Maximum Exact Match (BWA-MEM) and indexed with BWA-Index (BWA tools v7.1). Aligned WGS BAM files were visualized with Integrative Genome Viewer (v2.13.2). DNA used for WGS of Male, Female, and Stillbir was lost following WGS and genotypes could not be confirmed with PCR.

DNA Extraction

DNA from the offspring of the genome edited rams, including fetuses, was collected from tail samples removed during routine tail docking and DNA was extracted via phenol chloroform DNA extraction or the QIAGEN DNeasy Blood and Tissue® kit (QIAGEN, Hilden, Germany). Blood was collected from 7181, 7182, and 7183 with a purple top vacutainer and was kept on ice prior to centrifugation for 10’ at 2,000 RPM. Cells from the buffy coat were pipetted and DNA was extracted using the QIAGEN DNeasy Blood and Tissue® kit (QIAGEN). Semen was collected from rams 7182 and 7183, and DNA was extracted from a processed sperm pellet via organic extraction and alcohol precipitation of DNA.

WGS-Based Parentage and Sexing

The sire of each founder genome edited lamb was confirmed through a comparison of WGS genotypes at 23 SNPs where Ram 1 and Ram 2 were homozygous for different alleles (Appendix Table 2.7). A lamb was determined to be sired by a ram if it shared at least one allele

in common with a ram for all 23 SNPs. The sex of the genome edited lambs was determined visually and was confirmed by genotype at 5 SNPs of the ZFX gene in the whole genome sequencing data (Appendix Figure 2.4). Due to the lack of a Y chromosome reference sequence in the female ARS-UI_Ramb-v2.0 genome, ZFY gene reads align to the ZFX gene due to high sequence homology and males appear to be heterozygous at 5 SNPs in the ZFX gene that are only present in the ZFY gene. One of these SNPs, NC_056080.1:22,532,148 is used for restriction fragment length polymorphism based sexing of sheep and other eutherian mammals (Aasen & Medrano, 1990). Validation of the SNPs identified was performed by PCR and Sanger sequencing with primers that amplify the ZFX and ZFY regions showing heterozygosity at 4 SNPs in males, but not females (Figure 2.2A,B, Appendix Figure 2.5).

WGS Analysis of On-Target Edits

Deletions were indicated by sharp drops in coverage that spanned the cut site for either sgRNA in Integrative Genomics Viewer (version 2.13) visualization of the WGS results and at the read level by red colored reads indicating a deletion due to a larger than expected insert size. The length of deletions was determined by the locations of sharp drops in coverage and by matching reads to their read mates on either side of the deletion. Bases in IGV with minor allele frequencies (MAF) > 20% are colored green for adenine, red for thymine, orange for guanine, and blue for cytidines) and the relative proportion is indicated as the fraction of each read depth. Gray nucleotides match the reference genome with < 20% MAF. Alleles with > 20% MAF were used to predict heterozygosity. For mutations that did not span both target sites, two mutations were classified as belonging to the same allele if there were informative reads that had both mutations present on the same read.

PCR and Sanger Sequencing

PCR reactions were prepared with 10 µL of Promega GoTaq Green Master Mix (2x), 1 µLforward primer (10µM), 1 µL reverse primer (10µM), 100ng of genomic DNA, and molecular grade nuclease-free water to a final volume of 20 µL. The primer sequences and thermal cycler conditions for each amplicon are listed Table 2.1 and Table 2.2, and visualization of the primer binding sites for the ovine *Socs2* (oSOCS2) gene are shown in Figure 2.1 in purple. PCR products were run on 1% or 2% agarose gels (specified on gel image) prepared with 60 or 120 mL of 1% tris base acetic acid and EDTA (1% TBE), and SYBR™ Safe gel stain (10,000X) diluted to .6X. Visualization of bands was performed under blue and UV light. DNA fragments were cut out of gels individually using a scalpel and purified PCR products were gel-extracted by a freeze and squeeze method of placing the gel on top of a filter tip in a 1.5 mL conical tube, freezing the sample for 5' at -80 °C then centrifuging for 3 minutes at 14,000 RPM. Gel-purified PCR products were outsourced to GeneWiz (Azenta, South Plainfield, New Jersey) for purified PCR product Sanger sequencing. Visualization of Sanger sequencing results was performed in SnapGene (version 6.1.1).

Table 2.1: PCR primer sequences used in the current study.

Primer name	Sequence 5'-> 3'
Socs2 Forward	AGGGAGTGGTTTTGGGGTTC
Socs2 Reverse	AGCGCCCTAAGAGTCGATTT
Socs2 F-Internal	TCGAGTCCTCCGGGAATG
Socs2 F-Ext	CAGTAACTGGATGCTCGGGG
Socs2 R-2.3k	TGTCCGCTTATCCTTGCACA
ZFX/Y F	TTACCAGCAAGGCGGAGAAG
ZFX/Y R	TTCGCAGTACTGGCATTGGT

Table 2.2: Thermal cycler conditions used for PCR of each amplicon.

	Amplicons				
	Socs2 2.3K	Initial *Socs2*	*Socs2* Internal	*ZFX/Y* Sexing	*Socs2* 4K
Forward Primer	F Ext	Forward	F Internal	ZFX/Y F	F Ext
Reverse Primer	R-2.3K	Reverse	Reverse	ZFX/Y R	R-4K
Wild Type Amplicon Size	2397	479	359	308	4558
Initial Denaturation Time	4'	3'	3'	3'	3'
Denaturation Temp (°C)	95	95	95	95	95
Denaturation Time	30"	30	30	30"	30"
Annealing Time	30"	30	30	30"	15"
Annealing Temperature (°C)	59	58	58	59	59
Extension Time	2'30"	45	45	30"	5'30"
Extension Temperature (°C)	72	72	72	72	72
Final Extension Time	5'	5	5	5'	5'
Infinite Hold Temperature (°C)	4	4	4	4	4
Volume (μL)	20	20	20	20	20
Cycle #	35	34*	40	29	35

*35 cycles for comparison of founders

Variant Analysis

The consequence of each variant was assessed with ENSEMBL Variant effect predictor (release 108) for only the mutation created at target site 1 for Female, 7181, and Stillbir due to a limitation in the software for prediction of the effects of two variants on the same allele and all lambs being edited at the first target. Missense mutations that also result in frameshifts are not specified as high impact frameshifts, but as coding variants.

Results

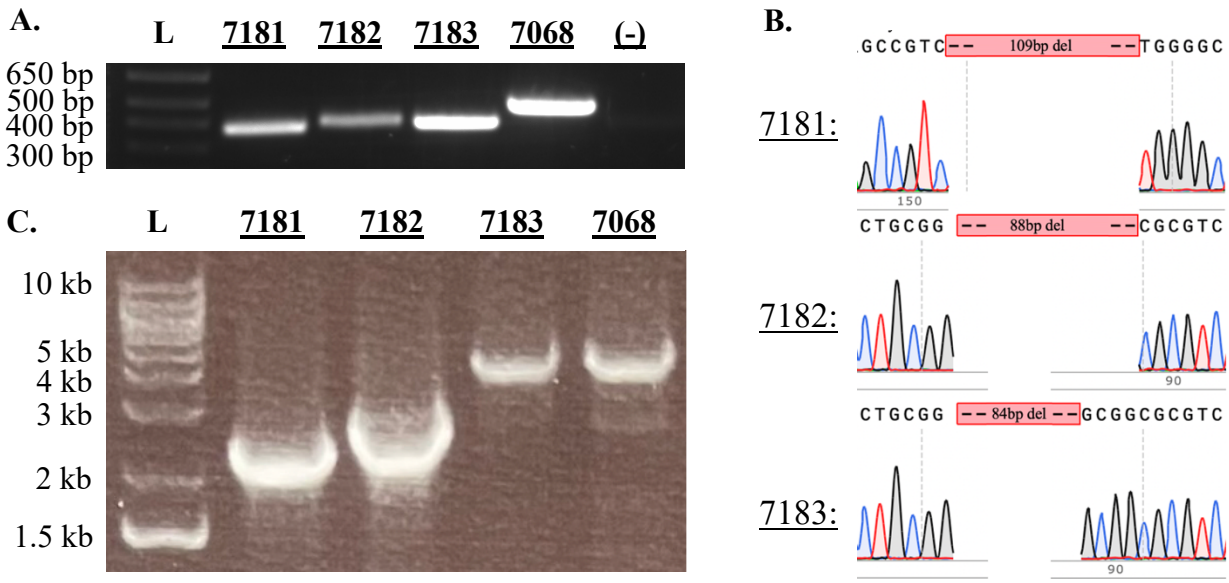
Initial Genotyping

The three live and healthy genome edited sheep, 7181, 7182, and 7183, were originally genotyped using the *Socs2* Forward and *Socs2* Reverse primers that yield a 479bp amplicon for the wild type *Socs2* gene. Sanger sequencing revealed that 7181 had a 1 bp Guanine insertion at the sgRNA 1 cleavage site and a 109bp deletion starting near the sgRNA 2 cleavage site; 7182 had an 84bp deletion, and 7183 had an 88bp deletion (Figure 2.2A,B). Based on the results of this PCR and Sanger sequencing analysis, the three healthy founders were genotyped as homozygous without any wild type DNA detectable with 35 cycles of PCR amplification. Rams 7182 and 7183 were then bred to 11 unedited ewes and the 21 offspring were genotyped using the same primer pair. 14 of the 21 offspring were genotyped as wild type, with only 7 sheep that were heterozygous for one of the deletions in their father (Figure 2.2D). Given that this wild type result was at odds with the homozygous genotype of the founder rams, WGS analysis of all genome edited sheep was performed to determine if there was allelic dropout. Additionally, a *Socs2* internal PCR amplicon was designed with the *Socs2* F Internal primer located within the 88bp and 84bp deletions of 7182 and 7183 and the Socs2 Reverse primer to test for trace amounts of wild type DNA in those fathers.

WGS Analysis of Genome Edited Sheep

All genomes had a Q30 greater than 93.9%. WGS results of 7182 were relatively low coverage with raw data of 38.3 GB compared to 94-120 GB for the other sheep. WGS indicated that both on-target sites for sgRNA 1 and sgRNA 2 were disrupted in each of the 12 alleles of the 6 genome-edited sheep resulting in 15 independent mutations (with most mutations spanning both cut sites, Figure 2.3). The size of deletion varied greatly from 30bp for Female allele 1 to

2127bp for 7181 allele 2 (Figure 2.3, Table 2.3). Three large deletions that spanned both target sites and eliminated one or multiple primer binding sites in the initial PCR were identified in lambs 7181, 7182, and 7183 (Figure 2.3, Table 2.3). Additionally, three intermediate-sized deletions of 30, 101, and 109 bp were identified, with the 101 bp deletion present in Male spanning both target sites and the other two initiating at target site 2 (Table 2.3). None of the mutations were the exact 85 bp deletion between the predicted cleavage sites, but 6 deletions were within a 5bp window of both cut sites (Figure 2.3). Additionally, three small insertions were observed, with a single base pair guanine insertion at cut site 1 occurring in allele 1 of ewe 7181 and allele 1 of Stillbir, as well as a "TTT" insertion occurring in Stillbir allele 1 cut site 2 (Table 2.3). After visualization of WGS results in IGV, primers were designed to amplify the *Socs2* 2.3K and 4K amplicons to detect on-target alleles potentially missed in the initial PCR. Large deletions visible by PCR and Sanger sequencing confirmed the presence of large previously unidentified deletions in the *Socs2* healthy founders and the offspring of the two founder rams (Figure 2.2C,D). All offspring were PCR and Sanger sequenced to determine *Socs2* genotype and all were genotyped as heterozygotes for one of two mutations present in the two founder rams (Figure 2.2D, Appendix Figure 2.8, Appendix Figure 2.9).


A.
L
7181
7182
7183
7068
(-)
650 bp
500 bp
400 bp
300 bp
B.
7181:
GCCGTC -- 109bp del -- TGGGGC
150
7182:
CTGCGG -- 88bp del -- CGCGTC
90
7183:
CTGCGG -- 84bp del -- GCGGCGCGTC
90
C.
L
7181
7182
7183
7068
10 kb
5 kb
4 kb
3 kb
2 kb
1.5 kb

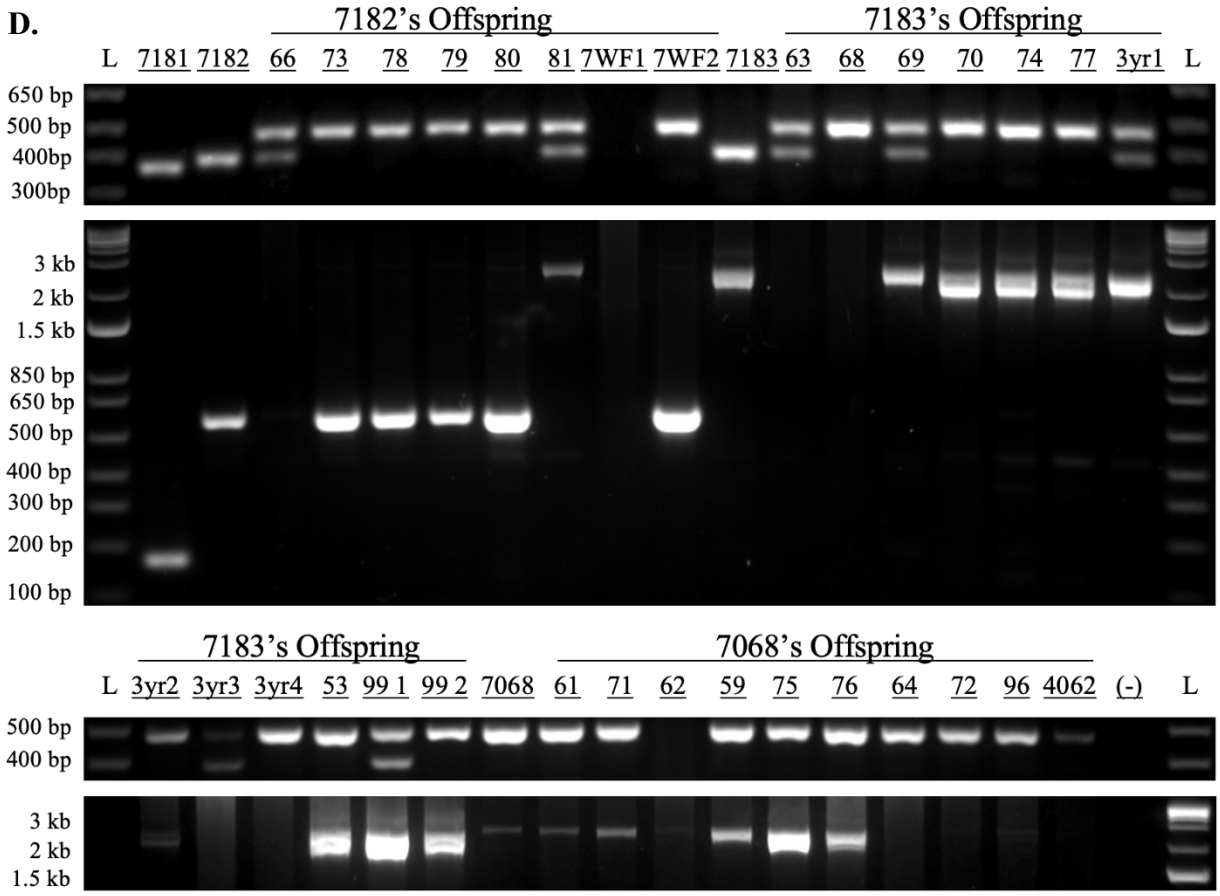

D.
7182's Offspring
7183's Offspring
L 7181 7182 66 73 78 79 80 81 7WF1 7WF2 7183 63 68 69 70 74 77 3yr1 L
650 bp
500 bp
400bp
300bp
3 kb
2 kb
1.5 kb
850 bp
650 bp
500 bp
400 bp
300 bp
200 bp
100 bp
7183's Offspring
7068's Offspring
L 3yr2 3yr3 3yr4 53 99 1 99 2 7068 61 71 62 59 75 76 64 72 96 4062 (-) L
500 bp
400 bp
3 kb
2 kb
1.5 kb

Figure 2.2: (A) Initial Socs2 PCR products from healthy genome edited lambs and control ram 7068, L indicates the 1 kb Plus Invitrogen Ladder, run on a 1% agarose gel, (B) Sanger sequencing chromatogram at breakpoints for deletions present in the healthy founders, (C) Socs2 4K PCR products indicating the presence of large deletions in 7181 and 7182, for complete gel and description see Appendix Figure 2.8, (D) PCR products from Initial and 2.3K Socs2 PCR amplicons showing the SOCS2 mutant alleles present in the healthy founders and the offspring of those founders, run on a 2% gel. Live offspring tag numbers shortened by the preceding "211", prenatal dead offspring were indicated by ewe tag # and number of fetus including 7WF 1 and 2, 3yr (ewe tag #3yrNT) 1-4, 53 (ewe tag #8153), 99 (ewe tag #9199) 1 and 2, 96 (ewe tag #6096), and 4062. Repeat of 2.3K Socs2 PCR was performed see Figure 2.9 to establish final genotypes.

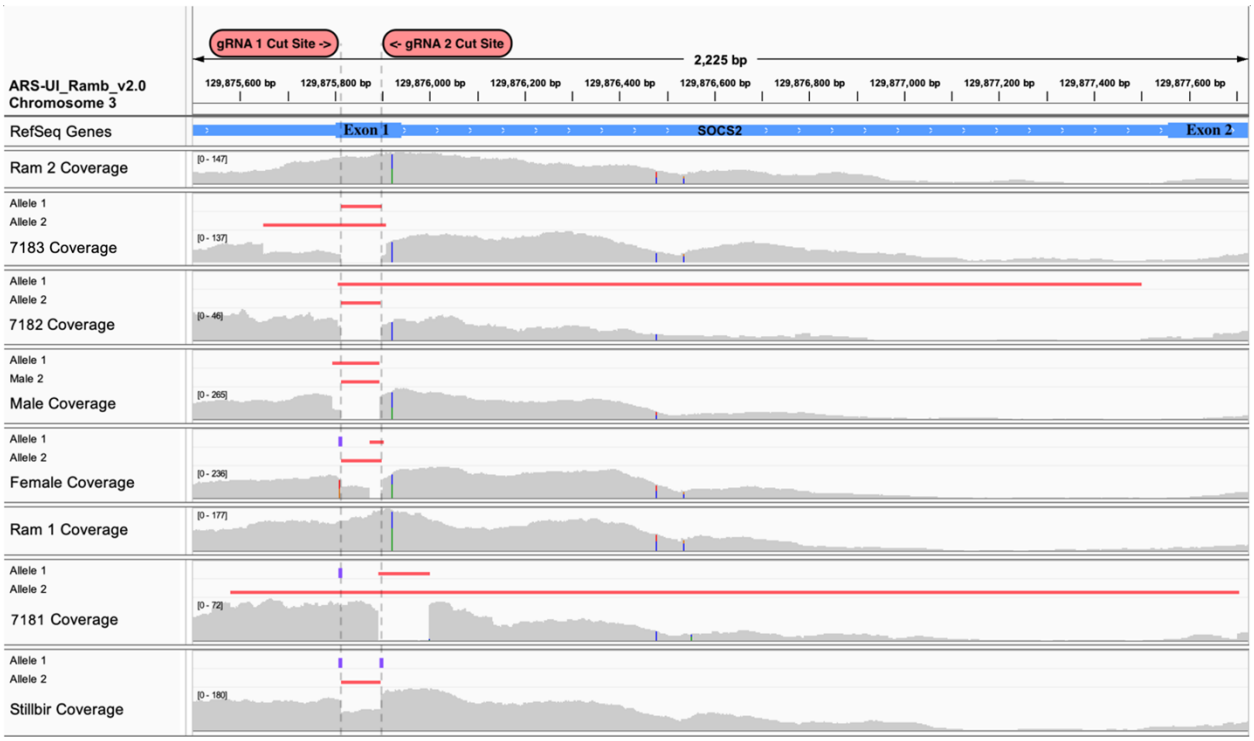
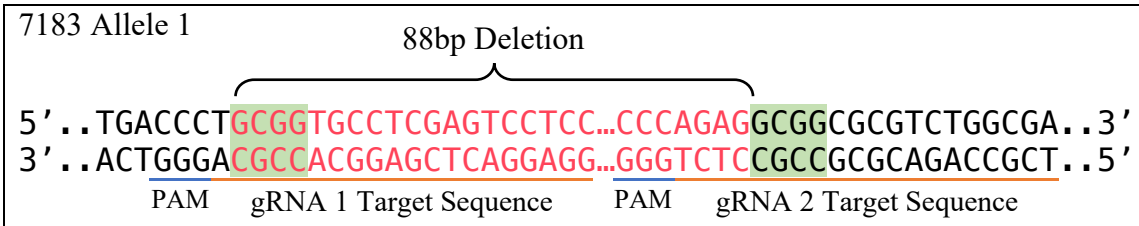
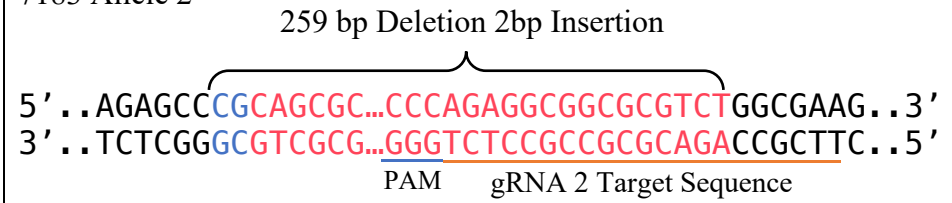
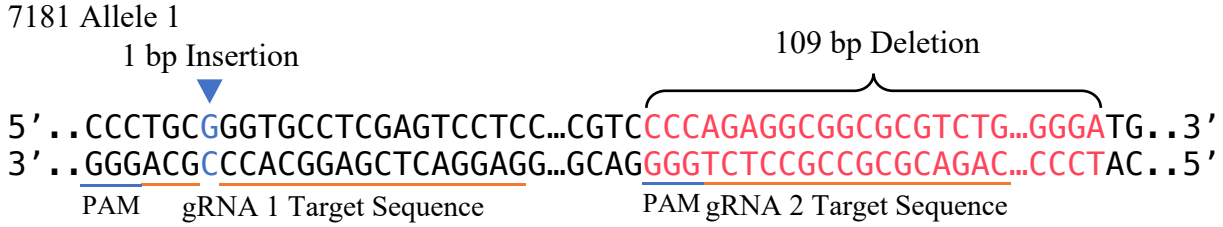


Figure 2.3: IGV view of on-target mutations with coverage (read depth) for each sheep showed in gray. Deletions are indicated by red bars spanning from one sharp drop in coverage to the next and inserions are indicated as purple rectangles.

Table 2.3: CRISPR/Cas9 genome editing breakpoints, descriptions of mutations, and their predicted effects. Breakpoints are annotated with red letters indicating deleted nucleotides, and blue letters indicating inserted nucleotides. Green highlighted sequences indicate homologous sequences potentially used by the MMEJ pathway to either delete one homology arm or use a nearby template sequence to repair a deletion, and yellow highlighted sequences indicate repeats present at one breakpoint and between a template sequence for synthesis-dependent strand annealing via the MMEJ pathway. Variant names are according to human genome variation society nomenclature guidelines for genomic variants and correspond to positions in the ARS-UI_Ramb_v2.0 reference genome. The predicted amino acid sequence for each mutant allele was determined through in silico translation of the Socs2 variant(s) in SnapGene with unaltered amino acids bolded.

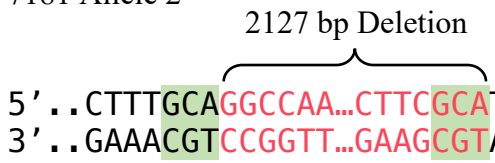
7183 Allele 1  Repair signature: MMEJ deletion Variant name: NC_056056.1:g.129875814_129875901del Predicted amino acid sequence: **MTLR**RVWRRP* Variant predictor consequence: Missense variant and coding sequence variant
7183 Allele 2  Repair signature: NHEJ Variant name: NC_056056.1:g.129875650_129875908delinsCG Predicted amino acid sequence: Start site deletion Variant predictor consequence: Coding sequence variant and 5' UTR variant

7181 Allele 1



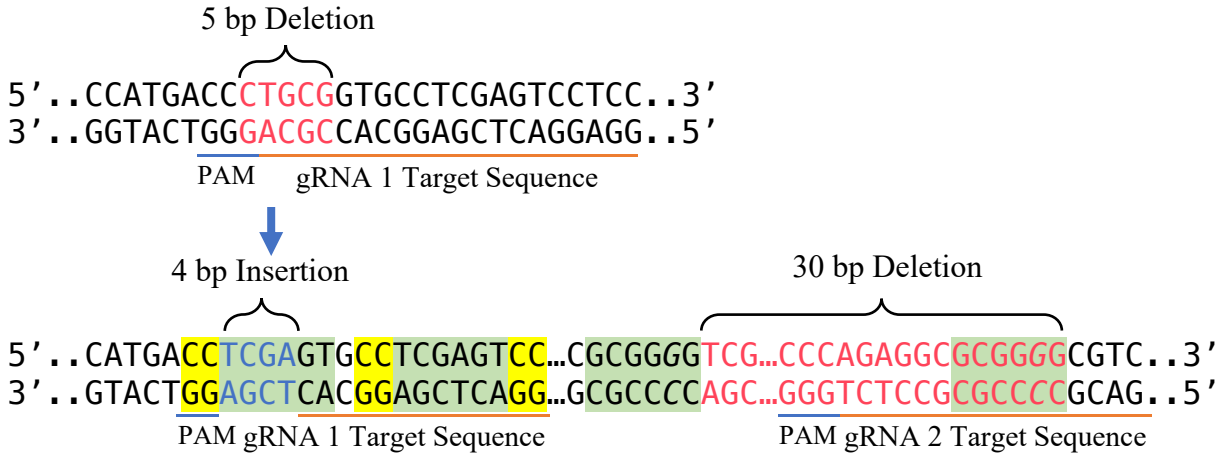
Repair signature: NHEJ, NHEJ
Variant name: NC_056056.1:g.129875811_129875812insG, NC_056056.1:g.129875579_129877705del
Predicted amino acid sequence:
MTLRVPRVLREWRGRGAEPVGDRGVGGGAVVGTGEI*
Variant predictor consequence: Frameshift variant

7181 Allele 2



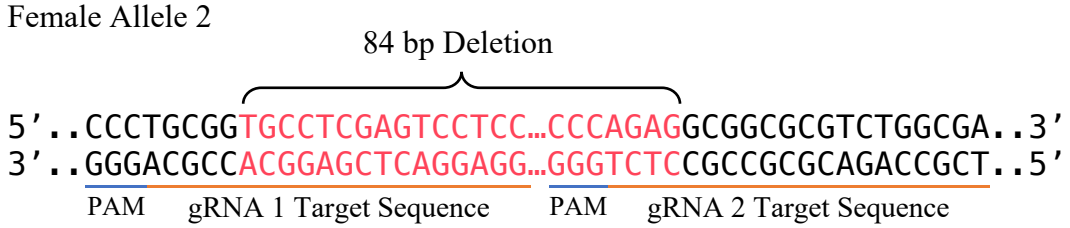
Repair signature: MMEJ
Variant name: NC_056056.1:g.129875578_129877705del
Predicted amino acid sequence: Start site deletion
Variant predictor consequence: Start lost, splice acceptor variant, splice donor variant, splice donor 5th base variant, splice polypyrimidine tract variant, coding sequence variant, 5’ UTR variant, and intron variant

Female Allele 1



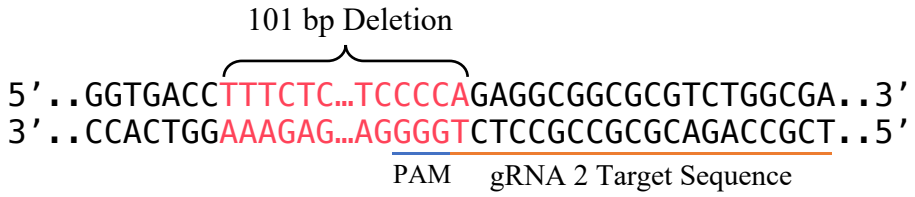
Repair signature: MMEJ mediated synthesis-dependent strand annealing, MMEJ
Variant name: NC_056056.1:g.129875808_129875812delinsTCGA, NC_056056.1:g.129875873_129875902del
Predicted amino acid sequence: **M**TSSASSPPGMARKGRRASGGPRGVWRRP*
Variant predictor consequence: Missense variant, and coding sequence variant
*The italicized guanine nucleotide, 6 nucleotides from the 3’ end is heterozygous G/C in Female

Female Allele 2



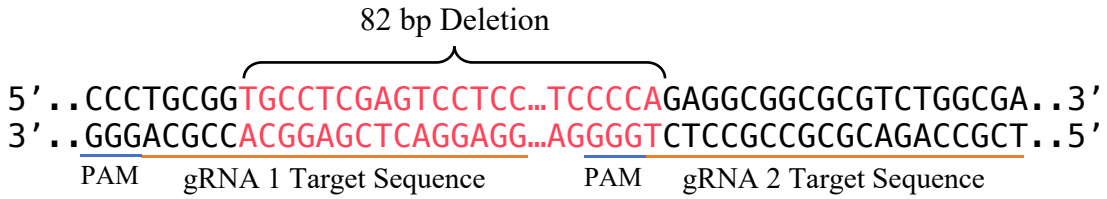
Repair signature: NHEJ
Variant name: NC_056056.1:g.129875814_129875901del
Predicted amino acid sequence: **MTLR-28 amino acid deletion-**
AARLAKALRELSHTGWYWGNM
TVNEAKEKLKEAPEGTFLIRDSSHSDYLLTISVKTSAGPTNLRIEYQDGKFRLDSII
CVKSKLKQFDSVVHLIDYYVQMCKDKRTGPEAPRNGTVHLYLTKPLYTSAPPLQ
HLCRLTINKCTSTIWGLPLPTRLKDYLEEYKFQV*
Variant predictor consequence: Missense variant

Male Allele 1



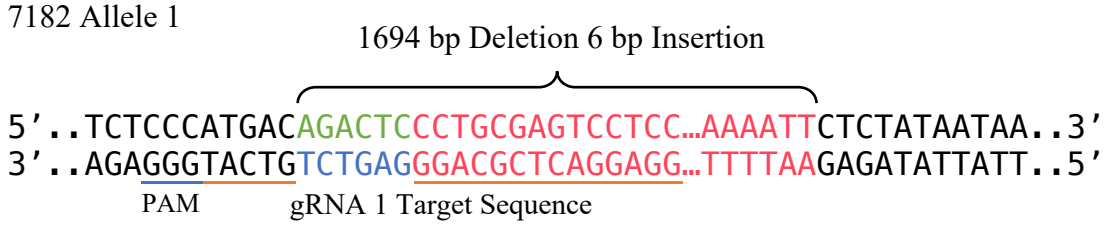
Repair signature: NHEJ
Variant name: NC_056056.1:g.129875794_129875894del
Predicted amino acid sequence: Start site deletion
Variant predictor consequence: Start lost and 5' UTR variant

Male Allele 2



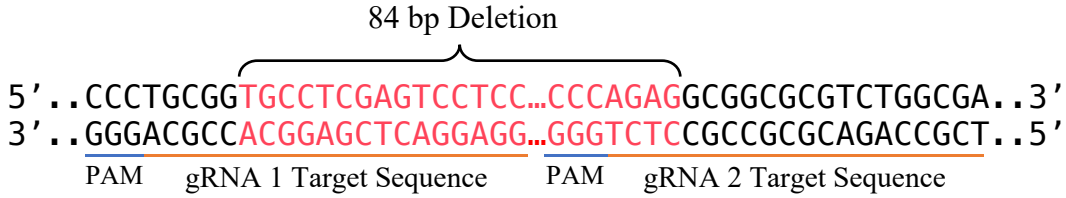
Repair signature: NHEJ
Variant name: NC_056056.1:g.129875813_129875894del
Predicted amino acid sequence: **MTLR**RRRVWRRP*
Variant predictor consequence: Missense variant and coding sequence variant

7182 Allele 1



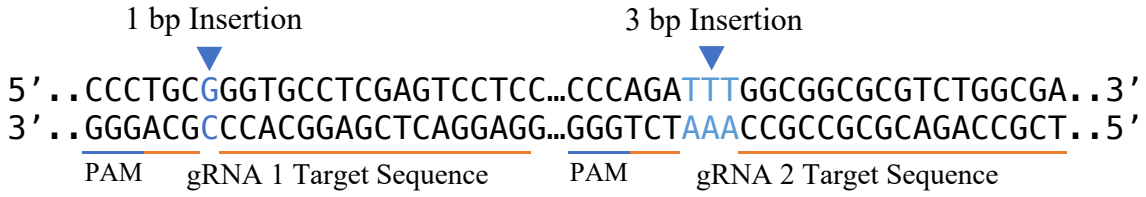
Repair signature: NHEJ
Variant name: NC_056056.1:g.129875807_129877500delinsAGACTC
Predicted amino acid sequence : Start site deletion
Variant predictor consequence: Start lost, splice donor variant, splice donor 5th base variant, coding sequence variant, and intron variant

7182 Allele 2



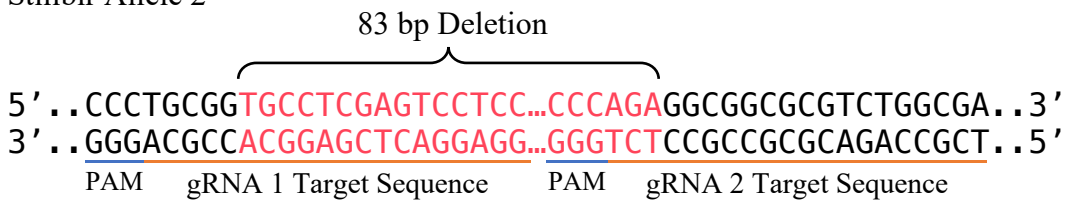
Repair signature: NHEJ
Variant name: NC_056056.1:g.129875814_129875897del
Predicted amino acid sequence: **MTLR-28 amino acid deletion-**
AARLAKALRELSHTGWYWGNM
TVNEAKEKLKEAPEGTFLIRDSSHSDYLLTISVKTSAGPTNLRIEYQDGKFRLDSII
CVKSKLKQFDSVVHLIDYYVQMCKDKRTGPEAPRNGTVHLYLTKPLYTSAPPLQ
HLCRLTINKCTSTIWGLPLPTRLKDYLEEYKFQV*
Variant predictor consequence: Missense variant

Stillbir Allele 1



Repair signature: NHEJ
Variant name: NC_056056.1:g.129875811_129875812insG,
NC_056056.1:g.129875875_129875876insTTT
Predicted amino acid sequence:
MTLRVPRVLREWRGRGAEPVGDRGVGGGAVPRFGGASGEGPEGT
QSHRLVLGKYDC*
Variant predictor consequence: Frameshift variant

Stillbir Allele 2



Repair signature: NHEJ
Variant name: NC_056056.1:g.129875813_129875895del
Predicted amino acid sequence: **MTLR**GGASGEGPEGTQSHRLVLGKYDC*
Variant predictor consequence: Missense variant and coding sequence variant

Analysis of the predicted effects of all allelic mutations present in the genome edited sheep were predicted to result in a protein reading frame that was either lacking a critical region of the SOCS2 protein, missing a start codon, or had a frameshift mutation within the first 5 amino acid residues (Table 2.3). The mutation predicted to have the least detrimental effect on protein translation was the in-frame NC_056056.1:g.129875814_129875897del mutation present in 7182 and Female (the only mutation that was not unique) that deleted an 84bp region of exon 1. While the mutation did not disrupt the SH2 or SOCS box domains, it did eliminate an 84 bp section of the extended SH2 domain of the SOCS2 protein, which is critical for the kinase inhibitory function of SOCS2 and for the structure and function of the SH2 and SOCS box domains (Bullock et al., 2006; Yasukawa et al., 1999). Based on current understanding of the function of the 84 bp deleted, the effect of deleting all but 5 of the wild type amino acids and the effect of deleting a start site, it's predicted that all of the mutant alleles will result in a nonfunctional SOCS2 protein.

Mosaicism

A small number of mosaic reads were observed in the WGS data, but all were present at below 5% MAF and may be artifacts of mismapped reads or index hopping, which according to

the manufacturer can occur at low rates in samples. Conflicting results were observed with PCR and Sanger sequencing of semen with a small fraction of wild type DNA observed in both rams from an initial collection of semen, but not in either from a second collection of blood (See Appendix Figure 2.6, Figure 2.7). Mosaic alleles with greater than one read are listed in Table 2.4.

Table 2.4: Mosaic reads and alleles occurring more than once in WGS results and spanning one of the two cut sites.

Lamb name	Position of reads	Number of reads	Allele
7182	NC_056056.1:129,875,612-129,877,500	4	1889bp deletion
7183	NC_056056.1:129,875,812-129,877,500	2	G insertion, Wild type from 3' side (doesn't cross to other cut site)
7181	NC_056056.1:129,875,814-129,875,894, NC_056056.1:129,875,975-129,875,999	3	81 and 21bp deletions

Off-Targets

Potential off-target binding sites within 4bp mismatches of the target sequence in the ARS-UI_Ramb_v2.0 genome were visually assessed for insertions at the cut site or drops in coverage spanning the cutsite and up to 10,000 bp away from the cut site (See appendix Table 2.6 for list). A more in depth analysis would be necessary to conclude that no off-target editing events occurred, but no major mutations were evident.

Offspring Genotypes

Following genotyping of the genome-edited sheep, PCR and Sanger sequencing of the offspring was performed and all offspring carried one edited allele from their father (Table 2.5).

Table 2.5: Socs2 genotypes of the genome edited rams 7182 and 7183, control ram 7068, and their offspring. Italicized sheep died prenatally.

Sire	**Tag #**	***Socs2* Genotype**
Ram 2	7182	NC_056056.1:g.129875814_129875897del/ NC_056056.1:g.129875807_129877500delinsAGACTC
7182	21166	NC_056056.1:g.129875814_129875897del/WT
7182	21173	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
7182	21178	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
7182	21179	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
7182	21180	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
7182	21181	NC_056056.1:g.129875814_129875897del/WT
7182	*7WF1*	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
7182	*7WF2*	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
Ram 2	7183	NC_056056.1:g.129875814_129875901del/ NC_056056.1:g.129875650_129875908delinsCG
7183	21163	NC_056056.1:g.129875814_129875901del /WT
7183	21168	NC_056056.1:g.129875650_129875908delinsCG/WT
7183	21169	NC_056056.1:g.129875814_129875901del /WT
7183	21170	NC_056056.1:g.129875650_129875908delinsCG/WT
7183	21174	NC_056056.1:g.129875650_129875908delinsCG/WT
7183	21177	NC_056056.1:g.129875650_129875908delinsCG/WT
7183	*3YRNT1*	NC_056056.1:g.129875814_129875901del /WT
7183	*3YRNT2*	NC_056056.1:g.129875650_129875908delinsCG/WT
7183	*3YRNT3*	NC_056056.1:g.129875814_129875901del/WT
7183	*3YRNT4*	259bp del, 2bp ins/WT
7183	*8153*	259bp del, 2bp ins/WT
7183	*9199 1*	NC_056056.1:g.129875814_129875901del/WT
7183	*9199 2*	259bp del, 2bp ins/WT
	7068	WT/WT
7068	21161	WT/WT
7068	21171	WT/WT
7068	21162	WT/WT
7068	21159	WT/WT
7068	21175	WT/WT
7068	21176	WT/WT
7068	21164	WT/WT
7068	21172	WT/WT

7068	*4062*	WT/WT
7068	*6096*	WT/WT

Discussion

Electroporation of Cas9 RNP is a recent advance in the genome engineering of livestock embryos that allows for the batched introduction of genome editing reagents for up to around 100 oocytes or embryos simultaneously, while microinjection can only be performed on individual oocytes (Lin & Van Eenennaam, 2021). Early evidence suggests that electroporation is highly efficient at creating genome edited embryos with a minor effect on embryo survival so long as electroporation is performed at the time of fertilization or at the pronuclear stage and electroporation settings are optimized for the species of interest (Lin & Van Eenennaam, 2021; Mahdi et al., 2022). In mice, electroporation of gRNA into Cas9 expressing embryos was shown to result in a 100% or close to 100% mutation efficiency with no mosaicism (Sakurai et al., 2020). In the current study, we found that electroporation of Cas9 RNP complexed with dgRNAs in sheep oocytes at the pronuclear stage resulted in a 100% mutation efficiency. This indicates that electroporation is a highly efficient method of introducing CRISPR/Cas9 gene editing reagents into zona intact embryos and has great potential for the generation of genome edited livestock. Additionally, using this approach, all the major alleles present in the genome-edited livestock were predicted to result in the intended KO of the *Socs2* gene. However, the intended deletion of 85 bp did not occur in any of the founder animals and there was a high degree of variability in mutations following editing.

Of particular importance to livestock genome engineers and regulators is that 25% of all alleles characterized by WGS were undetectable by PCR and Sanger sequencing of a standard

length amplicon using PCR primers close to the target site. This poses a major problem for the genotyping of genome edited livestock embryos where small quantities of DNA limit the possibilities for genotyping. One potential method of identifying a greater number of editing events is through designing primer sets that amplify a large genomic region around the cut sites that are more likely to identify large deletions. Additionally, the use of a ram that is homozygous for an SNP not present in the population of ewes used for oocyte collection can be used to determine if there is allelic dropout by analyzing chromatogram data for the absence, heterozygosity, or homozygosity of the ram's SNP indicating that either the ram's copy of the target sequence has not amplified, that both alleles are present, or the maternal allele has not amplified, respectively.

Unfortunately, PCR of large amplicons often requires a high degree of optimization for efficient amplification and when large deletions occur, the smaller PCR amplicon can outcompete the larger amplicon and distort the relative abundance of each amplicon or eliminate detection of the larger amplicon entirely. Additionally, it is unlikely that a mutually exclusive SNP will be located close to a target gene. Digital droplet PCR avoids the issue of PCR template heterogeneity and can be used to detect low-frequency alleles, but it is still limited to the detection of edits that fall within the range of the amplicon (Sorokina et al., 2022).

Recently, quantitative genotyping PCR was proposed as a relatively inexpensive method for detecting allelic dropout without requiring high amounts of DNA by comparing the quantity of a known two-copy gene on a different chromosome to the quantity of DNA from a target amplicon, however, there are no published works using this approach in blastocysts (Weisheit et al., 2021). Another potential remedy for the detection of on-target edits is the use of nicking Cas9 variants that make only single-stranded breaks, base editors, or prime editors that all have

reduced likelihoods of on-target edits that would evade detection through PCR (Chen & Liu, 2022). Thus, it is difficult to accurately assess the on-target edits in single-step generated genome edited livestock embryos and the ability to perform WGS or otherwise comprehensively genotype a cell line prior to introducing it to the germ line remains a major advantage for a two-step method of genome editing where the edit is created and characterized in a cell line then transferred to an oocyte or embryo in methods like somatic cell nuclear transfer. However, for the generation of a gene KO where a variety of indel mutations can result in the intended knockout phenotype, single-step introduction of genome editing reagents is effective at producing the intended knockout phenotype.

One major limitation to the adoption of CRISPR/Cas9 genome editing is the potential for off-target mutations and mosaicism. Off-target mutations can result in unintended deleterious effects in sheep, but their occurrence is relatively rare and can be predicted through the use of web-based tools like CasOFFinder that search reference genomes for potential off-target sites (Hennig et al., 2020; Zhang et al., 2015; Zhou et al., 2019). Our visual analysis of potential off-target sites with 4 mismatches or fewer from the target sequence did not identify any obvious off-target mutations. A more rigorous analysis of *de novo* variants present in the founder lambs was not possible due to the lack of maternal WGS data and the inability to distinguish between maternally inherited variants and CRISPR/Cas9 generated variants.

Another concern in genome editing is mosaicism, which can obscure the evaluation of founder animal phenotypes and result in a variety of offspring phenotypes (Hennig et al., 2020). While trace levels of mosaicism were detected in the WGS results of the three healthy lambs, none of the mosaic alleles were consistently detectable by PCR and only the two major alleles were inherited by the 21 offspring. The results from this paper support the mounting evidence

that off-target editing is rare with well-designed gRNAs and that low frequencies of mosaicism identified in WGS is unlikely to be disseminated to offspring (Atkins et al., 2021; Zhou et al., 2019).

The deletion of the exact region between the two Cas9 cleavage sites was not observed and a variety of interesting editing outcomes occurred. This may be due to the orientation of the dual gRNAs, which has been shown to dictate the rates of precise deletions, with 11% and 79% precise deletions observed with PAM external and PAM internal oriented dgRNAs respectively (Song et al., 2021). Additionally, there were discernable patterns at the breakpoints that indicated the utilization of the NHEJ and MMEJ repair pathways. While the NHEJ pathway is the most common double-strand break repair mechanism and was still found to mediate the majority of repair in our study, we found evidence for a modest frequency of MMEJ pathway utilization which is a less commonly reported repair pathway (Xue & Greene, 2021). One potential reason why the MMEJ repair pathway might have been preferred in this study was that a "GCGG" microhomologous sequence was located close to the cut sites of both gRNAs and was utilized in some cases for repair. While the gRNAs in this study were not intentionally designed to utilize the MMEJ pathway, some CRISPR/Cas9 genome engineers have intentionally designed gRNAs that create microhomologous sequences on either side of the double-strand break to bias the likelihood of a specific repair sequence that deletes one of the microhomology regions (Ata et al., 2018). This approach is particularly useful for creating targeted mutations and reducing mosaicism by increasing the likelihood of a specific repair.

This current study documents a comprehensive genotypic analysis of the effects of CRISPR/Cas9 genome editing on the genomes of six sheep electroporated with dual sgRNAs targeting the *Socs2* gene and shows that this method was highly efficient for generating KO

mutations. It also revealed that large mutations, greater than the expected 85 bp deletion, commonly evade standard methods of detection via PCR and Sanger sequencing. Additionally, low levels of mosaicism may have been present in some of the genome edited lambs, but at low frequency and none of the alleles were passed on to offspring by two of the genome edited founder rams. Further, examination of the mutations obtained suggests that the design of sgRNA orientation and location with respect to DNA homology can affect the repair pathways utilized by the cell to repair CRISPR/Cas9 mediated double-strand break.

Acknowledgments

We would like to express our deep gratitude to Superior Farms (Dixon, CA, USA) for providing sheep ovaries for this experiment, and to Dr. Darren E. Hagen, Oklahoma State University, for assistance with the WGS analyses.

Appendix - Chapter 2

Table 2.6: List of potential off-targets for the ARS-UI_Ramb_V2.0 genome. "MM" indicates the number of mismatches between the crRNA and potential target DNA.

gRNA	DNA	Chromosome	Position	Strand	MM
sgRNA1	GGAGGACTCGAGGCACCGCAGGG	CM028706.1	129875805	-	0
sgRNA1	GGAGGACTgGAGGCcCCtCATGG	CM028707.1	78675044	-	3
sgRNA1	GGAGGAgcCGAGGCACCGCgCGG	CM028714.1	10533418	+	3
sgRNA1	GGgGtACTCGgGGCACCGCAAGG	CM028723.1	7514399	-	3
sgRNA1	cGAGGACTgGAGGttCCGCAGGG	CM028704.1	33305754	+	4
sgRNA1	GaAGGACTCcAGtCACCtCATGG	CM028704.1	126076202	-	4
sgRNA1	tcAGGACTCGAGGgACCcCATGG	CM028704.1	213699613	-	4
sgRNA1	GGAGGACagGAGGCAgCGgAAGG	CM028704.1	278532031	-	4
sgRNA1	GGAGGcCTCacGGCACtGCAGGG	CM028705.1	943287	+	4
sgRNA1	GGAaGACTCcAcGtACCGCAGGG	CM028705.1	30746353	-	4
sgRNA1	GGAGGACTacAGaCACCGCgCGG	CM028705.1	107235354	-	4
sgRNA1	GGAGGACTgtgGGCACCcCAGGG	CM028705.1	244280549	-	4
sgRNA1	GGAGGACTgtgGGCACCcCAGGG	CM028705.1	244280528	-	4
sgRNA1	GGAGGACTgcgGGCACCtCAGGG	CM028705.1	244280442	-	4
sgRNA1	GGAGGACTgtgGGCACCcCAAGG	CM028705.1	244280571	-	4
sgRNA1	GaAGGACTgGAGGCACCcaAGGG	CM028705.1	246538513	-	4
sgRNA1	GGAGGcCTCGgGcCACCcCACGG	CM028705.1	248343737	+	4
sgRNA1	GtgGGACTCGAGGCtCCcCAGGG	CM028706.1	12410094	-	4
sgRNA1	GGAGGgCTgGAGGCAgCGaAGGG	CM028706.1	31449468	+	4
sgRNA1	GGAGGAaTCGAGaCACtGaAAGG	CM028706.1	33664649	+	4
sgRNA1	GGAGGACTgGAcaCACCaCAAGG	CM028706.1	97843722	-	4
sgRNA1	GGAGGACTCtgGGaACCaCAGGG	CM028706.1	100750234	+	4
sgRNA1	GGAGaACTgGAGGCAgaGCAAGG	CM028706.1	209727018	-	4
sgRNA1	tcAGGACTCGAGGgACCcCATGG	CM028706.1	218534004	-	4
sgRNA1	GGAGGgCTgGAGGCAgCcCAGGG	CM028706.1	220912924	-	4
sgRNA1	GGAGGACTCcAcGtgCCGCAGGG	CM028706.1	221389397	-	4
sgRNA1	GGAGGACaCGAGGCACaGagAGG	CM028707.1	77936562	-	4
sgRNA1	GGAGGAaTCGAtGgcCCGCAGGG	CM028708.1	59790498	+	4
sgRNA1	aGcGGAtTCGAGGCACgGCATGG	CM028708.1	105352459	-	4
sgRNA1	tGcaGACTCGgGGCACCGCAGGG	CM028709.1	46217940	-	4

sgRNA1	aGAGGACTCGAtGgACgGCAAGG	CM028709.1	73921886	-	4
sgRNA1	GGAGGcCTaGAGGCACtGgATGG	CM028709.1	104298668	+	4
sgRNA1	GGAGGACgtGcGGCACCGCcTGG	CM028709.1	118089899	+	4
sgRNA1	GGAGGACTCcAGGCAgCagATGG	CM028710.1	55768677	+	4
sgRNA1	GcAGGACTCtAGGCtCgGCATGG	CM028711.1	83698563	-	4
sgRNA1	GGAGGAggCGgGGCACCGCtGGG	CM028712.1	16207853	+	4
sgRNA1	GGAGGAggCGAGGCcCCGCcAGG	CM028712.1	44412563	-	4
sgRNA1	GGAaGAtgCGAGGCACtGCAGGG	CM028713.1	15662328	-	4
sgRNA1	GGAGaACTCGAcaCAtCGCAGGG	CM028713.1	22149648	+	4
sgRNA1	GGgGGACTCGAGtCACaGCcAGG	CM028713.1	33053766	-	4
sgRNA1	GGAGGACctGAGaCACaGCAAGG	CM028714.1	28323151	-	4
sgRNA1	aGAGGAgcCGAGGCACCGCtGGG	CM028714.1	34773080	+	4
sgRNA1	GGAGacCcCGAGGCACtGCAAGG	CM028714.1	52534274	-	4
sgRNA1	GGAGGACTCcAGGaAgCcCATGG	CM028714.1	57861666	-	4
sgRNA1	GcAGGACaaGAtGCACCGCAGGG	CM028715.1	3039110	+	4
sgRNA1	GGAGGgCTCtAaGCcCCGCATGG	CM028716.1	9264176	-	4
sgRNA1	tGAGGACcCGtGGCAgCGCAGGG	CM028716.1	40112002	-	4
sgRNA1	tcAGGACTCGAGGgACCcCATGG	CM028716.1	53088158	+	4
sgRNA1	GGAGGACTCGAGGgACCaggTGG	CM028716.1	56300445	+	4
sgRNA1	GGAGGgCTgcAGGCAgCGCATGG	CM028716.1	76940591	+	4
sgRNA1	GGAaGACcCcAcGCACCGCAGGG	CM028716.1	81103148	+	4
sgRNA1	GcAGGACTCGAGtCtCtGCATGG	CM028716.1	82436990	+	4
sgRNA1	GGAGGACgCctGGCACCGgAAGG	CM028717.1	17575607	+	4
sgRNA1	GGAGGAaTCGAaGCACaGgAAGG	CM028717.1	19107088	+	4
sgRNA1	GGAaGACTCcAGGgACCcCAGGG	CM028717.1	23091061	+	4
sgRNA1	GGAaGACTaGAGGCgCCGCgGGG	CM028717.1	43314749	-	4
sgRNA1	GGAGGACTtGcGGaACCGCcTGG	CM028717.1	52429174	+	4
sgRNA1	GGAGGACTCGAacCAaCaCAAGG	CM028718.1	5853666	+	4
sgRNA1	GcAGGACaCcAGGCcCCGCAGGG	CM028718.1	75148551	-	4
sgRNA1	GGAGGcCcCGAGGaACtGCAAGG	CM028719.1	4361816	-	4
sgRNA1	GGAGGACTtGgGGaACCaCAAGG	CM028719.1	22034918	-	4
sgRNA1	GGAGGACgaGAGGCtCtGCAGGG	CM028719.1	71407761	-	4
sgRNA1	acAGGACTtGAGGCtCCGCAGGG	CM028719.1	71660814	+	4
sgRNA1	GGAGGACTCaAGtCAatGCATGG	CM028720.1	36775229	-	4
sgRNA1	GGAGGACTCGAGGgACtcCcTGG	CM028720.1	38745925	+	4
sgRNA1	GGAGGACTgGAaGCtCCGCtCGG	CM028720.1	67417453	+	4
sgRNA1	GGAGGACTgGgGaCACCaCAGGG	CM028720.1	71157211	+	4
sgRNA1	GGtGGcCTCGcGGCtCCGCAGGG	CM028720.1	72928712	-	4

sgRNA1	GGAtaACTCGAGGCACacCATGG	CM028721.1	12675167	-	4
sgRNA1	GGAaGACggGAGGCACaGCAGGG	CM028721.1	24898855	-	4
sgRNA1	GGcGGACTCaAGGgACaGCATGG	CM028721.1	54417832	+	4
sgRNA1	GcAGGACTCGAtcCACCGCtGGG	CM028721.1	55184364	+	4
sgRNA1	GGAGGtaaCGAGGCACCaCAGGG	CM028721.1	65437512	-	4
sgRNA1	GGAGGACgCGAGGCACCcgcGGG	CM028721.1	67182468	-	4
sgRNA1	aGAGGgCcCGAGGCACCGCcTGG	CM028722.1	7597655	+	4
sgRNA1	GGAGGcCTCGAGGCAgCtCtTGG	CM028722.1	12055937	-	4
sgRNA1	tGAGGACTCcAGGgACCcCAGGG	CM028724.1	26144904	-	4
sgRNA1	aGtGGACTCGAGGaACaGCAGGG	CM028724.1	26972749	-	4
sgRNA1	cGAGGtCTgGAGGCACCcCAGGG	CM028724.1	29491693	+	4
sgRNA1	GaAGGACTCaAaGCACaGCAAGG	CM028724.1	41343187	+	4
sgRNA1	GGAGGAggCGcGGCACCaCAGGG	CM028725.1	7392237	+	4
sgRNA1	GGAGGACTCGtGGCtgtGCAAGG	CM028726.1	33443745	-	4
sgRNA1	GGAGGACTCGtGGCtgtGCAAGG	CM028726.1	33527622	-	4
sgRNA1	GGAGGcCTgGtGcCACCGCAGGG	CM028726.1	37666226	+	4
sgRNA1	GcAGGAgTCttGGCACCGCAGGG	CM028727.1	9843159	+	4
sgRNA1	GGgGGcCTgGtGGCACCGCAAGG	CM028727.1	41561047	-	4
sgRNA1	GGAGGACTCcAGGgAgtGCAGGG	CM028728.1	23119573	-	4
sgRNA1	GGgGGACcaGAGtCACCGCAGGG	CM028729.1	679349	-	4
sgRNA1	GGAGGACagGAcGCACgGCAGGG	CM028729.1	1589341	-	4
sgRNA1	GGgGGcCTgGAGGCcCCGCAGGG	CM028729.1	32220166	+	4
sgRNA1	cGAGGAgcCcAGGCACCGCAAGG	CM028729.1	36462741	-	4
sgRNA1	GGcGGACTCGgaGCACtGCAGGG	CM028730.1	30095518	-	4
sgRNA1	GGAGGACaCcAGGCACaGaAAGG	CM028730.1	47142407	-	4
sgRNA1	GGgaGgCTCGAGGCgCCGCAGGG	CM028730.1	63330112	-	4
sgRNA2	TCGCCAGACGCGCCGCCTCTGGG	CM028706.1	129875890	-	0
sgRNA2	TCGCCAGtCGCtCaGCCTCTCGG	CM028706.1	223235445	+	3
sgRNA2	gCGCCAGcCGCGCCcCCTCcCGG	CM028704.1	872317	-	4
sgRNA2	TCcCCAGACtCGCCaaCTCTAGG	CM028704.1	11968997	+	4
sgRNA2	TCaCCctACGgGCCGCCTCTGGG	CM028704.1	150018471	+	4
sgRNA2	TCGCCAGcCaCGCtGCCTCaGGG	CM028704.1	249463507	+	4
sgRNA2	TaGCCAGACGCcCaGCCcCTGGG	CM028704.1	264206587	-	4
sgRNA2	cCGgCcGcCGCGCCGCCTCTGGG	CM028705.1	1356378	-	4
sgRNA2	TCGCCcGcCGCGCCcCCTCcCGG	CM028705.1	43520264	-	4
sgRNA2	TCtCCAGcCGaGCCtCCTCTTGG	CM028705.1	244449328	+	4
sgRNA2	TCcCCAGACGCGaCGCtgCTCGG	CM028706.1	6001565	+	4
sgRNA2	TCagCAGACaCGCCGCCTCcTGG	CM028706.1	9058997	-	4

sgRNA2	TCcCCAGAaGaGCaGCCTCTGGG	CM028706.1	84477222	-	4
sgRNA2	gCGCCAGcCGCGCCcCCTCcTGG	CM028706.1	103935381	-	4
sgRNA2	TCGCaAGAgGCcCCGCCTCcGGG	CM028706.1	215397319	+	4
sgRNA2	TCcCCAGAgGCGgCGgCTCTGGG	CM028706.1	222117543	+	4
sgRNA2	gCtCCAGcCcCGCCGCCTCTGGG	CM028708.1	69266062	+	4
sgRNA2	TCGCCtGAgtCGgCGCCTCTTGG	CM028708.1	104646443	-	4
sgRNA2	TCGCCAGAgGCcCCcgCTCTGGG	CM028714.1	36450709	-	4
sgRNA2	TCGCCAGcCtCtCCaCCTCTAGG	CM028714.1	44638782	-	4
sgRNA2	TCGCCAGAgtCcCaGCCTCTGGG	CM028715.1	28168349	+	4
sgRNA2	TCcCCcGACGCGCtcCCTCTTGG	CM028716.1	12045681	-	4
sgRNA2	TgGCCAGACcCaCtGCCTCTAGG	CM028716.1	37696361	-	4
sgRNA2	TCcCCAGcCGCcCCGCCTgTTGG	CM028717.1	18885455	-	4
sgRNA2	TCaCCAGACcCGCtGCCcCTTGG	CM028717.1	35424386	-	4
sgRNA2	TgGCCAGAgcCGCCtCCTCTAGG	CM028717.1	52362968	+	4
sgRNA2	gCGgCgGACGCGCCGCCgCTCGG	CM028718.1	72596738	+	4
sgRNA2	TCGgCAGAatCGCCtCCTCTGGG	CM028718.1	79837975	+	4
sgRNA2	TCGCCgGcCGCGCCGaCTCgCGG	CM028719.1	8809938	-	4
sgRNA2	ggGCCtGACGCGCCGCCcCTCGG	CM028720.1	51514944	-	4
sgRNA2	TCtCCAGAaGgGCtGCCTCTTGG	CM028720.1	60513616	-	4
sgRNA2	TgGgCcGACGCGCCGCCTCgCGG	CM028720.1	71354519	-	4
sgRNA2	TgcCCAGACcCGCCGCCcCTGGG	CM028721.1	67534605	-	4
sgRNA2	cCtCCtGACGCGCaGCCTCTAGG	CM028723.1	11011757	-	4
sgRNA2	TCGgCgGAgGCGCCGCCTCcCGG	CM028723.1	46840280	-	4
sgRNA2	cCtCCAGACGCtCCGCCTCgGGG	CM028723.1	50152366	+	4
sgRNA2	TCtCCAGACcCGCaGCCTCgTGG	CM028724.1	39659942	-	4
sgRNA2	ggaCCAGACtCGCCGCCTCTCGG	CM028725.1	37014345	-	4
sgRNA2	TgGaCAGAgGCGCCGCtTCTTGG	CM028725.1	50880925	+	4
sgRNA2	gCGCCAGcCcCGCCGCtTCTCGG	CM028728.1	32580149	-	4
sgRNA2	aCGCCAGgCGCGCCctCTCTGGG	CM028729.1	35900929	+	4

Table 2.7: Genotypes of Rams and their offspring at SNPs identified where Ram 1 and Ram 2 were homozygous for different alleles. The vertical line separating Stillbir and Ram2 denotes Ram 1 and their offspring, and Ram 2 and their offspring, respectively.

ARS-UI_Ramb_V2.0 Genome Coordinates for SNPs Homozygous and Different Between Ram 1 and Ram 2	**Ram 1**	**7181**	**Stillbir**	**Ram2**	**7183**	**Female**	**7182**	**Male**
NC_056074.1:23,908,548	C/C	C/C	C/G	G/G	G/G	G/G	C/G	C/G
NC_056074.1:23,915,929	C/C	C/G	C/G	G/G	G/G	G/G	C/G	C/G
NC_056074.1:23,916,022-23,916,024	GGG/GGG	GGG/TTC	GGG/TTC	TTC/TTC	TTC/TTC	TTC/TTC	TTC/TTC	GGG/TTC
NC_056074.1:23,921,594	C/C	C/G	C/C	G/G	G/G	G/G	C/G*	G/G
NC_056074.1:23,922,264	T/T	T/G	T/G	G/G	G/G	G/G	T/G	G/G
NC_056069.1:35,930,427	C/C	C/C	C/C	T/T	C/T	C/T	C/T	C/T
NC_056070.1:46,557,953, 46,557,966, 46,557,969	T/T,G/G,C/C	T/C,G/A,T/C	T/C,G/A,T/C	C/C,A/A,T/T	T/C,A/A,T/T	T/C,G/A,T/C	C/C,A/A,T/T	C/C,A/A,T/T
NC_056055.1:131,058,201	T/T	T/C	T/C	C/C	C/C	T/C	C/C	C/C
NC_056056.1:3,414,481	T/T	T/T	T/T	C/C	C/C	C/C	C/T	C/C
NC_056058.1:57,247,260	C/C	C/C	T/C	T/T	T/C	T/T	T/T	T/C
NC_056059.1:88,177,713	A/A	A/A	A/A	G/G	A/G	A/G	G/G	A/G
NC_056065.1:44,363,310	C/C	C/C	T/C	T/T	T/C	T/T	T/T	T/C
NC_056067.1:33,257,985	C/C	C/T	C/T	T/T	T/T	T/T	C/T	C/T
NC_056068.1:33,835,699	A/A	A/G	GA	G/G	G/G	G/G	G/G	G/G
NC_056069.1:58,872,398	A/A	A/G	A/A	G/G	G/G	G/G	G/G	G/G
NC_056069.1:17,713,844	G/G	G/G	T/G	T/T	T/G	T/T	T/T	T/T
NC_056071.1:25,961,656	T/T	T/C	T/C	C/C	C/C	C/C	T/C	T/C
NC_056072.1:58,405,639	A/A	A/G	A/G	G/G	G/G	G/G	G/G	A/G
NC_056073.1:25,087,442	C/C	C/C	C/G	G/G	G/G	G/G	G/G	G/G

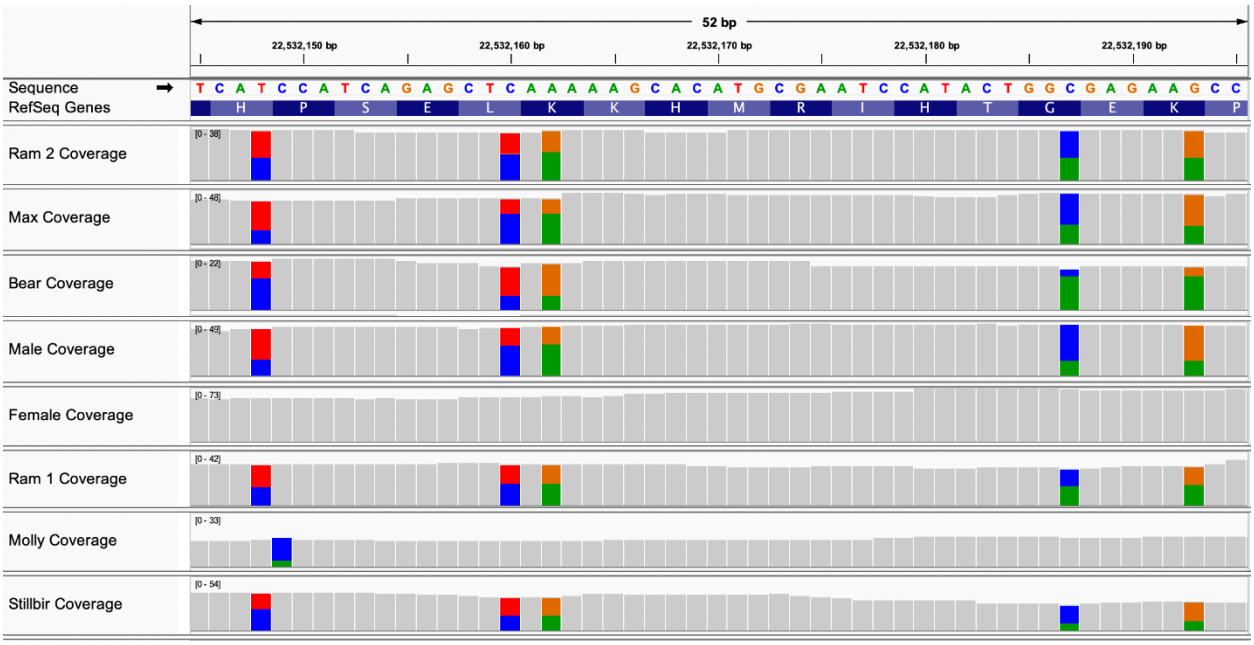


Figure 2.4: Visualization of WGS read alignments from the genome edited sheep and their fathers at ChrX:22,532,145-22,532,190 of the ARS-UI_Ramb_v2.0 female reference genome, a section of the ZFX gene. Nucleotides with a minor allele frequency greater than 20% are colored with the fraction of reads called as adenine (green), thymine (red), guanine (orange), and cytosine (blue). Females are homozygous, while males appear heterozygous at 5 SNPs in the ZFX gene ChrX:22,532,148, ChrX:22,532,160, ChrX:22,532,162, ChrX:22,532,160, ChrX:22,532,187, and ChrX:22,532,193 because reads from the homologous section of the ZFY gene (on the Y chromosome) with these 5 substitutions map to the ZFX gene due to the lack of ZFY gene in the female reference genome.

Figure 2.5: Screenshot of a section of the ZFX-Y Sexing amplicon Sanger sequencing chromatogram of offspring with 4 SNPs that differ between ZFX and ZFY gene highlighted blue. Males are heterozygous at each of these SNPs while females are homozygous. The "GAGCTC"

SacI restriction digest site used by (Aasen & Medrano, 1990) to cleave the ZFX, but not ZFY gene is indicated with the (C/T) SNP occurring at NC_056080.1:22,532,148 in the ARS_UI-Ramb_v2.0 reference genome.

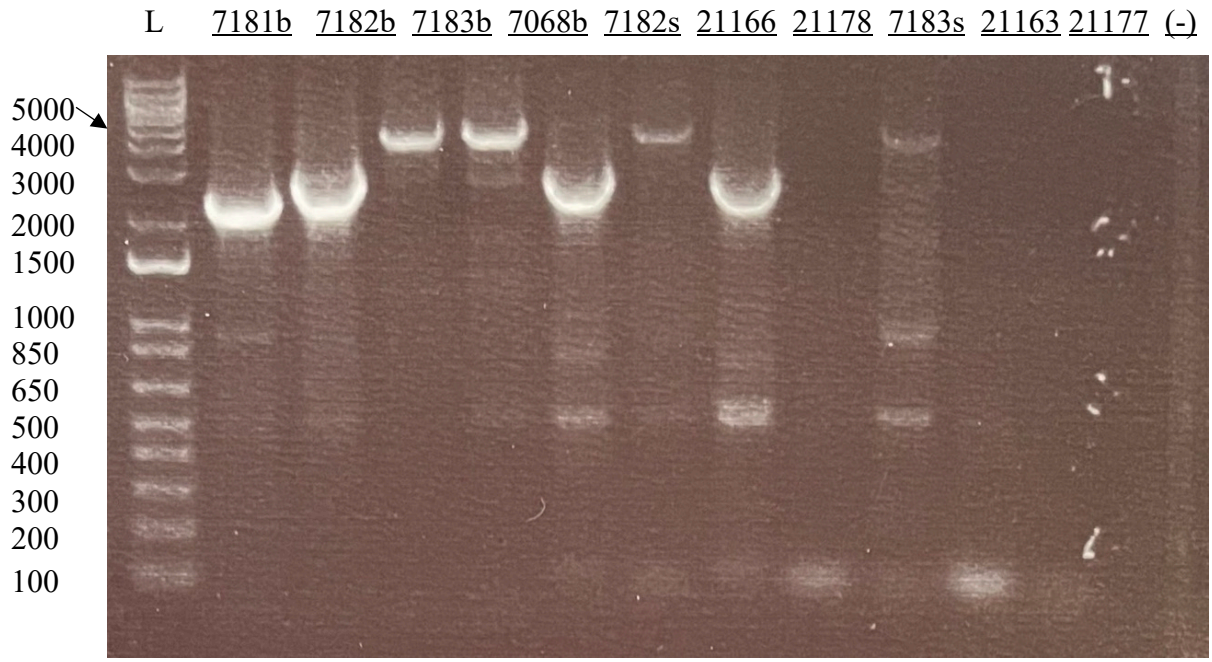
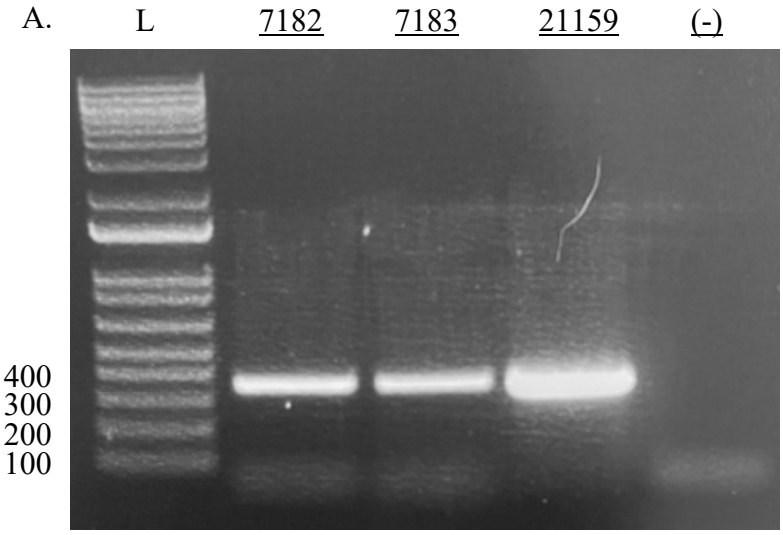


Figure 2.6: Socs2 4K PCR amplicon with DNA from blood (b) collected from the three healthy genome edited founders, control ram 7068, semen collected from 7182 and 7183, offspring that initially genotyped as wild type from 7182 (21166) and 7183 (21163), and a lamb sired by 7068 (21177).

A.


L
7182
7183
21159
(-)
400
300
200
100

B.

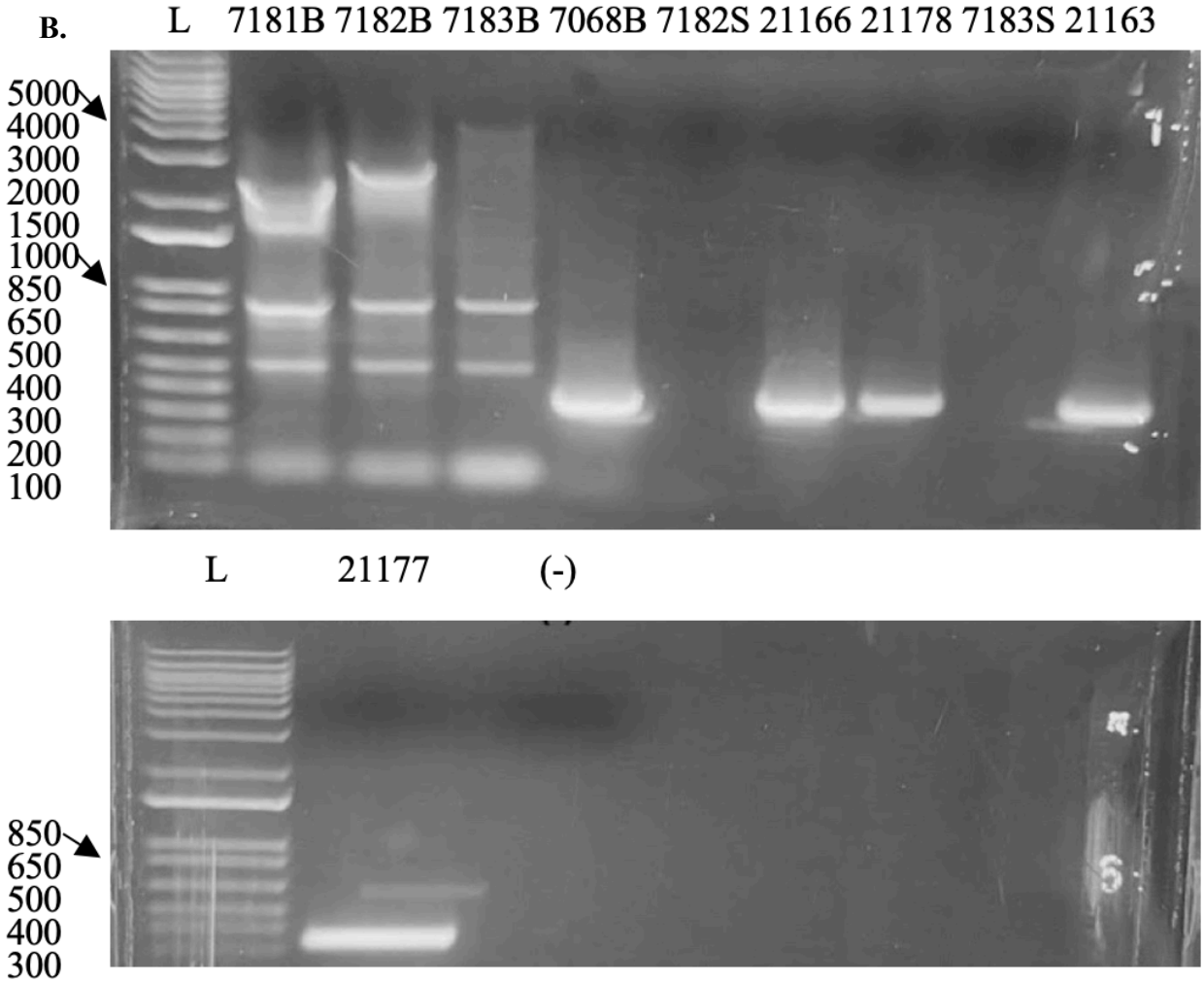
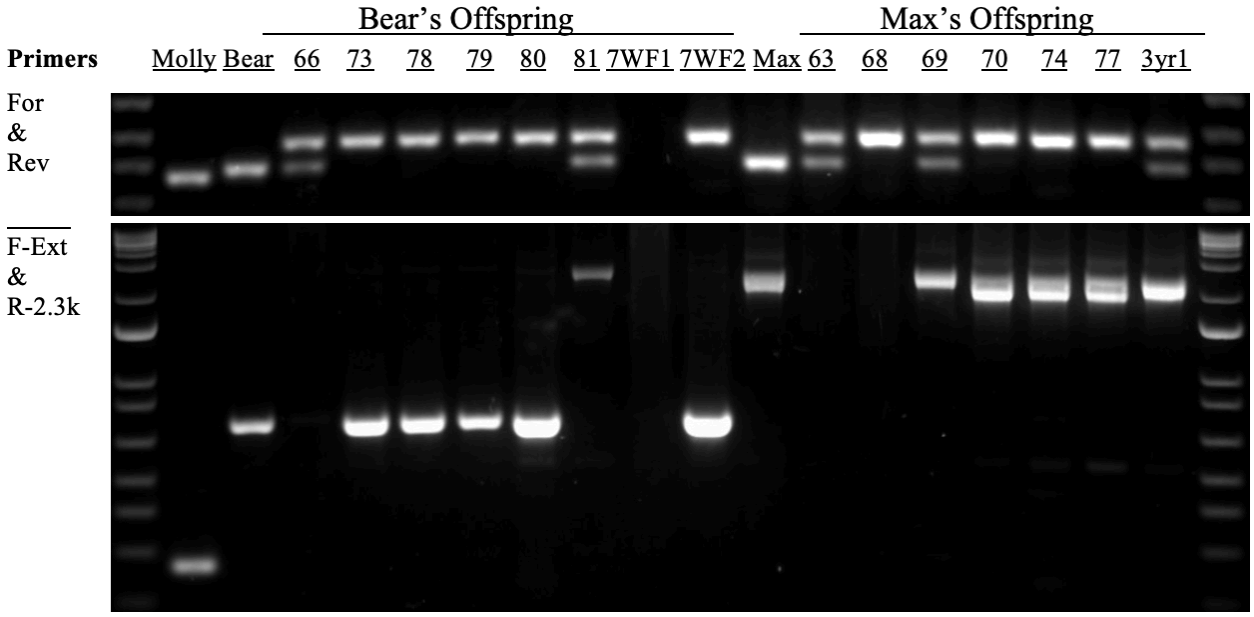
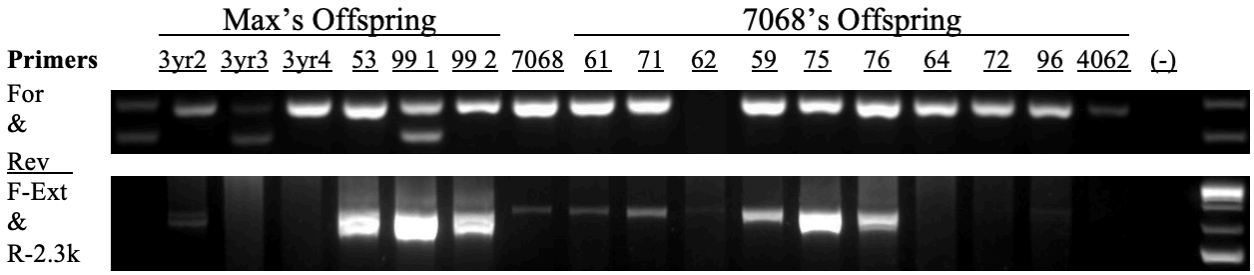
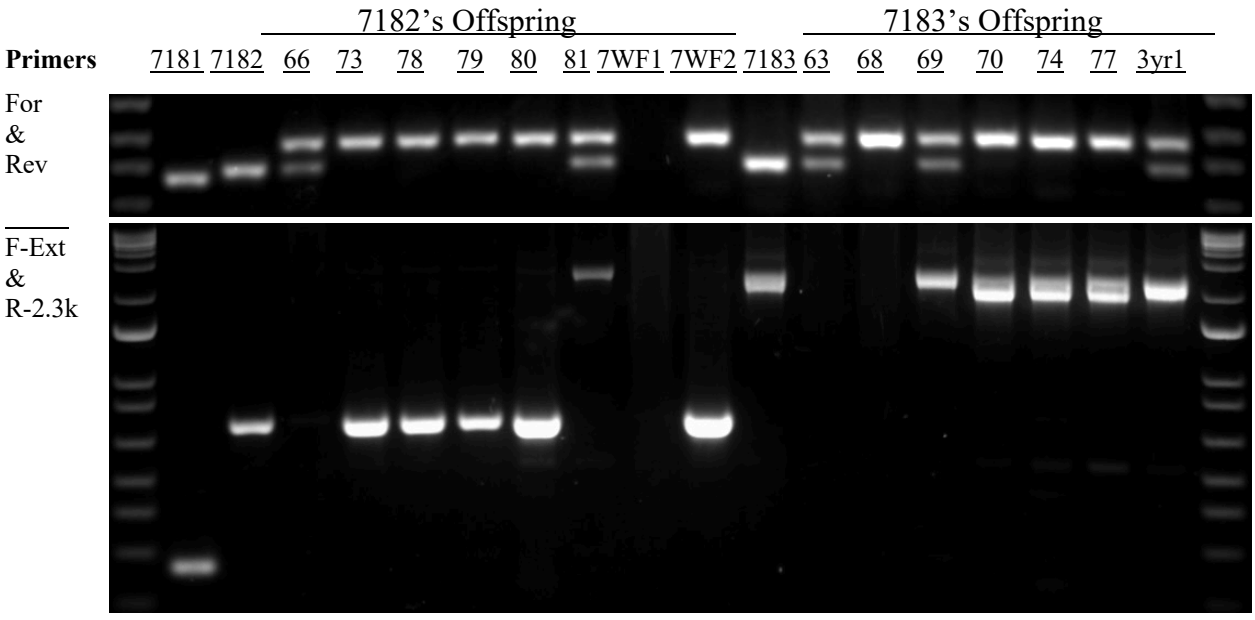

L 7181B 7182B 7183B 7068B 7182S 21166 21178 7183S 21163
5000
4000
3000
2000
1500
1000
850
650
500
400
300
200
100
L 21177 (-)
850
650
500
400
300

Figure 2.7: (A) PCR with Socs2 Internal amplicon with DNA collected from semen for rams 7182 and 7183 compared to control lamb 21159 (tail extracted DNA) with 100ng of DNA for all three samples and a no template negative control (-). (B) Subsequent PCR with Socs2 4k primers for samples 7181B, 7182B, and 7183B and Socs2 Internal primers for other samples. 100ng of DNA collected from blood "B" or semen "S", except no template negative control.


Bear's Offspring
Max's Offspring
Primers
Molly Bear 66 73 78 79 80 81 7WF1 7WF2 Max 63 68 69 70 74 77 3yr1
For & Rev
F-Ext & R-2.3k


Max's Offspring
7068's Offspring
Primers
3yr2 3yr3 3yr4 53 99 1 99 2 7068 61 71 62 59 75 76 64 72 96 4062 (-)
For & Rev
F-Ext & R-2.3k


7182's Offspring
7183's Offspring
Primers
7181 7182 66 73 78 79 80 81 7WF1 7WF2 7183 63 68 69 70 74 77 3yr1
For & Rev
F-Ext & R-2.3k

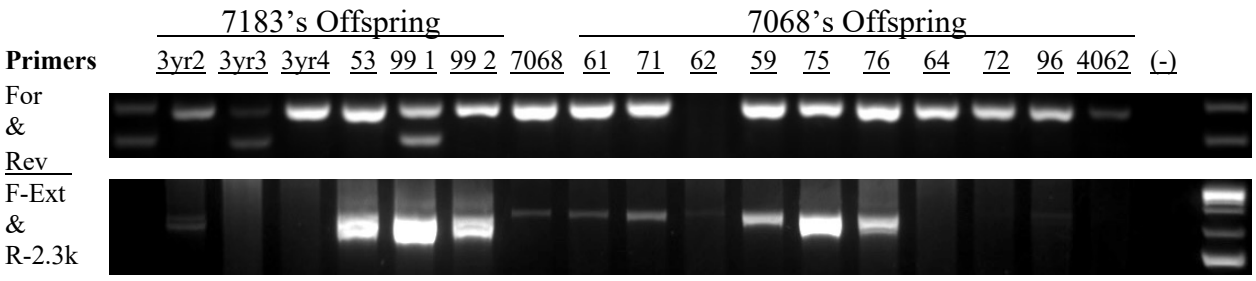
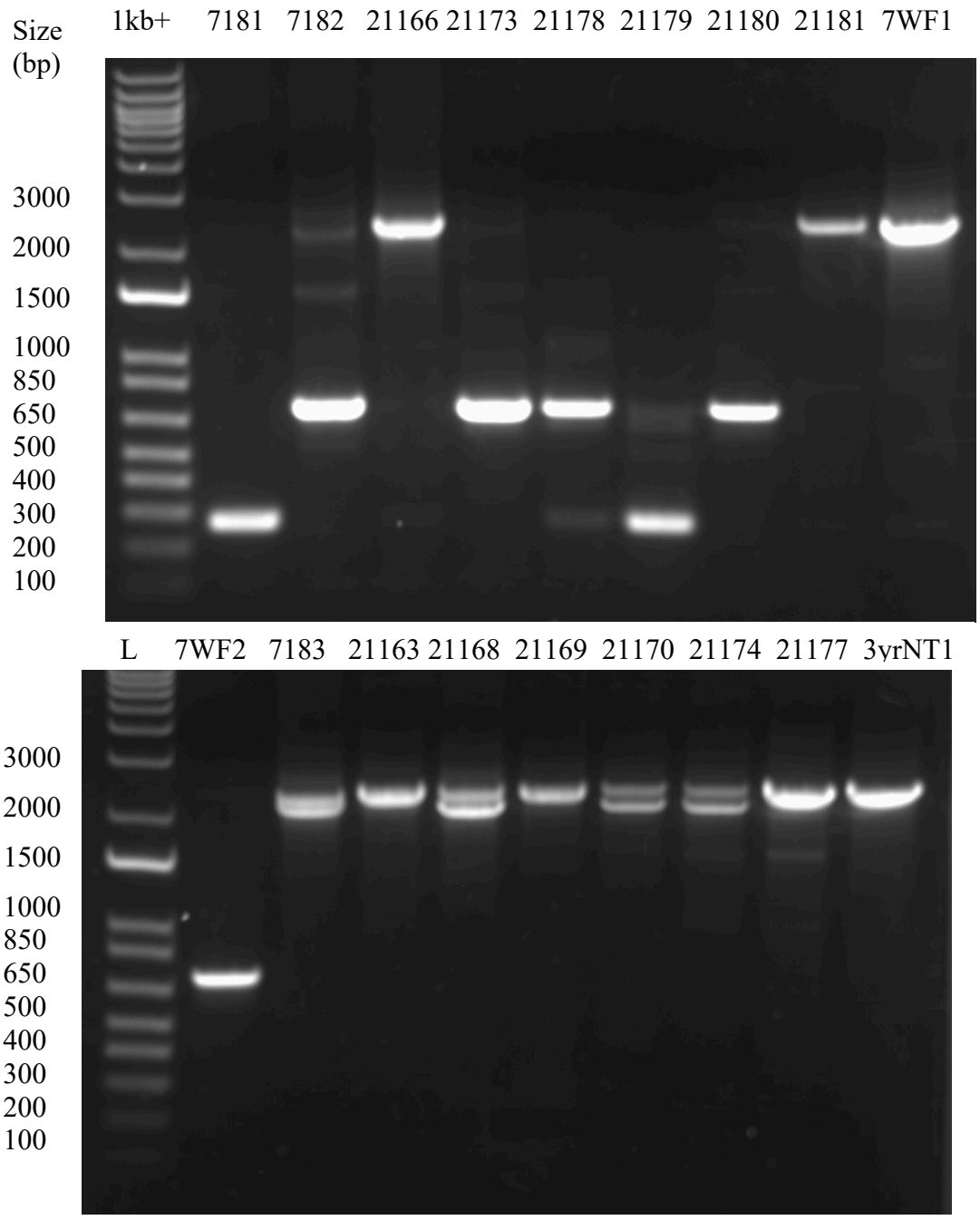


Figure 2.8: Initial Socs2 and Socs2 2.3k PCR amplicons for all of the offspring and healthy genome edited lambs with the same master mix used for each DNA sample. 2% agarose gel complete PCR from images shown in Figure 2.2D.


Size (bp)
1kb+
7181
7182
21166
21173
21178
21179
21180
21181
7WF1
3000
2000
1500
1000
850
650
500
400
300
200
100
L
7WF2
7183
21163
21168
21169
21170
21174
21177
3yrNT1
3000
2000
1500
1000
850
650
500
400
300
200
100

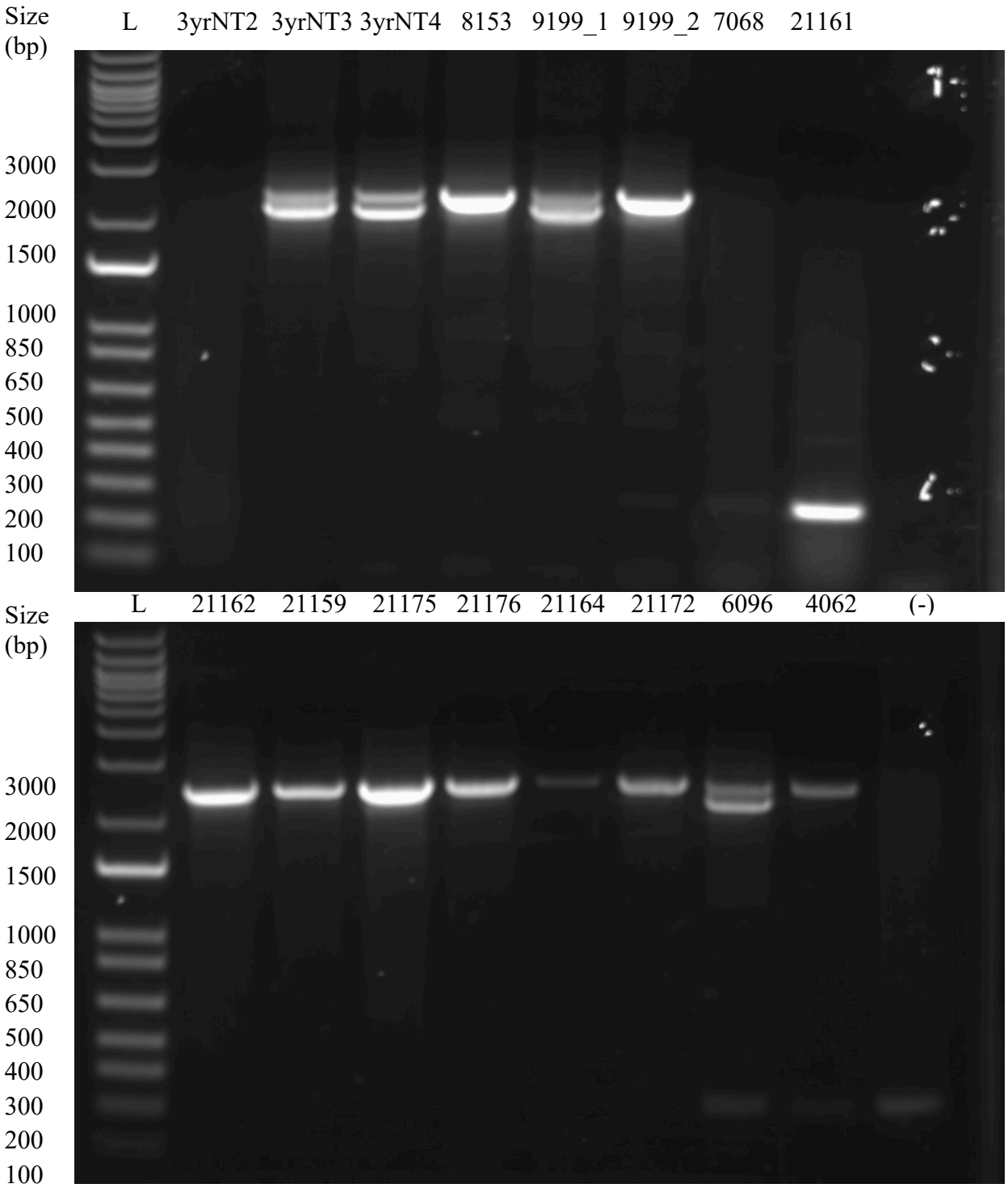


Figure 2.9: Repeat Socs2 2.3k PCR amplicon gel images shown below to confirm a genotype not observed in the first gel images.

Chapter 3: Pleiotropic Effects of *Socs2* on Reproduction, Survival, and Growth

Abstract

The *suppressor of cytokine signaling 2* (*Socs2*) gene is a major negative regulator of the growth hormone receptor signaling pathway. In *Socs2(-/-)* mice a 30-50% increase in post-weaning growth is observed, and in sheep, a naturally occurring point mutation in *Socs2* induces a p.R96C substitution is associated with increased mature size, weight, and milk yield, at the expense of increased susceptibility to mastitis in dairy ewes. While this p.R96C substitution is predicted to eliminate *Socs2* functionality, the effects of a complete *Socs2* knockout (KO) in sheep were not previously reported. Thus, we investigated the fertility of two *Socs2(-/-)* KO rams and one *Socs2* KO ewe as well as the health, growth, feed efficiency, and carcass characteristics of their heterozygous *Socs2(+/-)* offspring under meat lamb production conditions. A non-significant trend towards an increase in pre-weaning mortality rate was observed with 57% of *Socs2* heterozygous fetuses dying pre- or post-natally, as compared to 20% for controls (p=.052), with no consistent cause of death identified. At birth, the weight and size of *Socs2* heterozygous KO lambs did not differ significantly from sex-matched controls, with the exception of ram lambs that were 17% taller at the elbow at birth. During the pre-weaning period, heterozygous *Socs2* KO genotype was associated with a 22% increase in growth rate for males, and a 28% increase in growth rate in the post-weaning period for females. During the post-weaning period, *Socs2(+/-)* genotype was associated with 11% and 9% heavier male and female lambs, respectively. Additionally, the effect of *Socs2(+/-)* genotype on height was an increase of 8% and 4.3% for males and females, respectively as compared to controls. No significant differences in feed efficiency were observed and minor variation in carcass characteristics was observed. The modest improvements in the growth of *Socs2* heterozygous KO sheep and the negative trends observed in reproduction and

health suggest that introgression of the *Socs2* KO allele is unlikely to improve commercial sheep production unless the detrimental phenotypes are mitigated.

Introduction

The *Suppressor of cytokine signaling 2* (*Socs2*) gene is a member of the cytokine-inducible family of suppressor of cytokine signaling (SOCS) proteins that negatively regulate cytokine receptor signaling. SOCS2 potently inhibits growth hormone receptor (GHR) signaling by competing with Signal Transducers and Activators of Transcription 5b (STAT5b) for a binding site on the GHR and by tagging the GHR for proteasomal degradation through recruitment of the E3 ubiquitin ligase complex (Bullock et al., 2006; Greenhalgh et al., 2005). Knockout of the *Socs2* gene in mice results in a 30-50% increase in post-weaning growth, most often without increases in birth or pre-weaning weight and without excessive adiposity or leanness (Corva & Medrano, 2000; Metcalf et al., 2000). The dramatic increase in post-weaning growth observed in *Socs2* knockout (KO) mice suggests that *Socs2* KO may offer a production advantage for livestock grown for meat. However, the effects of *Socs2* deficiency in mice are pleiotropic with decreased fertility, prolonged inflammatory response to pathogens, and decreased lifespan also observed (Bradford & Famula, 1984; J. Casellas & J. F. Medrano, 2008; Machado et al., 2006).

Some of the well-characterized phenotypes of *Socs2* (KO) mice are also observed in sheep with a naturally occurring point mutation in *Socs2* that results in an arginine to cysteine substitution at the 96th amino acid (p.R96C) (Rupp et al., 2015). This substitution abrogates SOCS2 binding to its highest affinity binding site on the GHR (Rupp et al., 2015). In homozygous p.R96C SOCS2 Lacaune dairy sheep, weight, size, and milk yield are 18%, 24%, and 4.4% higher, respectively

(Rupp et al., 2015). Unfortunately, the point mutation was also highly associated with increased susceptibility to mastitis (Rupp et al., 2015). Ewes that were p.R96C SOCS2 heterozygous generally expressed intermediate phenotypes with increased weight at 3rd lambing, elbow height, milk yield, and susceptibility to mastitis (Rupp et al., 2015). The tradeoffs between production benefits and detriments of this mutation combined with a relatively high allele frequency of 21.7% suggest that the p.R96C allele is likely under balancing selection and that heterozygous sheep may offer a more optimal combination of characteristics than homozygotes in this environment (Rupp et al., 2015). The p.R96C point mutation or a *Socs2* KO allele may offer greater production benefits for meat breeds of sheep where improved growth is a primary breeding objective and mastitis is less of a production concern.

A primary breeding objective of the commercial lamb industry is to decrease the time to market weight, increase carcass yield, and maintain carcass quality. Growth traits of birth weight, growth rate, weaning weight, post-weaning weight, adult weight, and fat depth are all positively associated and highly heritable (Safari et al., 2005). However, there is a negative association between body weight and the number of lambs born/ewe lambing and fecal egg count indicating a tradeoff between growth traits and the traits of lambing ease and disease resistance (Safari et al., 2005). Growth hormone receptor signaling is involved in all these traits and transgenic overexpression of growth hormone was found to increase the rate of growth and feed efficiency at the expense of increased leanness and fecal egg count (Adams et al., 2002). The consideration of traits that are known to be associated with increased growth and growth hormone receptor signaling are necessary to determine whether *Socs2* KO or the p.R96C SOCS2 mutation has an holistic production benefit.

The introgression of this allele from a dairy breed of sheep to a meat breed of sheep using traditional breeding strategies of crossbreeding and repeated backcrossing would require multiple generations of selection, but genome editing technology has enabled the introduction of the p.R96C point mutation and *Socs2* KO mutations into meat breeds of sheep within a single generation and without altering genetic background. A cytosine base editor was used to recreate the p.R96C point mutation by substituting the wild type cytosine nucleotide for thymine, but no phenotypic data have been reported from these sheep beyond the founder generation which had a variety of mutations and a maximum substitution efficiency below 40% (Zhou et al., 2019). The generation of *Socs2* KO sheep was performed efficiently by Clustered Regularly Interspersed Short Palindromic Repeats/CRISPR associated protein 9 (CRISPR/Cas9) genome editing of sheep zygotes with dual guide RNAs (gRNA) targeting an 85 base pair section of exon 1 (Mahdi, 2021). This study aims to characterize the reproductive outcomes of three healthy *Socs2* KO lambs and the survival, growth, feed efficiency, and carcass characteristics of heterozygous *Socs2* KO lambs to provide an initial evaluation of the effects of *Socs2* deficiency on production traits in a meat breed of sheep.

3.3. Materials and Methods:

3.3.1 Animal Management

All experiments involving animals were approved and performed in accordance with the University of California Davis Institutional Animal Care and Use Committee (IACUC Protocol #18343). Data on the weight and size of *Socs2(-/-)* founders, *Socs2(+/-)* offspring, and control sheep were obtained from the birth of the first lamb on April 1st 2021 to the last carcass

measurements performed on December 14th 2021. All stillborn and neonatal dead lambs with a suspected infectious cause of death were sent to the California Animal Health & Food Safety Lab System (CAHFS), Davis, CA, for necropsy. The lambs were born over a span of 40 days and weaned when the youngest lambs reached 10 weeks of age on July 19th, 2021. During the pre-weaning period, lambs were kept in group pens with their mothers and segregated into two pens based on sex after weaning to prevent breeding and compare the effects of genotype on growth for each sex. During the entire study, lambs had access to clean water, shade, shelter, and the outdoors.

3.3.2. Reproduction

Two genome edited *Socs2* KO whiteface rams tag # 7182 and 7183, and one unedited whiteface ram tag # 7068, were each bred to six whiteface ewes of primarily Dorset background from the UC Davis research flock. All ewes were *Socs2* wild type, except for one *Socs2* KO ewe #7181 that was bred to ram #7182 (Figure 3.1). When she did not become pregnant, she was bred with control ram #7068. In this case, a progesterone CIDR sponge (0.3 g of progesterone; CIDR-G; Zoetis) was inserted from 9.5-16 hours after heat detection to compensate for potential luteal insufficiency until 50 days after introduction, replaced with a new CIDR every 14 days to try to prevent luteal insufficiency observed in some *high growth Socs2* KO mice (Cargill et al., 1999).

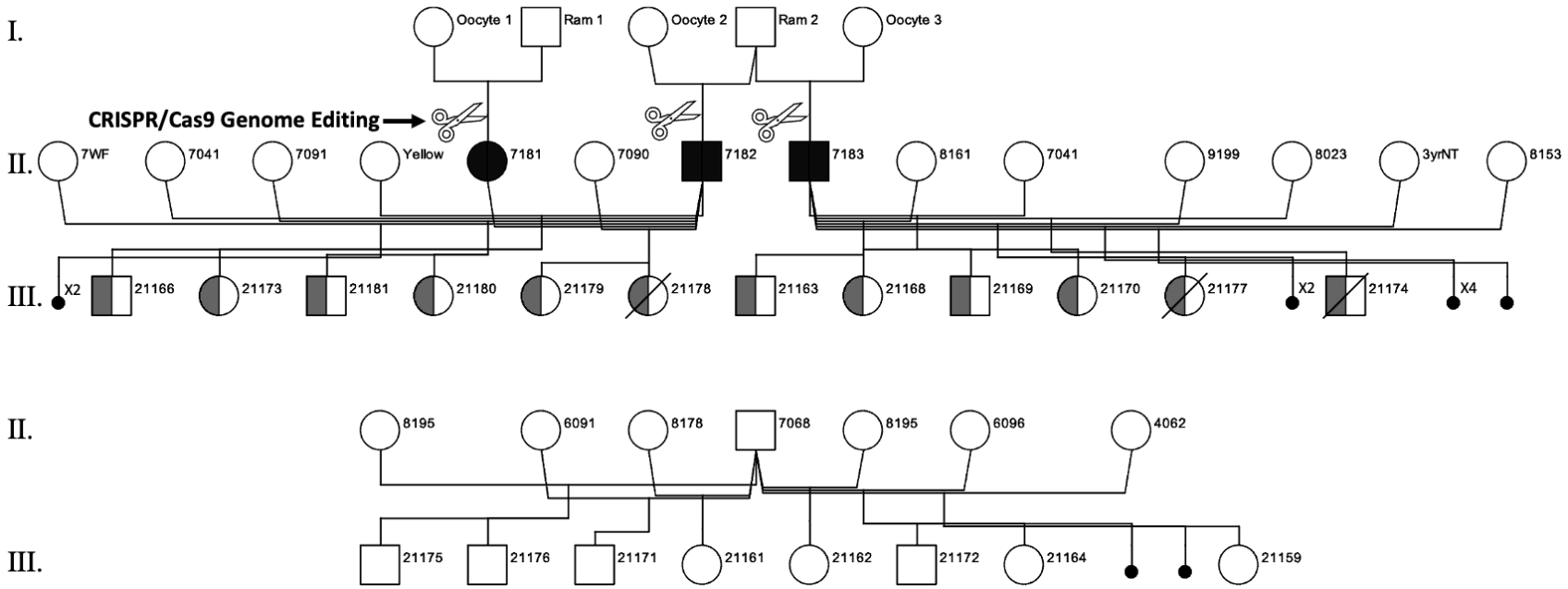


Figure 3.1: Pedigree of Socs2 KO and wild type comparators. Scissors indicate germline genome editing of Socs2, shape fill indicates Socs2 genotype, dots at the ends of lines indicate prenatal deaths, shapes bisected by a diagonal line indicate neonatal deaths, circles represent females, and square represent males. Roman numerals indicate generation with matings between sheep in generation II occurring simultaneously. Pedigree designed with CeGaT pedigree chart designer.

Ewes with no visible fetuses at the time of ultrasound pregnancy check at 70 days of gestation were considered infertile (Appendix Table 16). The prolificacy rate was defined as the number of lambs or fetuses born dead or alive per fertile ewe. The lambing rate was calculated as the percentage of lambs born per ewe exposed to a ram. The pre-weaning mortality rate was calculated as the percent of lambs born that died prenatally or before weaning. The live litter size included lambs that died neonatally, while weaned litter size only included lambs that were weaned. The relative birth date refers to the number of days relative to the date that the first lamb was born (Appendix Table 9). P values for each rate in the fertility results were calculated with a Fisher's exact test, and comparisons of birth date were performed with a Wilcoxon rank sum test with continuity correction.

Growth Measurements

From birth to 20 weeks post-weaning, lamb weight was measured weekly, with size measurements taken every two weeks. A Brecknell PS1000 scale was used to measure weight during the pre-weaning period, and a Transcell tech TI 500E scale was used to measure weight during the post-weaning period. A T-square adjustable ruler was used to measure height at the withers, sacrum, and elbow; width of the chest and hips; and crown to rump length (Appendix Figure 3.5). During the post-weaning period, all measurements were performed in the morning from 5:00-7:30.

For comparisons of birth weight and size, measurements from all lambs that were born alive were included, while comparisons of growth only included measurements of lambs that were weaned. Pre-weaning growth measurements were performed relative to birth date, and post-

weaning measurements were performed relative to weeks post-weaning. Size measurements in the post-weaning period switched from even to odd numbered weeks from weeks 6 to 7 post-weaning when the two-week introductory period for the feed efficiency study began. To compensate for variation in age at weaning and project growth from birth to 30 weeks of age, an approximate age for each lamb was assigned for each post-weaning measurement. The approximate age was the age in weeks closest to each lamb's actual age while maintaining measurements for all lambs for every week (Appendix Table 3.7).

Feed Composition and Measurement of Feed Intake

Feed intake measurements began with a pretrial period from 7 to 9 weeks post-weaning to get the sheep adjusted to the total mixed ration (TMR) diet and the electronic feeders. An automated electronic feeder (Smartfeed; C-Lock Inc., Rapid City, SD) was set up for each pen. The device consisted of a feed bin suspended on two weigh cells and a radio frequency identifier (RFID) reader attached to a feeding window large enough for only one lamb to enter at a time. All lambs were tagged with an RFID and each time a lamb entered the feeding window, the visit duration and mass difference from entry to exit was recorded.

Lambs were given *ad libitum* access to clean drinking water and TMR during the pretrial adjustment period from 7 to 9 weeks post-weaning and the trial period from 9 to 19 weeks post-weaning. The ingredient formulation and chemical composition of the TMR is detailed in Table 3.1. Alfalfa was chopped 2 inches long, except for week 19 (after the trial) where an issue with feed processing resulted in a much finer feed than in previous weeks.

Analysis of Feed Efficiency

Feed data generated from the SmartFeed system was filtered for negative feed intake values and high feed intake rates, finalized by C-Lock, and further removal of visits with feed intake amounts >1.36 kg (3lbs) removed as outliers (the average daily feed intake (ADFI) was 2.30 kg) and intakes of this magnitude almost always returned to a baseline indicating no actual feed intake (Appendix Table 13). The average daily feed intake was calculated as the total feed intake over the 70 day feed efficiency trial with missing data from November 13th to November 19th 2021 imputed as the average feed intake for the remaining 7 days of weeks 17 and 18 post-weaning (Appendix Table 12A). The average daily dry matter intake (ADDMI) was then calculated as the product of ADFI and %DM in kg (Appendix Table 12B) (Alemu et al., 2017). The average daily gains (ADG) were calculated as the final – initial weight divided by the number of days encompassed by these measurements. The Gain:DMI (G:DMI) was calculated as ADG divided by ADDMI for the days preceding the final measurement (Appendix Table 12C). The mean metabolic bodyweight (MetBW) was calculated as the mid-trial body weight to the power of .75 ((Week 14 Weight)$^{.75}$) (Appendix Table 12D). The RFI was calculated by linear regression of MetBW and ADG on ADDMI (lm(ADDMI ~ MetBW + ADG)) to get the coefficients of MetBW and ADG, and the intercept. Then, the expected ADDMI for each lamb was calculated as MetBW*the coefficient of MetBW + ADG*the coefficient of ADG + the intercept of the regression (MetBW*-0.02803+ADG*-0.29763+2.65553). The residual feed intake (RFI) was then calculated as the actual ADDMI – expected ADDMI for each lamb Appendix Table 12E). P values for the comparisons between genotypes when controlling for sex were calculated with a Wilcoxon rank-sum test.

Table 3.1 A,B: Formulation of TMR ingredients (A) and chemical composition (B) of the TMR offered during the feed efficiency trial.

A.

Ingredient	% of TMR
Alfalfa Hay	35
Rolled Barley	19
Flaked corn	23
Soybean Meal	17
Molasses	5.45
Sodium Bicarbonate	0.125
White salt	0.25
Decox	0.025
Ammonium Sulfate	0.125
Ammonium Molybdate	0.025

B.

Chemical composition	% of TMR
Crude Protein	18.28
Dry Matter	86.59
Neutral Detergent Fiber	26.62
Acid Detergent Fiber	17.72
Starch	23.9
Fat	2.32
Calcium	0.73
Phosphorous	0.34

Analysis of Growth

Growth was analyzed in the pre-weaning period from 0 to 10 weeks of age and in the post-weaning period from 16 to 30 weeks of age for weight and from 16 to 29 weeks of age for size (the maximum age spans where all measurements were performed in either the pre-weaning or post-weaning periods). Each repeated growth measurement in the pre-weaning and post-weaning periods served as a response variable for a linear mixed effects model in R. Linear mixed effects models are more accurate than linear regression models for characterizing the growth in weight and size observed in people during the rapid growth that occurs early in life (Gularte-Mérida et al., 2014; Johnson et al., 2013). The base model consisted of the fixed effects of sex, genotype, age, the interactions between those fixed effects, weaned litter size, and the random effect for each lamb (base model: "lmer(Response ~ Sex*Genotype*Age + Weaned Litter Size + (1 | Lamb Tag))"). Each model was assessed for meeting linear regression assumptions through visual analysis of residuals vs. fitted, scale location, standardized residuals

vs. norm quartiles and normal Q-Q plots. Due to the curvilinear growth observed during the pre-weaning period for size measurements (Figure 3.4B-G), “Age” was square root transformed for width models, raised to the .6 power for sacrum and withers height models, and raised to the .75 power for elbow height and length models. On top of this base model, the interaction between weaned litter size and age (transformed age if age is transformed in the base model) was added to the model if it significantly improved the goodness of fit when tested against the base model with the likelihood ratio test. This weaned litter size * age term was not included in post-weaning models, but was included for all pre-weaning growth models except for withers and sacrum height. The estimated marginal mean (emmean) for the effect of each group (genotype/sex combination) on the response variable was calculated via the emmeans() function in R, and comparisons between heterozygous *Socs2* KO sheep and sex-matched controls was carried out with the pairs() function. To get the estimated marginal trend (emtrend) for the growth rate of each group on each response and during both periods, the emtrends function was used (response_lmer, specs=~interaction(Sex,Genotype), var="Age"). Then comparisons of the effect of genotype on the estimated marginal growth rate for each sex, was performed with the pairs() function (pairs(response_emtrend, by = "Sex"). A likelihood ratio test was also performed for genotype in all models to determine if it did not significantly increase the goodness of model fit (anova(resonse_lmer, reduced_response_lmer)). Model summaries including the marginal and conditional R^2 were generated by the tab_model() function of the sjPlot package (Appendix Figure 10A-N).

Carcass Measurements

The heterozygous *Socs2(+/-)* and control groups were sent to slaughter at 147 and 156 days post-weaning, respectively (Supplementary table 10). Due to the regulatory status of genome-

edited animals, we were not able to process the *Socs2(+/-)* lambs at a commercial abattoir, hence they were processed at the UC Davis meat laboratory, whereas the control lambs were processed at the Superior Farms slaughterhouse (Dixon, CA). Live weight, hot carcass weight, heart weight, kidney weight, lung weight, and testicular weight were measured shortly after slaughter. Live weights were measured on the same scale as post-weaning measurements; hot and cold carcass weights were measured on monorail carcass scales at the respective abattoirs; combined weights of both kidneys and both testicles were recorded with the ureters removed from the kidneys and the spermatic cord cut directly above the epididymis of the testis, and lungs were cut to remove the trachea down to a stub proximal to the lungs. Cold carcass weight, carcass length, cannon bone length, rack weight, loin weight, leg weight, backfat thickness, loineye depth, loineye area, shoulder + breast weight, and loin + flank weight was measured the day after slaughter. The carcass length was measured by hanging the carcass by the left Achilles tendon and measuring the length from the end of the left tibia to the end of the left cannon bone; cannon bone length was measured from the visible end of the bone to the end felt on the carcass. Careful attention was made to replicate the cuts made at Superior Farms and at the UC Davis meat laboratory, but certain cuts could not be replicated at the meat lab resulting in the grouping of multiple cuts. The same team performed measurements at both locations. The backfat thickness was measured over the center of the ribeye muscle between the 12^{th} and 13^{th} ribs. The loin muscle area was measured by using a grid to measure the cross-sectional area at the 12^{th}-13^{th} rib. The yield grade was calculated as 10 X backfat thickness (inches) + .4.

Results

Survival and Reproduction

The first attempt to breed the homozygous *Socs2* KO ewe 7181 to *Socs2* KO ram 7182 was unsuccessful, and a second attempt was made to mate ewe 7181 with control ram 7068. The *Socs2* KO ewe 7181 remained open when pregnancy checked with an ELISA pregnancy assay (CAHFS) 50 days post-breeding.

There were no significant differences observed in the fertility or prolificacy of ewes bred by either *Socs2* KO ram. The *Socs2* KO and control bred ewes had similar prolificacy rates of 210% and 167%, respectively (Table 3.2). However, there was a trend towards a higher rate of pre-weaning mortality for heterozygous *Socs2* KO lambs compared to control lambs (Fisher's exact test p=.052). Prenatal mortality was observed in 2/10 control fetuses and 9/21 heterozygous fetuses with an additional 3/12 live *Socs2(+/-)* lambs dying preweaning and no control lambs dying neonatally. All three lambs that died preweaning died in the first two weeks of life (Appendix Table 9)

Table 3.2: Pregnancy results by genotype. For individual pregnancy results see Supplementary Table 7.

Parameter	Ram Genotype	
	Socs2 KO	Wild Type
Number of WT ewes mated	11	6
Infertility rate	9.1%	0%
Number of conceptuses	21	10
Prolificacy rate	210%	167%
Number of prenatal deaths	9	2
Number of neonatal deaths	3	0
Total conceptuses lost	12	2
Number of lambs weaned	9	8
Number of males weaned	4	4
Number of females weaned	5	4
Pre-weaning mortality rate	57%	20%
Lambing rate	82%	133%

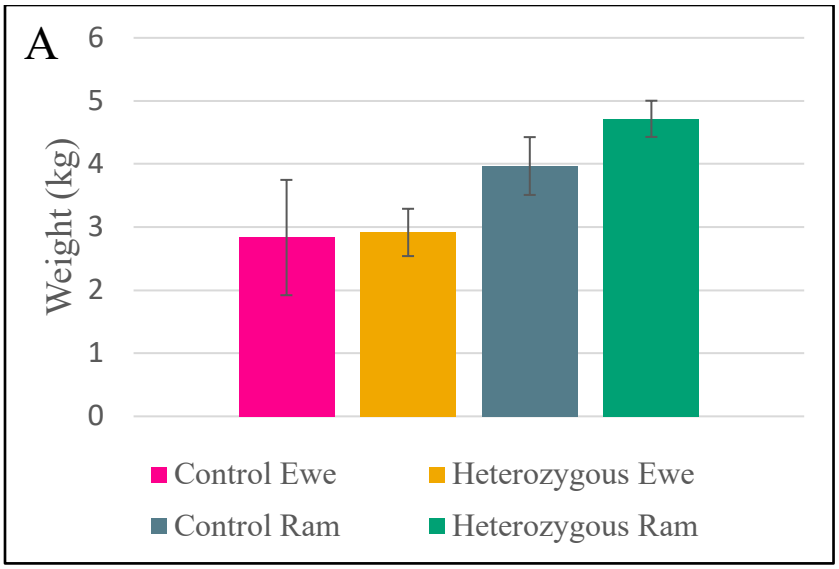
Necropsy results revealed no consistent cause of death across lambs. Fetal stress was commonly observed among lambs of both groups and evidence of mineral deficiency was observed in lambs of both groups, but different mineral deficiencies were observed among the offspring of two different mothers. Amongst the heterozygous lambs, dystocia, intrahepatic cholestasis, respiratory issues, thyroid disorders, muscular autolysis, and weakness were observed in multiple lambs (supplementary Table 7). However, a potentially confounding factor for the comparisons between groups was that a single ewe bred by *Socs2* KO ram #7183 carried quadruplets that all aborted, and quadruplets have a 62% pre-weaning mortality rate independent of *Socs2* genotype (Hinch et al., 1985).

The weaned litter size and birth dates varied between genotypes, and especially between ewe lambs of different genotypes. The mean parturition date of the *Socs2* KO bred ewes was 12 days later than ewes bred by the control ram ($p<.05$). This particularly influenced comparisons of

growth between *Socs2(+/-)* and control ewe lambs because the controls were on average 15.2 days older than the heterozygotes at weaning, compared to a .5 day difference between ram lamb groups (Appendix Table 8A,B). The mean weaned litter size was 2.2 for the heterozygous ewe lambs, 1.25 for the control ewe lambs, 2 for the heterozygous ram lambs, and 1.75 for the control ram lambs. Weaned litter size was included as a fixed effect in models of growth and the post-weaning measurements were age adjusted to provide continuous measurements from birth to approximately 30 weeks of age and compare sheep of more similar ages (Appendix Table 7).

Birth Size

There were no significant effects of genotype on the weight, width, or length of male or female lambs at birth, except that *Socs2(+/-)* males were born 3.02 cm taller than the control males at the elbow ($p<.05$) (Figure 3.2A,B). Additionally, while not statistically significant, the heterozygous lambs were on average heavier and larger than sex-controlled comparators for all measurements except for ewe lamb hip width, even though the heterozygous *Socs2(+/-)* lambs were born on average to larger litters (Figure 3.2A,B, Table 3.14).



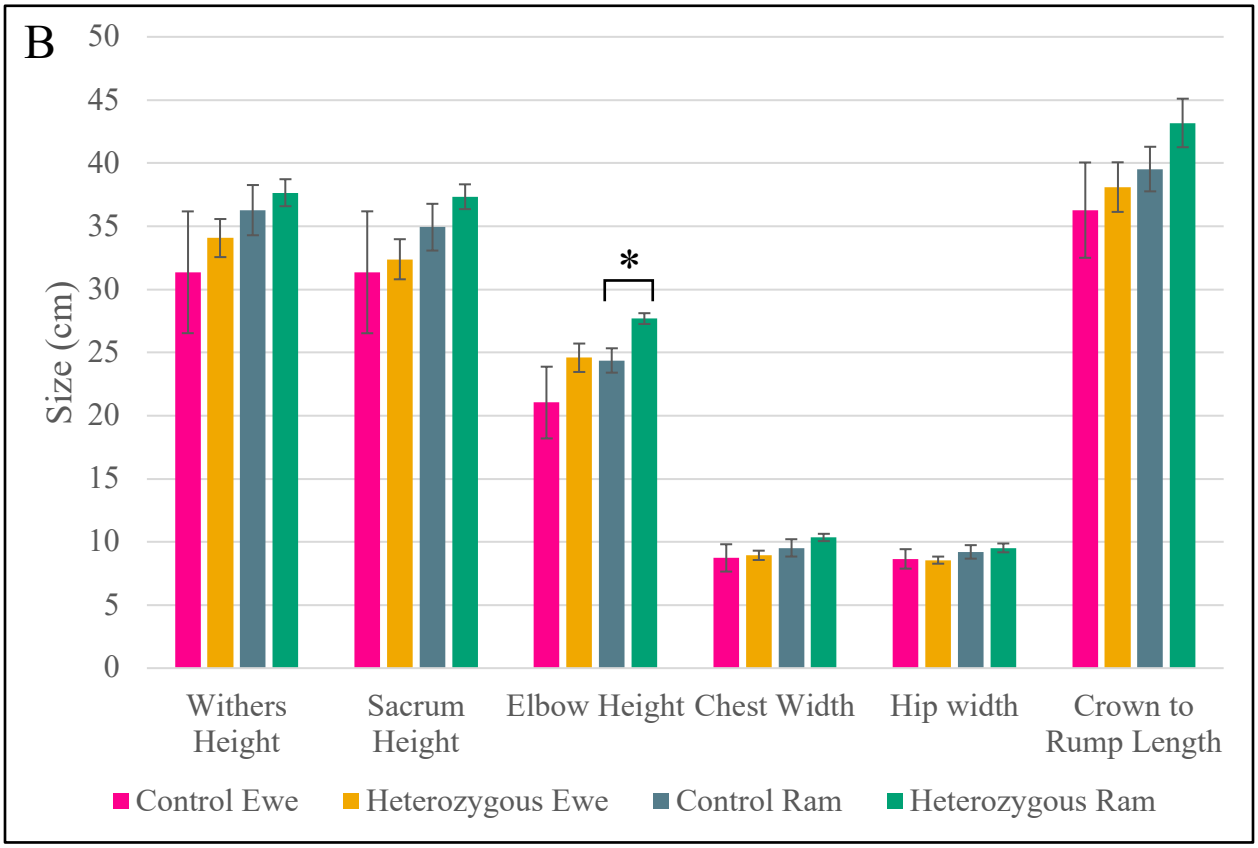


Figure 3.2 A,B: Birth Measurements of weight (A) and size (B) for all groups with error bars indicating the standard error of the mean.

Pre-Weaning and Post-Weaning Growth

The growth curves for each genotype sex combination for each growth measurement (Figure 3.3A-G) show in general that the male sheep grew faster than females in the post-weaning period for weight and that the heterozygous *Socs2* KO males grew to be taller than the other groups. During the pre-weaning period, there were no significant differences in growth measurements between genotypes, except that heterozygous rams had an increased elbow height, which was a difference that was present at birth (Table 3.3A). However, the heterozygous *Socs2(+/-)* rams had a 22% increase in pre-weaning growth rate for weight ($p<.0001$), and an increase in withers height growth rate relative to control rams ($p<.05$) (Table 3.3B).

From 16 to 29 weeks of age, the effect of *Socs2(+/-)* genotype on ram height was an additional 4.7 cm at the withers ($p<.05$), 5.6 cm at the sacrum ($p<.05$), and 3.5 cm at the elbow ($p<.01$) as compared to the controls (Table 3.3C). For ewes, the effect of *Socs2(+/-)* genotype on elbow height was also significant at 2.8cm ($p<.05$). However, there was no significant effect of genotype on the width of chest or hips with no more than a 3% difference observed between genotypes of either sex. The effect of genotype was also not significant for the mean weight or length, but the effect sizes tended to be positive for weight with an effect of 3.8 kg for ewes and 5.9 kg for rams. For length, *Socs2(+/-)* genotype had a nonsignificant positive effect of 2.5 cm for ewes and 3.6 cm for rams ($p>.1$) (Table 3.3C). Additionally, the *Socs2* heterozygous knockout genotype was associated with a 28% increase in the rate of weight gain ($p<.0001$) and a .49 cm/week increase in the crown to rump length ($p<.0001$) for ewes (Table 3.3D). In ewes, the heterozygous genotype was also associated with a 34% and 38% increase in the rate of sacrum and elbow height growth ($p<.05$ and $p<.01$, respectively, Table 3.3D).

The significance of genotype as a predictor of growth was tested with a likelihood ratio test that showed that genotype did improve the goodness of fit for models of pre-weaning weight and elbow height, as well as post-weaning weight, height, and length ($p<.05$) (Supplementary Table 12). Additionally, the proportion of variance explained by each model (conditional R^2) was greater than .91 for all models of pre-weaning growth and post-weaning weight and height, but was .430, .276, and .795 for post-weaning chest width, hip width, and length, respectively (Appendix Table 10 A-N). The relatively low proportion of variance explained by these models may be attributable to low variability in the growth rates for these latter measurements, increased fat thickness making hip width more difficult to accurately measure, and variation in animal posture influencing chest width and length more than height.

A significant increase in the height of the heterozygous rams compared to control rams was first observed at birth for elbow height and at 8 weeks of age for withers and sacrum height (Figure 3.3 B,C). For ewes, chest width was first significantly narrower at 8 weeks of age and hip width was narrower only at 23 weeks of age (Figure 3.3 D,E). Growth measurements of lamb #21173 on week 6 and lamb #21176 on week 12 pre-weaning were not recorded and were treated as absent values.

Table 3.3 A-D: Estimated marginal means and standard error for the effect size of Socs2(+/-) genotype on growth measurements during the pre- (A) and post-weaning periods (B). Estimated marginal means of linear trends for the effects of Socs2(+/-) genotype on growth rates during the pre- (B) and post-weaning periods (D). P value for comparisons between genotypes and % difference between heterozygotes and controls. P-values with an asterisk indicate statistical significance (p<.05).

A

			Heterozygous		Control				
Category	Measurement	Sex	emmean	SE	emmean	SE	Effect	p.value	% Difference
Weight (kg)	Weight	Ewe	14.5	1.2	13.4	1.4	1.1	0.5972	8%
		Ram	16.8	1.3	14.7	1.3	2.1	0.2805	14%
Height (cm)	Withers	Ewe	48.0	1.7	44.8	2.0	3.2	0.2709	7%
		Ram	52.3	1.8	48.3	1.8	4.0	0.1471	8%
	Sacrum	Ewe	46.9	1.7	44.0	2.0	2.9	0.3154	7%
		Ram	51.6	1.8	47.1	1.8	4.5	0.1024	10%
	Elbow	Ewe	32.5	1.0	29.1	1.2	3.4	0.0621	12%
		Ram	**34.7**	1.1	**31.4**	1.1	**3.4**	**0.0471 ***	11%
Width (cm)	Chest	Ewe	15.1	0.5	15.2	0.6	-0.1	0.9304	0%
		Ram	16.2	0.5	15.6	0.5	0.6	0.4126	4%
	Hips	Ewe	13.5	0.3	13.4	0.4	0.1	0.7884	1%
		Ram	14.3	0.3	13.6	0.3	0.7	0.164	5%
Length (cm)	Crown to Rump	Ewe	61.9	1.8	59.1	2.0	2.8	0.3436	5%
		Ram	63.5	1.9	60.8	1.9	2.7	0.3226	4%

B

Category	Measurement	Sex	Heterozygous emtrend	SE	Control emtrend	SE	Effect	p-value	% Difference
Weight (kg)	Weight	Ewe	2.18	0.053	2.05	0.061	0.121	0.1603	6%
		Ram	**2.52**	0.056	**2.07**	0.056	**0.446**	**<.0001*****	22%
Height (cm)	Withers	Ewe	5.86	0.289	6.04	0.322	-0.19	0.6691	-3%
		Ram	**6.32**	0.322	**5.40**	0.322	**0.92**	**0.0465***	17%
	Sacrum	Ewe	5.96	0.313	6.03	0.349	-0.07	0.88	-1%
		Ram	6.29	0.349	5.64	0.349	0.644	0.1952	11%
	Elbow	Ewe	2.21	0.124	2.36	0.144	-0.15	0.4684	-6%
		Ram	2.31	0.133	2.29	0.132	0.021	0.9132	1%
Width (cm)	Chest	Ewe	2.67	0.186	3.16	0.216	-0.49	0.1088	-16%
		Ram	2.76	0.198	2.67	0.197	0.095	0.7364	4%
	Hips	Ewe	2.14	0.119	2.11	0.138	0.036	0.8547	2%
		Ram	2.19	0.127	1.95	0.126	0.241	0.1838	12%
Length (cm)	Crown to Rump	Ewe	6.81	0.330	7.26	0.384	-0.45	0.4065	-6%
		Ram	6.53	0.353	6.30	0.35	0.234	0.6407	4%

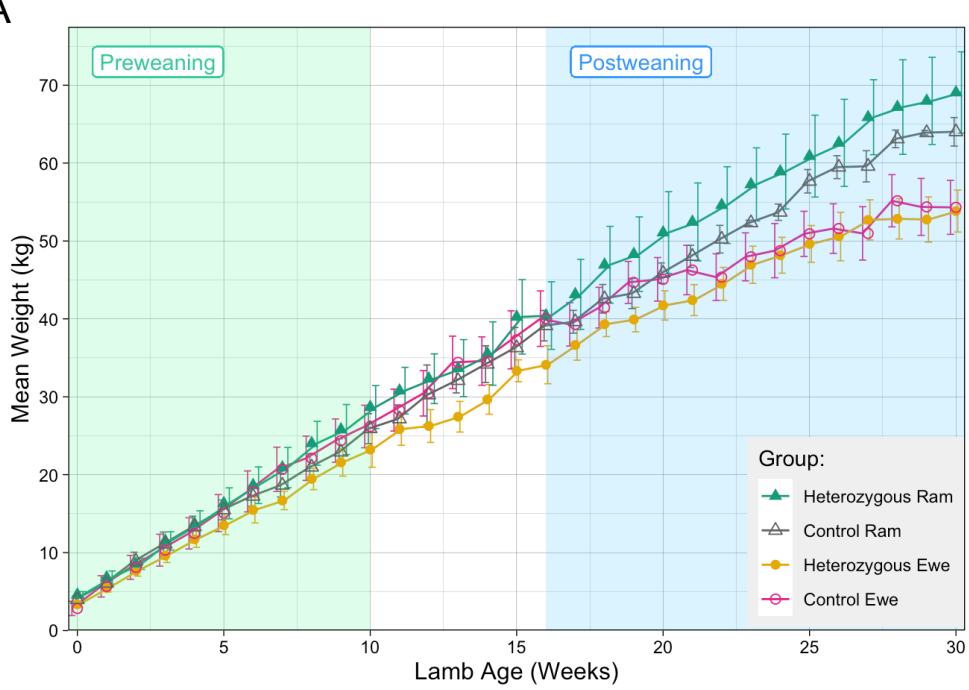
C

Category	Measurement	Sex	Heterozygous emmean	SE	Control emmean	SE	Effect	p-value	% Difference
Weight (kg)	Weight	Ewe	47.8	2.2	44.0	2.6	3.8	0.3224	9%
		Ram	57.2	2.4	51.4	2.4	5.9	0.1092	11%
Height (cm)	Withers	Ewe	66.6	1.1	64.4	1.2	2.2	0.2242	3%
		Ram	**71.9**	1.1	**67.2**	1.1	**4.7**	**0.0117 ***	7%
	Sacrum	Ewe	65.3	1.3	63.7	1.5	1.7	0.4391	3%
		Ram	**70.6**	1.4	**65.0**	1.3	**5.6**	**0.0124 ***	9%
	Elbow	Ewe	**42.6**	0.6	**39.9**	0.7	**2.8**	**0.0132 ***	7%
		Ram	**45.4**	0.6	**41.9**	0.6	**3.5**	**0.0019 ****	8%
Width (cm)	Chest	Ewe	19.5	0.4	19.5	0.5	0.0	0.9975	0%
		Ram	20.6	0.5	20.0	0.5	0.6	0.3532	3%
	Hips	Ewe	17.4	0.2	17.2	0.3	0.2	0.5760	1%
		Ram	17.8	0.3	17.5	0.3	0.3	0.4450	2%
Length (cm)	Crown to Rump	Ewe	86.2	1.2	83.8	1.4	2.5	0.2286	3%
		Ram	89.9	1.3	86.3	1.3	3.6	0.0681	4%

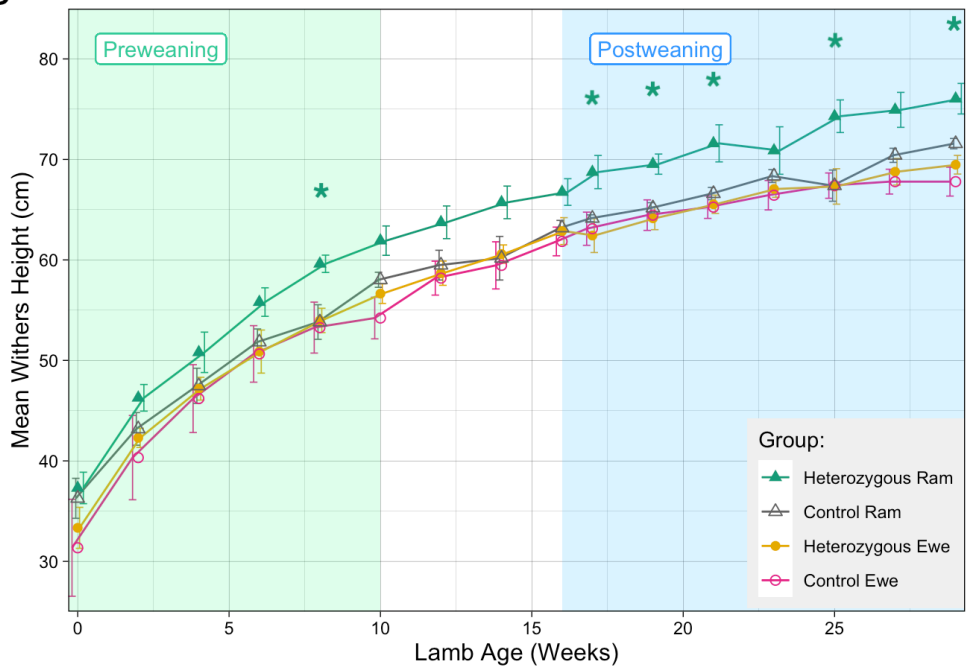
D

			Heterozygous		Control				
Category	Measurement	Sex	emtrend	SE	emtrend	SE	Effect	p-value	% Difference
Weight (kg)	Weight	Ewe	**1.45**	0.054	**1.13**	0.063	**0.32**	**0.0001*** **	28%
		Ram	2.06	0.061	1.99	0.06	0.079	0.3539	4%
Height (cm)	Withers	Ewe	0.576	0.049	0.48	0.054	0.096	0.1884	20%
		Ram	0.713	0.054	0.628	0.054	0.085	0.2695	14%
	Sacrum	Ewe	**0.662**	0.049	**0.494**	0.054	**0.168**	**0.0229***	34%
		Ram	0.678	0.054	0.599	0.054	0.078	0.3109	13%
	Elbow	Ewe	**0.353**	0.021	**0.256**	0.024	**0.097**	**0.0026****	38%
		Ram	0.381	0.024	0.398	0.024	-0.02	0.6164	-4%
Width (cm)	Chest	Ewe	0.037	0.05	-0.1	0.055	0.14	0.0619	-136%
		Ram	0.013	0.055	0.114	0.055	-0.1	0.1988	-88%
	Hips	Ewe	0.15	0.04	0.035	0.045	0.115	0.0613	325%
		Ram	0.059	0.045	0.128	0.045	-0.07	0.2827	-54%
Length (cm)	Crown to Rump	Ewe	**0.701**	0.088	**0.215**	0.098	**0.485**	**0.0004*****	226%
		Ram	0.804	0.098	0.703	0.098	0.101	0.4688	14%

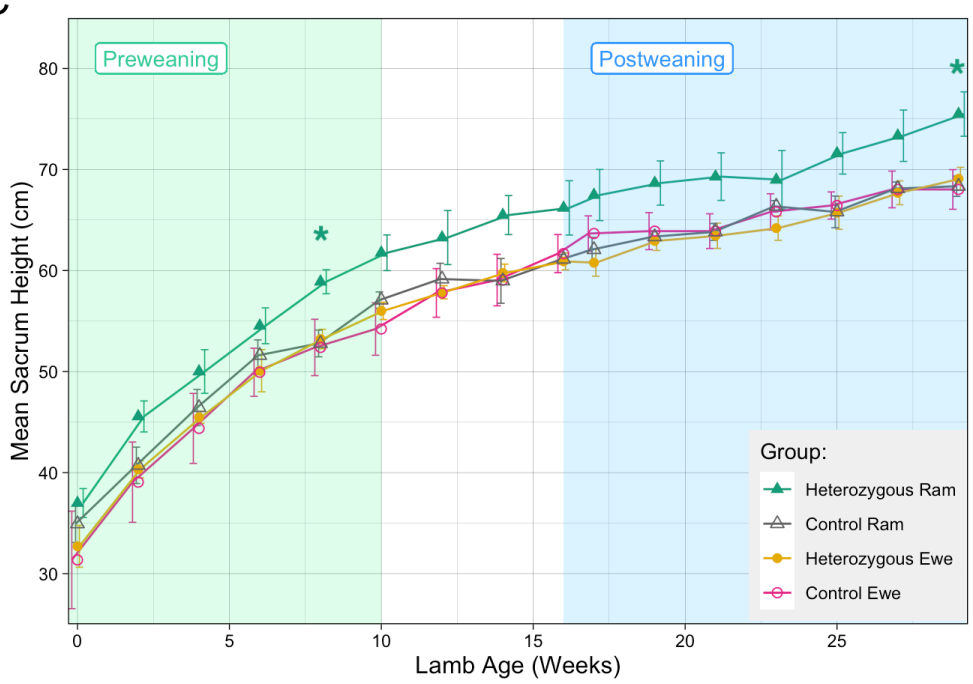
A


Preweaning
Postweaning
Mean Weight (kg)
Lamb Age (Weeks)
Group:
Heterozygous Ram
Control Ram
Heterozygous Ewe
Control Ewe

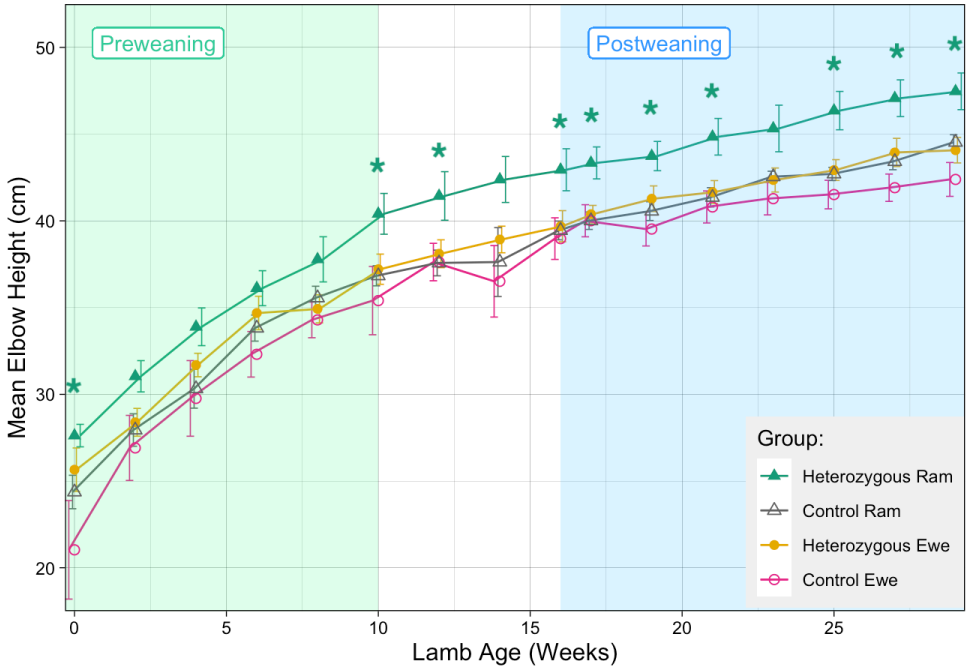
B


Preweaning
Postweaning
Mean Withers Height (cm)
Lamb Age (Weeks)
Group:
Heterozygous Ram
Control Ram
Heterozygous Ewe
Control Ewe

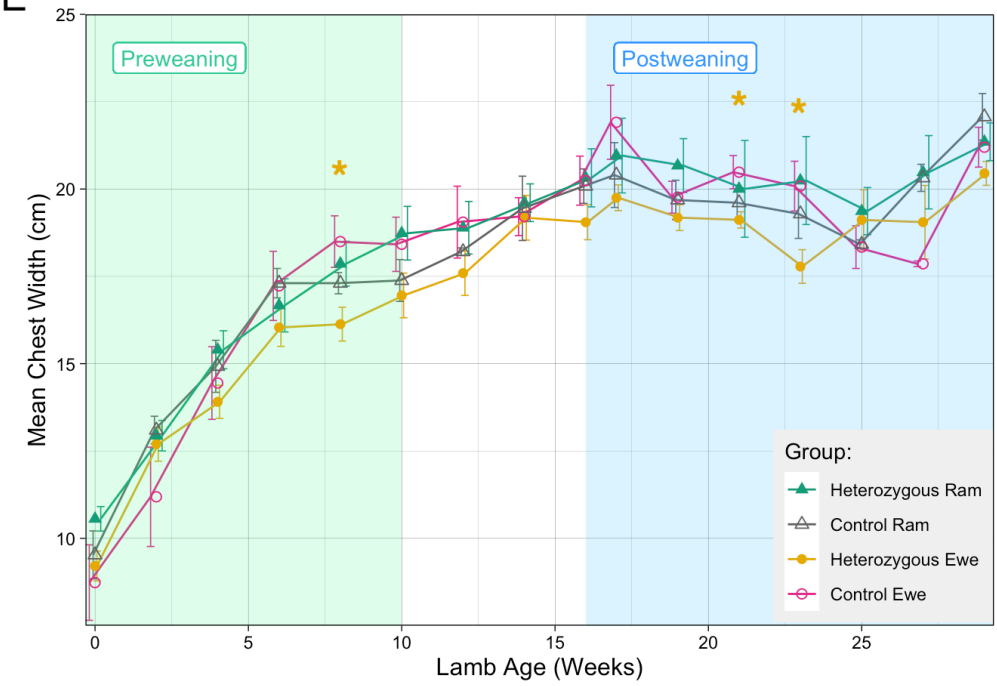
C


Preweaning
Postweaning
Mean Sacrum Height (cm)
Lamb Age (Weeks)
Group:
Heterozygous Ram
Control Ram
Heterozygous Ewe
Control Ewe

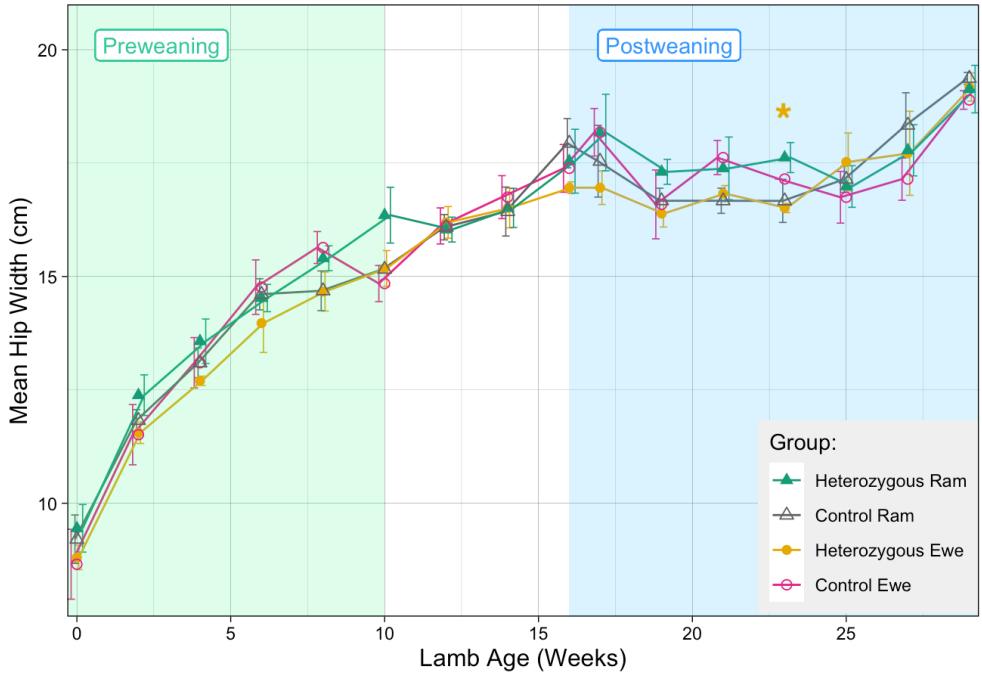
D


Preweaning
Postweaning
Mean Elbow Height (cm)
Lamb Age (Weeks)
Group:
Heterozygous Ram
Control Ram
Heterozygous Ewe
Control Ewe

E


Preweaning
Postweaning
Mean Chest Width (cm)
Lamb Age (Weeks)
Group:
Heterozygous Ram
Control Ram
Heterozygous Ewe
Control Ewe

F


Preweaning
Postweaning
Mean Hip Width (cm)
Lamb Age (Weeks)
Group:
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Control Ram
Heterozygous Ewe
Control Ewe

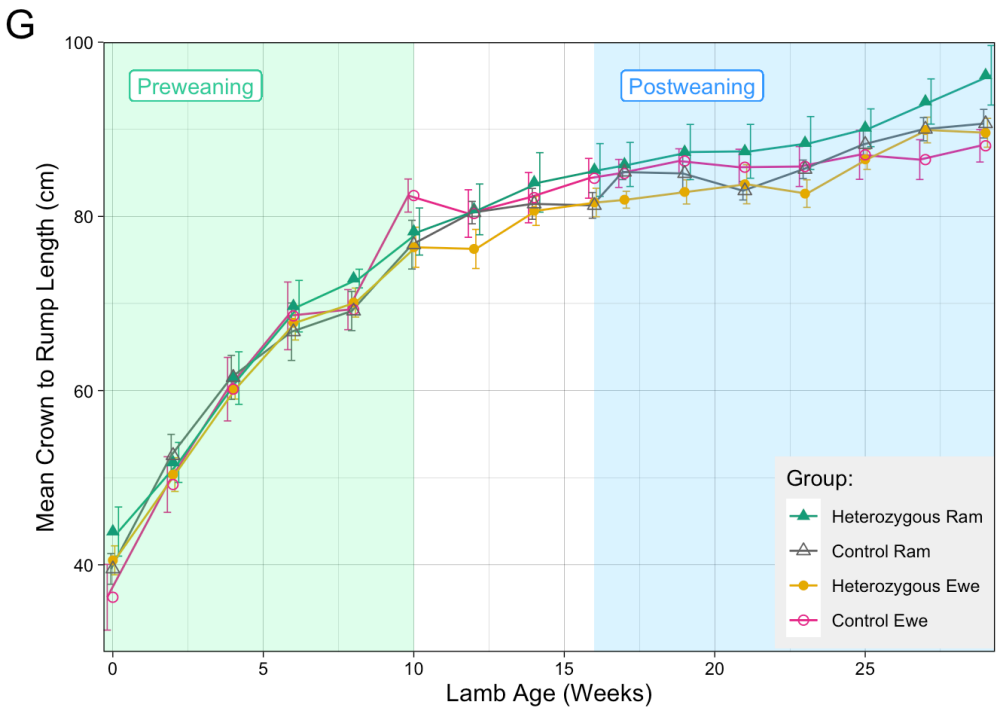


Figure 3.3 A-G: Group mean lamb weights from birth to 30 weeks of age (A) and group mean lamb size from birth to 29 weeks of age for withers height (B), sacrum height (C), elbow height (D), chest width (E), hip width (F), and crown to rump length (G). Error bars indicate standard errors of the mean, asterisks indicate student's t-test p<.05 for comparisons between genotypes of rams (green) or ewes (gold), green and blue shaded regions indicate ages when all measurements were of lambs in the pre-weaning or post-weaning periods, respectively. See Appendix Figures 6A-G for individual lamb growth charts.

Feed Efficiency

From week 9 to 19 post-weaning, genotype was not significantly associated with ram or ewe lamb ADG, DMI, G:DMI, or RFI (p>.05). Out of all the measures of feed efficiency, the largest effect size was for RFI where the heterozygotes tended to have lower RFI than controls, but the difference was not statistically significant (p>.05) (Table 3.4). Additionally, the mean gain to dry matter

intake ratio (G:DMI) was nearly identical between heterozygotes and controls of each sex indicating that the gains in weight were proportional to the amount of feed eaten (Table 3.4).

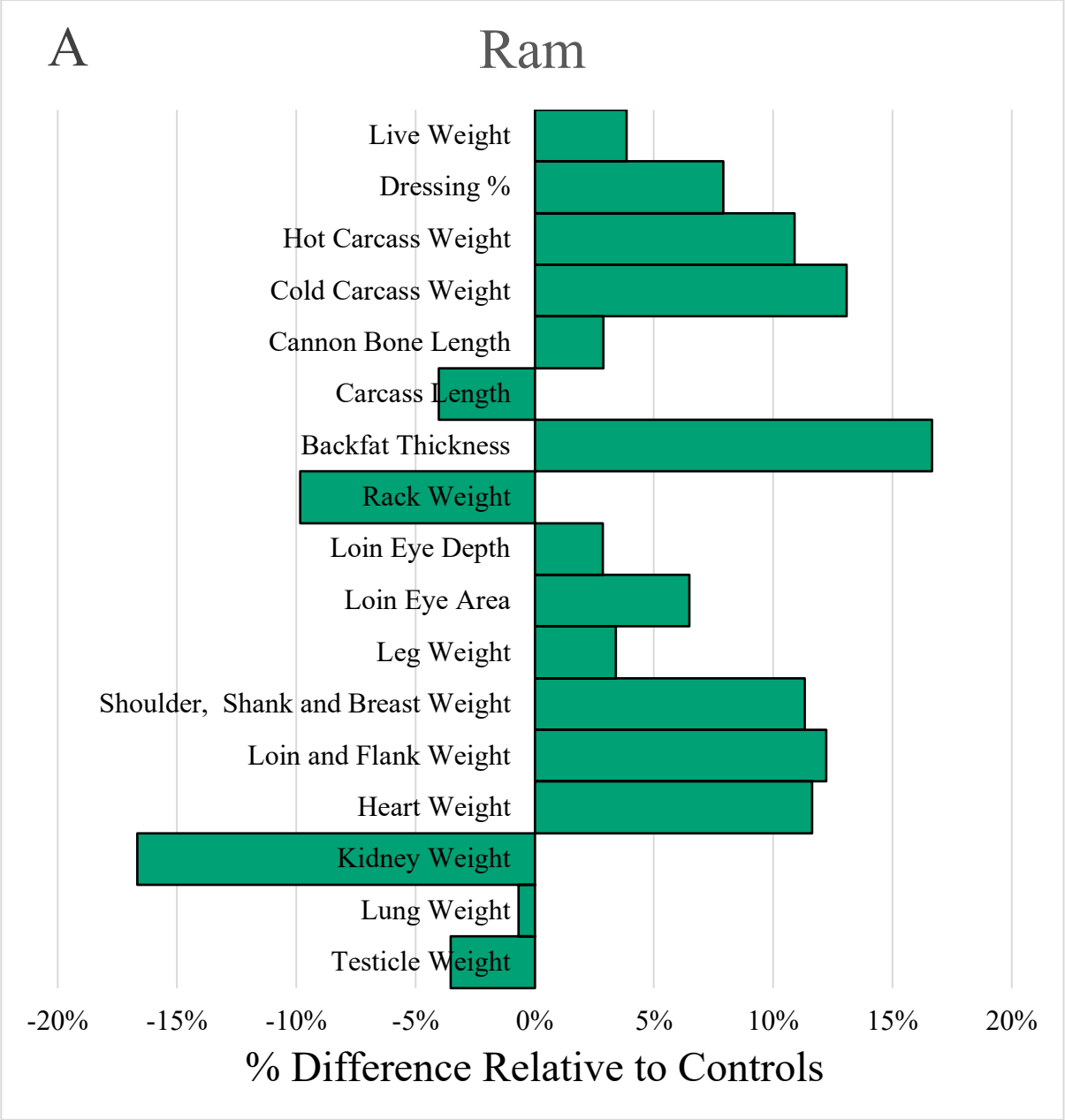
Table 3.4: Feed efficiency trial results. Heterozygous and control means, standard errors (SE), the difference between groups, and Wilcoxon rank sum test with continuity correction p-values for the comparison between genotypes.

		Heterozygous		Control			
Measurement	Sex	Mean	SE	Mean	SE	Effect	p.value
Average daily gains (kg/day)	Ewe	0.203	0.030	0.197	0.026	0.006	0.9048
	Ram	0.292	0.012	0.307	0.026	-0.015	1
Average daily DMI (kg)	Ewe	1.956	0.093	1.753	0.151	0.202	0.2663
	Ram	2.104	0.163	2.159	0.031	-0.055	0.7702
Gain:DMI (kg/kg)	Ewe	0.102	0.012	0.111	0.007	-0.009	0.5556
	Ram	0.141	0.011	0.142	0.011	-0.001	1
Residual feed intake	Ewe	0.103	0.037	-0.148	0.166	0.252	0.2857
	Ram	0.122	0.168	-0.103	0.096	0.226	0.3429

Carcass Characteristics

The *Socs2(+/-)* and control rams did not differ significantly in carcass characteristics, but the effect sizes were moderate for some characteristics. The *Socs2(+/-)* rams weighed 13% more than control rams for cold carcass weight and were 17% fatter than controls (Figure 3.4A, Table 3.5). Additionally, the kidneys of the *Socs2(+/-)* rams weighed 17% less and a decrease in testicle weight was observed as well, despite an increased carcass weight (Figure 3.4A, Table 3.5). The lack of significance of these observations may partially be attributed to the 36-day span of ages amongst the *Socs2(+/-)* rams and the nine day delay in slaughter date for the controls (the mean age at slaughter was 6 days younger for *Socs2(+/-)* rams, and 24.2 days younger for *Socs2(+/-)* ewes relative to sex-matched controls) (Appendix Table 3.9, Table 3.5). Hot carcass weights of

the *Socs2(+/-)* ewes were 7% lighter than control ewes (Figure 3.4B). The heterozygous ewes were shorter in length by 7% ($p<.05$), had 28% lighter racks ($p<.01$), and trended towards larger loin eye areas ($p<.1$). The discrepancy between low rack weight and large loin eye area may be attributable to unaccounted for differences in how the lamb rack was cut between abattoirs (figure 3.4B). While not statistically significant, the *Socs2(+/-)* ewes were 32% leaner than the controls. The backfat thickness of the heterozygous *Socs2* KO lambs was intermediate to the low backfat thickness of the control rams and the high backfat thickness of the control ewes, with no lambs fetching discounted prices except for a control ewe tag # 21162 that was too fat.


A
Ram
Live Weight
Dressing %
Hot Carcass Weight
Cold Carcass Weight
Cannon Bone Length
Carcass Length
Backfat Thickness
Rack Weight
Loin Eye Depth
Loin Eye Area
Leg Weight
Shoulder, Shank and Breast Weight
Loin and Flank Weight
Heart Weight
Kidney Weight
Lung Weight
Testicle Weight
-20%
-15%
-10%
-5%
0%
5%
10%
15%
20%
% Difference Relative to Controls

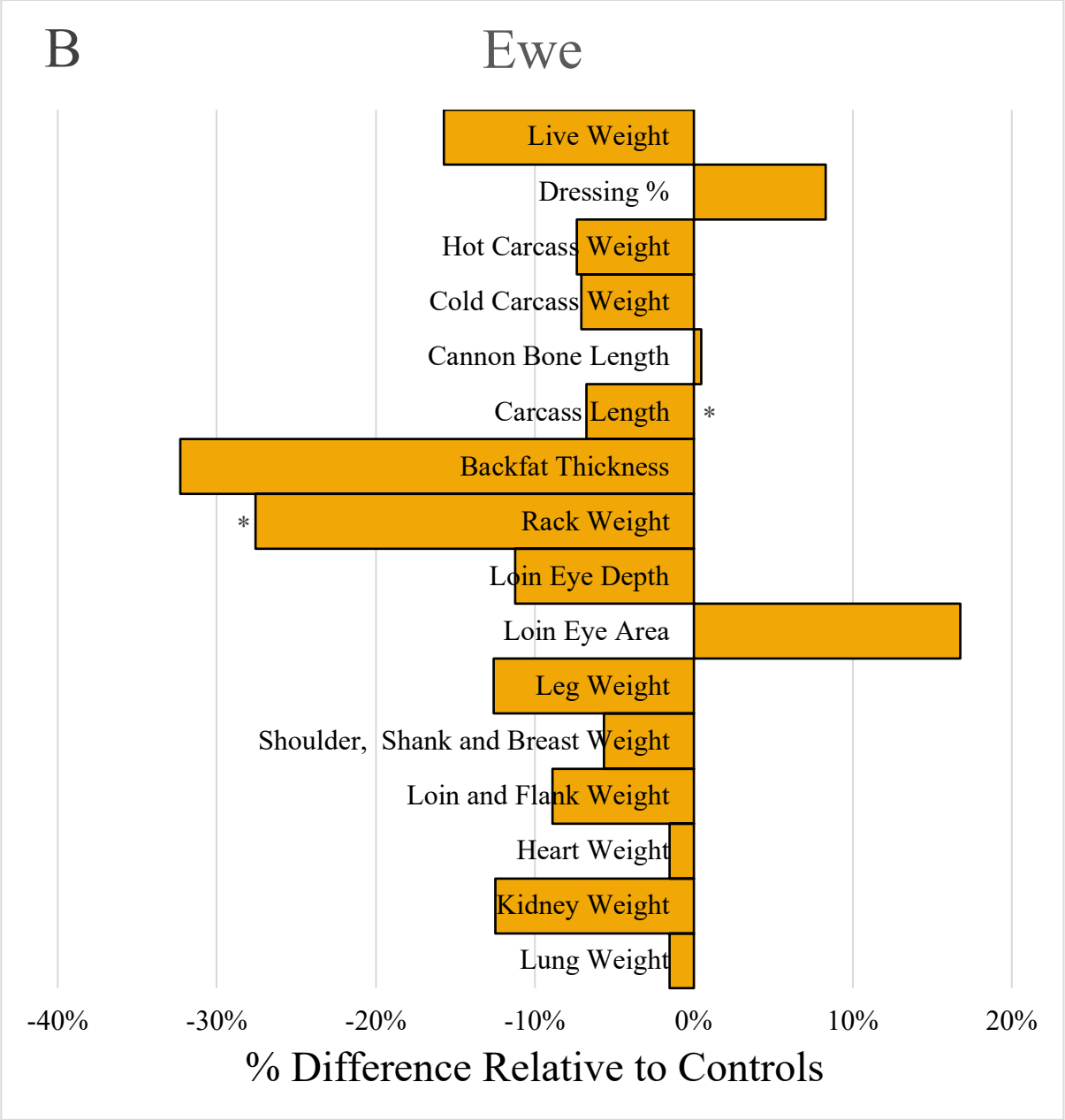


Figure 3.4: The percent difference between heterozygous and control rams (A) and ewes (B) for carcass characteristics. Differences that are statistically significant by Wilcoxon rank sum test with continuity correction p<.05 are indicated "".*

Table 3.5:Mean carcass measurements.

Carcass Characteristic	Sex	Heterozygous Mean	Heterozygous SEM	Control Mean	Control SEM	% Difference	p-value
Live Weight (kg)	Ram	76.26	5.61	73.43	2.98	4%	1.000
	Ewe	56.19	2.80	66.68	4.33	-16%	0.114
Hot Carcass Weight (kg)	Ram	45.47	4.82	41.00	1.79	11%	0.686
	Ewe	35.83	1.56	38.68	3.55	-7%	0.556
Heart Weight (g)	Ram	302.77	11.62	271.25	8.51	12%	0.110
	Ewe	241.31	4.63	245.00	17.91	-2%	0.902
Kidney Weight (g)	Ram	179.17	7.75	215.00	11.37	-17%	0.114
	Ewe	143.34	9.36	163.75	8.98	-12%	0.286
Lung Weight (g)	Ram	641.83	19.47	646.25	47.14	-1%	0.886
	Ewe	610.53	78.79	620.00	24.15	-2%	0.730
Testicle Weight (g)	Ram	683.79	28.59	708.75	50.92	-4%	1.000
Cold Carcass Weight (kg)	Ram	44.79	4.90	39.61	1.23	13%	0.686
	Ewe	35.06	1.55	37.73	3.25	-7%	0.539
Carcass Length (cm)	Ram	141.76	3.69	147.70	1.71	-4%	0.486
	Ewe	**129.16**	1.03	**138.49**	2.79	-7%	**0.016**
Cannon bone length (cm)	Ram	13.85	0.25	13.46	0.37	3%	0.486
	Ewe	13.21	0.15	13.14	0.42	0%	0.711
Rack Weight (kg)	Ram	4.48	0.56	4.97	0.34	-10%	0.686
	Ewe	**3.62**	0.16	**5.00**	0.32	-28%	**0.016**
Leg Weight (kg)	Ram	13.31	1.11	12.88	0.43	3%	1.000
	Ewe	10.83	0.40	12.39	1.25	-13%	0.556
Backfat Thickness (cm)	Ram	0.44	0.12	0.38	0.13	17%	0.739
	Ewe	0.56	0.10	0.83	0.16	-32%	0.241
Loin Eye Depth (cm)	Ram	4.57	0.29	4.45	0.22	3%	0.640
	Ewe	4.11	0.28	4.64	0.12	-11%	0.132
Loin Eye Area (cm)	Ram	23.87	1.21	22.42	1.25	6%	0.384
	Ewe	23.35	0.69	20.00	1.05	17%	0.061
Shoulder + Shank + Breast (kg)	Ram	19.32	1.95	17.36	0.66	11%	0.686
	Ewe	13.97	0.53	14.81	0.83	-6%	0.556
Loin and Flank Weight (kg)	Ram	5.66	0.84	5.04	0.35	12%	0.686
	Ewe	4.84	0.36	5.32	0.58	-9%	0.730

Discussion

Socs2 is a major negative regulator of growth hormone receptor signaling and suppression of *Socs2* has pleiotropic effects on growth, the immune system, and reproduction. Currently, it's unknown if the naturally occurring p.R96C substitution observed in sheep disrupts all functionality of the *Socs2* gene and how much of the phenotype observed in *Socs2(-/-)* and *high growth* mice translates to sheep. In this study, we observed the fertility of two homozygous *Socs2 KO* rams anda single homozygous *Socs2* KO ewe. Additionally, we analyzed the effects of the heterozygous *Socs2* genotype on growth, survival, feed efficiency, and carcass characteristics. This will help to elucidate the effects of *Socs2* KO on production characteristics relevant to lamb production, answer questions about the sex-dependent effects of *Socs2,* and serve as a comparator for sheep with the p.R96C Socs2 substitution.

In ewe lambs, the effects of heterozygous *Socs2* KO on weight and size was consistent with the effects of the p.R96C substitution found in French Lacaune dairy ewes (Rupp et al., 2015). Both *Socs2(+/-)* and p.R96C SOCS2mutations significantly affected elbow height ($p<.05$), but not other measurements of size. The weight of heterozygous p.R96C sheep was not significantly heavier than controls until third lambing, and the heterozygous *Socs2* KO ewes in this study did not differ from controls over the 30 weeks of age studied. However, the *Socs2(+/-)* genotype was associated with a 28% greater rate of gain from 16 to 30 weeks of age ($p<.0001$), as compared to controls, though this may be due to compensatory gains affected by the larger weaned litter size of the heterozygous females. For rams, *Socs2(+/-)* genotype had an earlier and larger effect on weight than in ewes. In male *Socs2(+/-)* mice, weight gain was not significantly different from controls until 11 weeks of age (in comparison to 6 weeks of age for the homozygous *Socs2* KO

males), while no significant difference in weight was observed up to 12 weeks of age in female *Socs2(+/-)* mice (Metcalf et al., 2000).

For size, heterozygous *Socs2* KO males were significantly taller at the elbow at birth and this difference was maintained throughout most of the 30 weeks of growth, while the differences in height at the withers and sacrum were first significantly different at 8 weeks of age. The effect of heterozygous *Socs2* KO genotype on the size of rams is similar to the effects of the homozygous p.R96C SOCS2 point mutation on the size of ewes where a significant positive effect of genotype is seen on the height at the withers, sacrum, and elbow, a positive trend is observed on length, and no significant effect is observed for hip width (Rupp et al., 2015). The greater and earlier effect of the *Socs2* KO mutation on male lamb growth observed this study is consistent with the growth observed in *Socs2(+/-)* mice and inconsistent with the heterozygous p.R96C mice (Li et al., 2022; Metcalf et al., 2000).

Other characteristics of growth like feed efficiency did not differ greatly between genotypes. There were no significant differences in DMI, ADG, G:DMI, or RFI between genotypes during the feed efficiency study and slight variation in DMI and ADG between groups still resulted in very similar G:DMI. Homozygous high growth (*Socs2* KO) mice with a C57BL/6J genetic background ate more in proportion to C57BL/6J controls, but it was proportional to the 51% increase in mature body weight observed, which was a much greater effect on growth than what was observed in this study (Corva & Medrano, 2000). Additionally, the effect of homozygous *Socs2* KO in *high growth* mice on fat was that they had larger fat deposits than controls, but the increase was proportional to body weight. When fed a high energy diet however, control C57BL/6J mice became obese and high growth mice did not and instead had less restricted growth (Corva & Medrano, 2000). A proportional increase in backfat thickness was observed in the *Socs2(+/-)* rams

and the *Socs2(+/-)* ewes were potentially protected from the obesity observed in one of the control ewes when on a relatively high energy and protein TMR.

The reproductive phenotypes of the *Socs2* homozygous KO rams were similar in many ways to the phenotypes observed in *high growth* mice and suggest that reproduction would likely be a major hurdle for the introduction of the *Socs2* KO allele into commercial lamb production operations. A delay in parturition date and increased variation was observed in ewes bred by *Socs2* KO rams and while this may be partially attributable to this being the first time breeding for the rams, it is also consistent with the increased interval between mating and increased gestation length observed in *high growth* mice (Cargill et al., 2000). Additionally, there was a 57% pre-weaning mortality rate and increased fetal number that trended towards significance, which are both consistent with the higher fetal number at 17 days of gestation and smaller litter sizes observed in *high growth* mice (Cargill et al., 2000; Joaquim Casellas & Juan F Medrano, 2008). Additionally, the infertility observed in the one *Socs2* KO ewe when bred by both a fertile homozygous *Socs2* KO ram and a control ram may be due to anovulation, which was found to be the cause of complete female infertility when the *high growth* mutation was introgressed into female FVB/NJ and A/J lines (Lathan, 2012).

Identification of additional effects of heterozygous *Socs2* KO on growth, reproduction and carcass quality would likely be identified with a larger sample size. Additionally, we could not control for background genetic variation in this study, partially because the generation of genome edited sheep requires large numbers of oocytes that were collected from a local slaughterhouse where the genetic background of the oocyte cannot be known prior to birth. Additionally, greater synchrony in birth dates of lambs would have eliminated the need for age adjustment of the post-weaning data, and reduced the variation due to age for feed efficiency and carcass characteristic

analyses. As a result of the variation in ages and in abattoirs, it is difficult to draw strong conclusions from the carcass observations in this study.

Future studies are needed to determine if the detrimental effects of *Socs2* genotype on reproduction observed in this study are observed in different genetic backgrounds and if heterozygous *Socs2* KO ewes are infertile. Additionally, further comparison of *Socs2* heterozygous KO and p.R96C sheep is necessary to determine if the earlier occurring and larger effects of *Socs2* genotype on growth in heterozygous *Socs2* KO males is present in p.R96C rams. Other traits such as internal and external parasite resistance have also been associated with *Socs2* and *STAT5b* genotype, and *Socs2(-/-)* male mice have nearly twice as much collagen tissue in the skin at 9 weeks of age indicating that *Socs2* genotype may also affect pelt characteristics in sheep (Estrada-Reyes et al., 2019; Reiser et al., 1996). Additionally, *Socs2(-/-)* sheep may serve as good large animal models for growth disorders and certain cancers (Braz et al., 2014; Hoefer et al., 2014).

When the first *Socs2* KO mouse was studied in 1984, the authors noted that the value of the gene for increasing the growth of livestock species would be limited unless the adverse effects of the mutation on reproduction could be overcome (Bradford & Famula, 1984). In the present study, *Socs2* KO genotype was found to have a negative impact on reproduction that likely outweighs the relatively minor improvements in growth. Additionally, the greatest increases in size observed in *Socs2(+/-)* lambs were for measurements of height, which is not a common breeding goal for most producers and large improvements in feed efficiency or carcass characteristics were not observed. This study did show for the first time that increased height was present at birth for the male *Socs2(+/-)* lambs and that the improved growth rate of *Socs2(+/-)* males occurred prior to that of ewes.

Acknowledgments

We would like to express our deep gratitude to Matthew Hayes and residents of the UC Davis sheep barn, including Paige Condy for their help with animal husbandry and establishing the feed efficiency infrastructure, to Dr. Lee Allen Pettey for formulating a diet for the sheep, and to Xiang (Crystal) Yang and her lab for lending many hands for the carcass analysis.

Appendix - Chapter 3

Table 3.6: Individual pregnancy results. ♂ indicates a male, ♀ indicates a female, blank sex indicates the sex of the lamb is unknown the genotype represents coordinated in the ARS-UI_Ramb_V2.0 reference genome. Complete autopsy results are available upon request.

Ram	Ewe	Prenatal dead	Neonatal dead (death date)	Weaned	Sex of lamb	Necropsy summary	*Socs2* genotype
7182	7WF	7WFA			♂	Dystocia, fetal stress, myocardial necrosis	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
		7WFB			♂	Dystocia/preterm, fetal stress, myocardial necrosis	NC_056056.1:g.129875814_129875897del/WT
	7041 (Red)						
	7091			21180	♀		NC_056056.1:g.129875807_129877500delinsAGACTC/WT
				21181	♂		NC_056056.1:g.129875814_129875897del/WT
	7090		21178 (4/29)		♀	Weak, unable to nurse	NC_056056.1:g.129875807_129877500delinsAGACTC/WT
				21179	♀		NC_056056.1:g.129875807_129877500delinsAGACTC/WT
	Blank Yellow			21166	♂		NC_056056.1:g.129875814_129875897del/WT
				21173	♀		NC_056056.1:g.129875807_1298775

							00delinsAGACTC /WT
	7181						NC_056056.1:g.129875811_129875812insG, NC_056056.1:g.129875579_129877705del/ NC_056056.1:g.129875578_129877705del
7183	8023		21174 (4/28)		♂	Respiratory issues	NC_056056.1:g.129875650_129875908delinsCG/WT
	8161			21163	♂		NC_056056.1:g.129875814_129875901del/WT
	7041 (Yellow)			21168	♀		NC_056056.1:g.129875650_129875908delinsCG/WT
				21169	♂		NC_056056.1:g.129875814_129875901del/WT
				21170	♀		NC_056056.1:g.129875650_129875908delinsCG/WT
	3yrNT	3yrNTA				Intrahepatic cholestasis, fetal stress	NC_056056.1:g.129875814_129875901del/WT
		3yrNT B				Intrahepatic cholestasis, fetal stress	NC_056056.1:g.129875650_129875908delinsCG/WT
		3yrNT C				Intrahepatic cholestasis, renal tubular epithelial bile, fetal stress	NC_056056.1:g.129875814_129875901del /WT
		3yrNT D				Fetal stress	NC_056056.1:g.129875650_129875908delinsCG/WT
	8153	8153			♂	Stillborn, goiter, fetal stress	NC_056056.1:g.129875650_129875908delinsCG/WT

	9199	9199 1			♂	low selenium, inactive thyroid follicles	NC_056056.1:g.129875814_129875901del/WT
		9199 2			♂	low selenium, inactive thyroid follicles, contraction band necrosis	NC_056056.1:g.129875650_129875908delinsCG/WT
			21177 (4/28)		♀	failure of passive transfer, respiratory issues, Mannheimia hemolytica muscular autolysis	NC_056056.1:g.129875650_129875908delinsCG/WT
7068	8178			21161	♀		WT/WT
	6091			21171	♂		WT/WT
	8185			21162	♀		N/A
	4062	4062			♂	Fetal stress, staphylococcus equorum	WT/WT
				21159	♀		WT/WT
	8195			21175	♂		WT/WT
				21176	♂		WT/WT
	6096	6096				Low liver zinc, copper	WT/WT
				21164	♀		WT/WT
				21172	♂		WT/WT

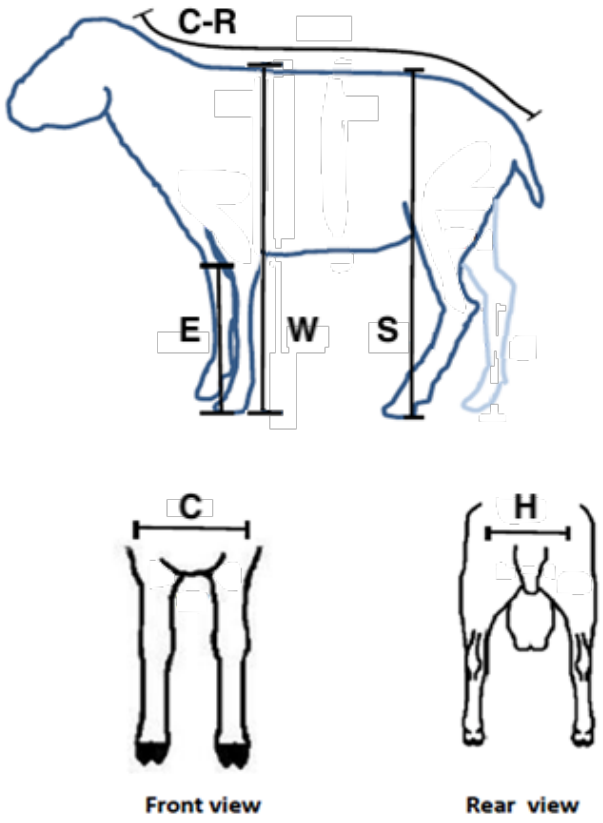


Figure 3.5:Diagram of size measurements: crown to rump length (C-R) measured along spine from the back of head to the end of the sacrum, elbow height (E), withers height (W), sacrum height (S) measured in line with back leg, chest width (C) measured from left to right head of humerus, hip width (H) distance between hip bones. Adapted from (Rupp, Tosser-Klopp, 2015).

Table 3.7: The measurement of lamb #21176 on week 12 pre-weaning is missing, but was still adjusted as if it were measured on week 12.

Tag #	Group	Relative Birth Date	Number of Pre-weaning Measurements Beyond 10 Weeks of Age		Number of Days Older Than Adjusted Age		
			For Weight	For Size	For Weight	For Size	Size For Weeks 17-19
21159	Control Ewe	0	5	2	5	12	5
21161	Control Ewe	1	5	2	4	11	4
21162	Control Ewe	3	5	2	2	9	2
21164	Control Ewe	4	5	2	1	8	1
21172	Control Ram	4	5	2	1	8	1
21171	Control Ram	10	4	2	2	2	-5
21175	Control Ram	22	2	1	4	4	-3
21176	Control Ram	22	1*	0*	4	4	4
21173	Heterozygous Ewe	6	4	2	6	6	-1
21168	Heterozygous Ewe	6	4	2	6	6	-1
21170	Heterozygous Ewe	6	4	2	6	6	-1
21179	Heterozygous Ewe	28	1	0	5	12	12
21180	Heterozygous Ewe	40	0	0	0	0	0
21163	Heterozygous Ram	4	5	2	1	8	1
21166	Heterozygous Ram	6	4	2	6	6	-1
21169	Heterozygous Ram	6	4	2	6	6	-1
21181	Heterozygous Ram	40	0	0	0	0	0

Table 3.8A,B: Mean and standard deviation of birthdates and the number of days older than the adjusted age in weeks for post-weaning measurements (A). Litter sizes, birth and slaughter dates and the standard deviations of birthdates and adjusted ages for weaned lambs.

A.	Mean				Standard Deviation			
		Days Older Than Age				Days Older Than Age		
Group	Relative Birth Date	Weight	Size	Weeks 17-19 Size	Relative Birth Date	Weight	Size	Weeks 17-19 Size
Control Ewe	2.0	3	10	3	1.83	1.83	1.83	1.83
Control Ram	14.5	2.75	4.5	-0.75	9.00	1.50	2.52	4.03
Heterozygous Ewe	17.2	4.6	6	1.8	15.91	2.61	4.24	5.72
Heterozygous Ram	14.0	3.25	5	-0.25	17.36	2.22	1.00	3.20

B. Group	Live Litter Size	Mean Weaned Litter Size	Mean Weaned Lamb Birth Date	σ Weaned Lamb Birth Date	Mean Days Older Than Adjusted Age	σ Days Older Than Adjusted Age	Mean Age at Slaughter
Control Ewe	1.25	1.25	2.0	1.8	3	1.8	263
Heterozygous Ewe	2.14	2.2	17.2	15.9	4.6	2.6	238.8
Control Ram	1.75	1.75	14.5	9.0	2.75	1.5	242
Heterozygous Ram	1.8	2	14.0	17.4	3.25	2.2	236

Table 3.9:Individual litter size, birth date, and age at death.

Sire	Lamb Tag #	Group	Litter Size	Weaned Litter Size	Days Born After First Born	Neonatal Dead?	Age at Death (Days)	Slaughter Date
7068	21159	Control Ewe	1	1	0	No	265	12/22/21
7068	21161	Control Ewe	1	1	1	No	264	12/22/21
7068	21162	Control Ewe	1	1	3	No	262	12/22/21
7068	21164	Control Ewe	2	2	4	No	261	12/22/21
7068	21172	Control Ram	2	2	4	No	261	12/22/21
7068	21171	Control Ram	1	1	10	No	255	12/22/21
7068	21175	Control Ram	2	2	22	No	243	12/22/21
7068	21176	Control Ram	2	2	22	No	243	12/22/21
7183	21163	Heterozygous Ram	1	1	4	No	252	12/13/21
7182	21166	Heterozygous Ram	2	2	6	No	250	12/13/21
7183	21169	Heterozygous Ram	3	3	6	No	250	12/13/21
7182	21181	Heterozygous Ram	2	2	40	No	216	12/13/21
7182	21173	Heterozygous Ewe	2	2	6	No	250	12/13/21
7183	21168	Heterozygous Ewe	3	3	6	No	250	12/13/21
7183	21170	Heterozygous Ewe	3	3	6	No	250	12/13/21
7182	21179	Heterozygous Ewe	2	1	28	No	228	12/13/21
7182	21180	Heterozygous Ewe	2	2	40	No	216	12/13/21
7183	21174	Heterozygous Ram	1	0	14	Yes	14	
7183	21177	Heterozygous Ewe	1	0	25	Yes	2	
7182	21178	Heterozygous Ewe	2	1	28	Yes	0	

Table 3.10: Summary of mixed effect models for lamb weight (A), withers height (B, sacrum height (C , elbow height (D), chest width (E), hip width (F), and crown to rump length (G) from 0 to 10 weeks of age and lamb weight (H), withers height (I), sacrum height (J), elbow height (K), chest width (L), hip width (M), and crown to rump length (N) from 16 to 30 weeks of age. Week exp is Week^.75, Week exp2 is Week^.6, Age is adjusted age in weeks. Heterozygous males, control males, heterozygous females and control females are indicated by green, gray, yellow, and pink points respectively.

A

Weight				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	3.98	-1.64 – 9.60	0.150	13.14
Sex [Ram]	0.62	-3.24 – 4.48	0.734	13.14
Genotype [WT]	-0.48	-4.88 – 3.92	0.819	13.14
Week	3.15	2.94 – 3.37	**<0.001**	164.00
Weaned Litter Size	-0.20	-2.47 – 2.08	0.856	13.14
Sex [Ram] * Genotype [WT]	0.62	-5.17 – 6.41	0.821	13.14
Sex [Ram] * Week	0.34	0.19 – 0.49	**<0.001**	164.00
Genotype [WT] * Week	-0.12	-0.29 – 0.05	0.160	164.00
Week * Weaned Litter Size	-0.54	-0.62 – -0.45	**<0.001**	164.00
(Sex [Ram] * Genotype [WT]) * Week	-0.33	-0.55 – -0.10	**0.005**	164.00
Random Effects				
σ^2	1.37			
$\tau_{00\ Lamb.Tag.}$	6.57			
ICC	0.83			
$N_{Lamb.Tag.}$	17			
Observations	186			
Marginal R^2 / Conditional R^2	0.873 / 0.978			

B

Withers				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	37.93	30.04 – 45.81	**<0.001**	12.87
Sex [Ram]	3.16	-2.47 – 8.80	0.252	16.41
Genotype [WT]	-3.68	-10.02 – 2.67	0.237	15.34
Week exp2	5.86	5.28 – 6.43	**<0.001**	80.01
Weaned Litter Size	-2.05	-5.21 – 1.11	0.182	11.98
Sex [Ram] * Genotype [WT]	1.82	-6.61 – 10.25	0.653	16.11
Sex [Ram] * Week exp2	0.46	-0.40 – 1.32	0.288	80.00
Genotype [WT] * Week exp2	0.19	-0.67 – 1.05	0.669	80.00
(Sex [Ram] * Genotype [WT]) * Week exp2	-1.11	-2.35 – 0.14	0.082	80.00
Random Effects				
σ^2	4.34			
$\tau_{00\ Lamb.Tag.}$	12.52			
ICC	0.74			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.803 / 0.949			

C

Sacrum				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	36.52	28.73 – 44.31	**<0.001**	13.05
Sex [Ram]	3.89	-1.72 – 9.51	0.162	17.39
Genotype [WT]	-3.07	-9.38 – 3.24	0.319	16.08
Week exp2	5.96	5.33 – 6.58	**<0.001**	80.01
Weaned Litter Size	-2.01	-5.12 – 1.11	0.186	11.98
Sex [Ram] * Genotype [WT]	0.06	-8.34 – 8.45	0.989	17.03
Sex [Ram] * Week exp2	0.33	-0.60 – 1.26	0.482	80.01
Genotype [WT] * Week exp2	0.07	-0.86 – 1.00	0.880	80.01
(Sex [Ram] * Genotype [WT]) * Week exp2	-0.72	-2.07 – 0.64	0.296	80.00
Random Effects				
σ^2	5.10			
$\tau_{00\ Lamb.Tag.}$	12.03			
ICC	0.70			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.805 / 0.942			

D

Elbow				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	26.14	21.39 – 30.89	**<0.001**	15.79
Sex [Ram]	1.91	-1.35 – 5.17	0.231	15.79
Genotype [WT]	-3.82	-7.54 – -0.10	**0.045**	15.79
Week exp	2.85	2.34 – 3.37	**<0.001**	79.00
Weaned Litter Size	-0.29	-2.21 – 1.63	0.753	15.78
Sex [Ram] * Genotype [WT]	0.54	-4.35 – 5.43	0.819	15.78
Sex [Ram] * Week exp	0.09	-0.26 – 0.45	0.598	79.00
Genotype [WT] * Week exp	0.15	-0.26 – 0.55	0.468	79.00
Week exp * Weaned Litter Size	-0.35	-0.56 – -0.14	**0.001**	79.00
(Sex [Ram] * Genotype [WT]) * Week exp	-0.17	-0.70 – 0.36	0.530	79.00
Random Effects				
σ^2	1.47			
$\tau_{00\ Lamb.Tag.}$	4.25			
ICC	0.74			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.797 / 0.948			

E

Chest				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	8.72	6.07 – 11.36	**<0.001**	27.25
Genotype [WT]	-1.03	-3.09 – 1.04	0.318	27.25
Sex [Ram]	0.92	-0.90 – 2.73	0.309	27.25
Week [sqrt]	3.54	2.77 – 4.31	**<0.001**	79.01
Weaned Litter Size	0.22	-0.85 – 1.29	0.674	27.25
Genotype [WT] * Sex [Ram]	0.60	-2.12 – 3.32	0.657	27.25
Genotype [WT] * Week [sqrt]	0.49	-0.11 – 1.10	0.109	79.01
Sex [Ram] * Week [sqrt]	0.09	-0.44 – 0.62	0.736	79.01
Week [sqrt] * Weaned Litter Size	-0.48	-0.79 – -0.16	**0.003**	79.00
(Genotype [WT] * Sex [Ram]) * Week [sqrt]	-0.59	-1.38 – 0.21	0.145	79.01
Random Effects				
σ^2	1.02			
$\tau_{00\ Lamb.Tag.}$	0.94			
ICC	0.48			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.828 / 0.911			

F

Hips				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	8.72	7.08 – 10.36	**<0.001**	28.68
Genotype [WT]	-0.06	-1.34 – 1.23	0.927	28.68
Sex [Ram]	0.63	-0.49 – 1.76	0.259	28.68
Week [sqrt]	2.55	2.06 – 3.04	**<0.001**	79.01
Weaned Litter Size	0.01	-0.65 – 0.67	0.976	28.68
Genotype [WT] * Sex [Ram]	-0.09	-1.78 – 1.60	0.912	28.68
Genotype [WT] * Week [sqrt]	-0.04	-0.42 – 0.35	0.855	79.02
Sex [Ram] * Week [sqrt]	0.05	-0.29 – 0.38	0.787	79.02
Week [sqrt] * Weaned Litter Size	-0.22	-0.42 – -0.03	**0.028**	79.00
(Genotype [WT] * Sex [Ram]) * Week [sqrt]	-0.20	-0.71 – 0.30	0.424	79.01
Random Effects				
σ^2	0.42			
$\tau_{00\ Lamb.Tag.}$	0.35			
ICC	0.46			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.870 / 0.929			

G

Length				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	40.95	32.18 – 49.73	**<0.001**	21.32
Sex [Ram]	2.44	-3.59 – 8.46	0.410	21.32
Genotype [WT]	-4.21	-11.08 – 2.66	0.217	21.32
Week exp	8.60	7.23 – 9.96	**<0.001**	79.01
Weaned Litter Size	-0.12	-3.68 – 3.43	0.943	21.31
Sex [Ram] * Genotype [WT]	2.21	-6.83 – 11.26	0.616	21.31
Sex [Ram] * Week exp	-0.28	-1.22 – 0.66	0.556	79.01
Genotype [WT] * Week exp	0.45	-0.62 – 1.52	0.407	79.01
Week exp * Weaned Litter Size	-0.98	-1.54 – -0.43	**0.001**	79.00
(Sex [Ram] * Genotype [WT]) * Week exp	-0.68	-2.09 – 0.73	0.338	79.00
Random Effects				
σ^2	10.40			
$\tau_{00\ Lamb.Tag.}$	11.95			
ICC	0.53			
$N_{Lamb.Tag.}$	17			
Observations	101			
Marginal R^2 / Conditional R^2	0.884 / 0.946			

H

Weight				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	25.95	15.61 – 36.29	**<0.001**	13.67
Sex [Ram]	-4.60	-12.28 – 3.07	0.226	21.32
Genotype [WT]	3.54	-5.04 – 12.12	0.399	19.22
Age	1.45	1.34 – 1.56	**<0.001**	239.01
Weaned Litter Size	-6.19	-10.30 – -2.09	**0.007**	12.00
Sex [Ram] * Genotype [WT]	-7.58	-19.04 – 3.88	0.183	20.77
Sex [Ram] * Age	0.61	0.45 – 0.77	**<0.001**	239.01
Genotype [WT] * Age	-0.32	-0.48 – -0.16	**<0.001**	239.01
(Sex [Ram] * Genotype [WT]) * Age	0.24	0.01 – 0.47	**0.044**	239.01
Random Effects				
σ^2	4.38			
$\tau_{00\ Lamb.Tag.}$	22.09			
ICC	0.83			
$N_{Lamb.Tag.}$	17			
Observations	260			
Marginal R^2 / Conditional R^2	0.767 / 0.961			

I

Withers				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	57.04	51.90 – 62.18	**<0.001**	18.48
Sex [Ram]	2.22	-2.24 – 6.69	0.322	49.39
Genotype [WT]	-0.05	-4.84 – 4.74	0.984	39.90
Age	0.58	0.48 – 0.67	**<0.001**	115.00
Weaned Litter Size	-1.81	-3.75 – 0.12	0.064	12.00
Sex [Ram] * Genotype [WT]	-2.78	-9.37 – 3.81	0.401	46.82
Sex [Ram] * Age	0.14	-0.01 – 0.28	0.063	115.00
Genotype [WT] * Age	-0.10	-0.24 – 0.05	0.188	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.01	-0.20 – 0.22	0.916	115.00
Random Effects				
σ^2	1.65			
$\tau_{00\ Lamb.Tag.}$	4.78			
ICC	0.74			
$N_{\ Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.675 / 0.916			

J

Sacrum				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	55.24	49.20 – 61.27	**<0.001**	16.39
Sex [Ram]	4.92	-0.02 – 9.86	0.051	36.94
Genotype [WT]	2.12	-3.25 – 7.48	0.428	30.49
Age	0.66	0.57 – 0.76	**<0.001**	115.00
Weaned Litter Size	-2.60	-4.92 – -0.27	**0.032**	12.00
Sex [Ram] * Genotype [WT]	-5.98	-13.30 – 1.34	0.106	35.17
Sex [Ram] * Age	0.02	-0.13 – 0.16	0.834	115.00
Genotype [WT] * Age	-0.17	-0.31 – -0.02	**0.023**	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.09	-0.12 – 0.30	0.397	115.00
Random Effects				
σ^2	1.66			
$\tau_{00\ Lamb.Tag.}$	6.97			
ICC	0.81			
$N_{\ Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.625 / 0.928			

K

Elbow				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	37.68	34.90 – 40.45	**<0.001**	15.82
Sex [Ram]	2.15	-0.08 – 4.38	0.058	33.49
Genotype [WT]	-0.60	-3.03 – 1.84	0.618	27.93
Age	0.35	0.31 – 0.39	**<0.001**	115.00
Weaned Litter Size	-1.61	-2.68 – -0.53	**0.007**	12.00
Sex [Ram] * Genotype [WT]	-3.28	-6.59 – 0.02	0.052	31.96
Sex [Ram] * Age	0.03	-0.03 – 0.09	0.379	115.00
Genotype [WT] * Age	-0.10	-0.16 – -0.03	**0.003**	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.11	0.02 – 0.21	**0.015**	115.00
Random Effects				
σ^2	0.31			
$\tau_{00\ Lamb.Tag.}$	1.50			
ICC	0.83			
$N_{\ Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.755 / 0.958			

L

Chest				
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	20.42	17.56 – 23.28	**<0.001**	60.54
Sex [Ram]	1.58	-1.92 – 5.09	0.373	125.81
Genotype [WT]	3.13	-0.45 – 6.70	0.086	121.92
Age	0.04	-0.06 – 0.13	0.456	115.00
Weaned Litter Size	-0.94	-1.74 – -0.14	**0.025**	12.00
Sex [Ram] * Genotype [WT]	-6.03	-11.14 – -0.91	**0.021**	125.14
Sex [Ram] * Age	-0.02	-0.17 – 0.12	0.749	115.00
Genotype [WT] * Age	-0.14	-0.29 – 0.01	0.062	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.24	0.03 – 0.45	**0.027**	115.00
Random Effects				
σ^2	1.71			
$\tau_{00\ Lamb.Tag.}$	0.63			
ICC	0.27			
$N_{\ Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.220 / 0.430			

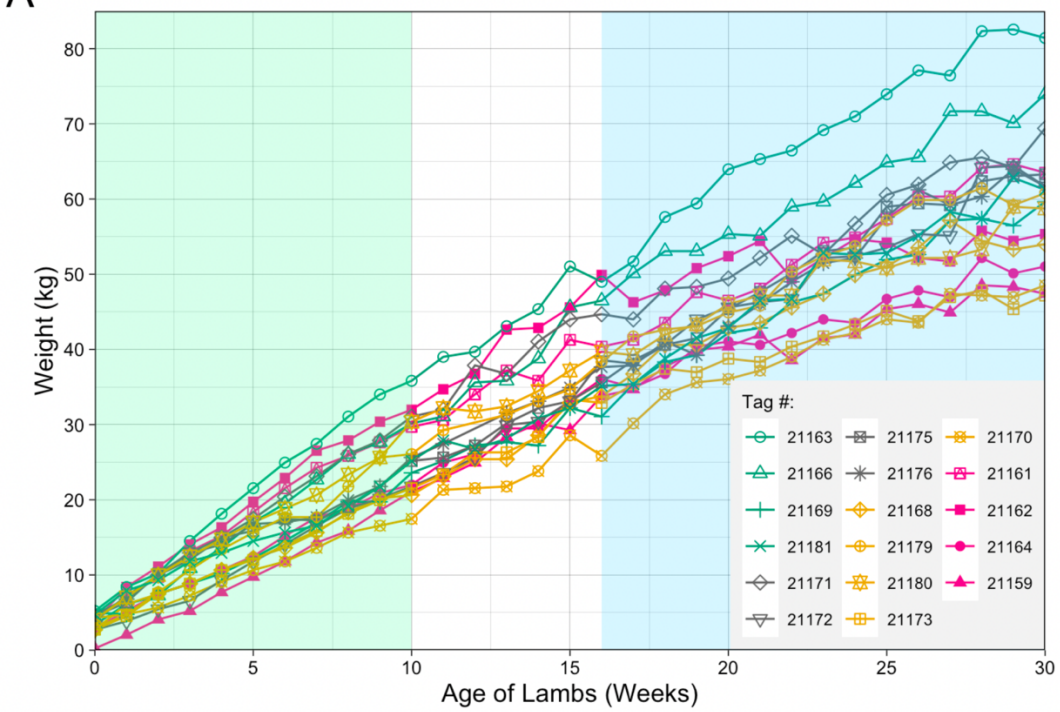
M	Hips			
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	14.89	12.83 – 16.94	**<0.001**	101.89
Sex [Ram]	2.43	-0.34 – 5.20	0.085	125.45
Genotype [WT]	2.33	-0.46 – 5.13	0.101	126.65
Age	0.15	0.07 – 0.23	**<0.001**	115.00
Weaned Litter Size	-0.45	-0.90 – 0.00	0.050	12.00
Sex [Ram] * Genotype [WT]	-4.17	-8.20 – -0.13	**0.043**	125.79
Sex [Ram] * Age	-0.09	-0.21 – 0.03	0.136	115.00
Genotype [WT] * Age	-0.11	-0.23 – 0.01	0.061	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.18	0.01 – 0.36	**0.039**	115.00
Random Effects				
σ^2	1.14			
$\tau_{00\ Lamb.Tag.}$	0.12			
ICC	0.10			
$N_{Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.197 / 0.276			

N	Length			
Predictors	*Estimates*	*CI*	*p*	*df*
(Intercept)	77.34	70.92 – 83.77	**<0.001**	30.84
Sex [Ram]	1.38	-5.36 – 8.12	0.685	99.70
Genotype [WT]	8.41	1.41 – 15.41	**0.019**	85.15
Age	0.70	0.53 – 0.87	**<0.001**	115.00
Weaned Litter Size	-3.72	-5.89 – -1.55	**0.003**	12.00
Sex [Ram] * Genotype [WT]	-9.73	-19.61 – 0.15	0.053	96.26
Sex [Ram] * Age	0.10	-0.16 – 0.36	0.435	115.00
Genotype [WT] * Age	-0.49	-0.75 – -0.22	**<0.001**	115.00
(Sex [Ram] * Genotype [WT]) * Age	0.38	0.01 – 0.76	**0.047**	115.00
Random Effects				
σ^2	5.39			
$\tau_{00\ Lamb.Tag.}$	5.59			
ICC	0.51			
$N_{Lamb.Tag.}$	17			
Observations	136			
Marginal R^2 / Conditional R^2	0.583 / 0.795			

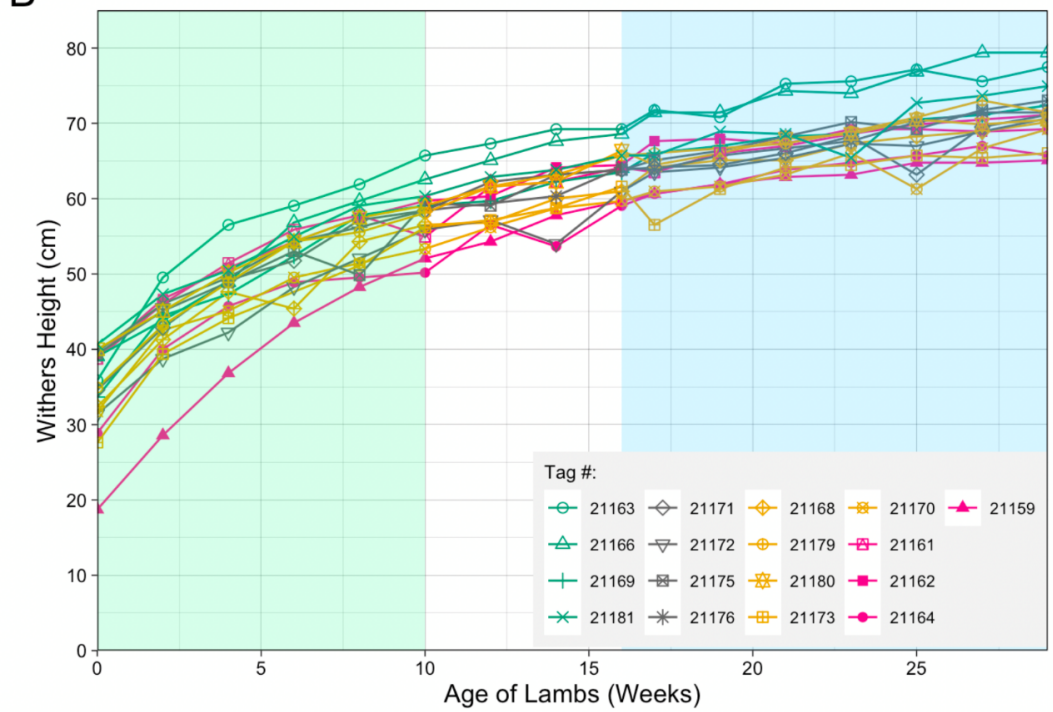
Table 3.11: Likelihood ratio tests for Genotype and weaned litter size in linear mixed effect models of growth.

		Genotype		Weaned Litter Size * Age	
Category	Measurement	Pre-weaning	Post-weaning	Pre-weaning	Post-weaning
Weight (kg)	Weight	1.50E-06	0.0004255	2.20E-16	0.09496
Height (cm)	Withers	0.06804	0.009749	0.07758	0.1414
	Sacrum	0.1537	0.003142	0.101	0.7494
	Elbow	0.04523	1.46E-05	0.0008351	0.1963
Width (cm)	Chest	0.4222	0.1606	0.002294	0.8651
	Hips	0.3608	0.1971	0.02271	0.7661
Length (cm)	Crown to Rump	0.4719	0.0005035	0.0004528	0.8672

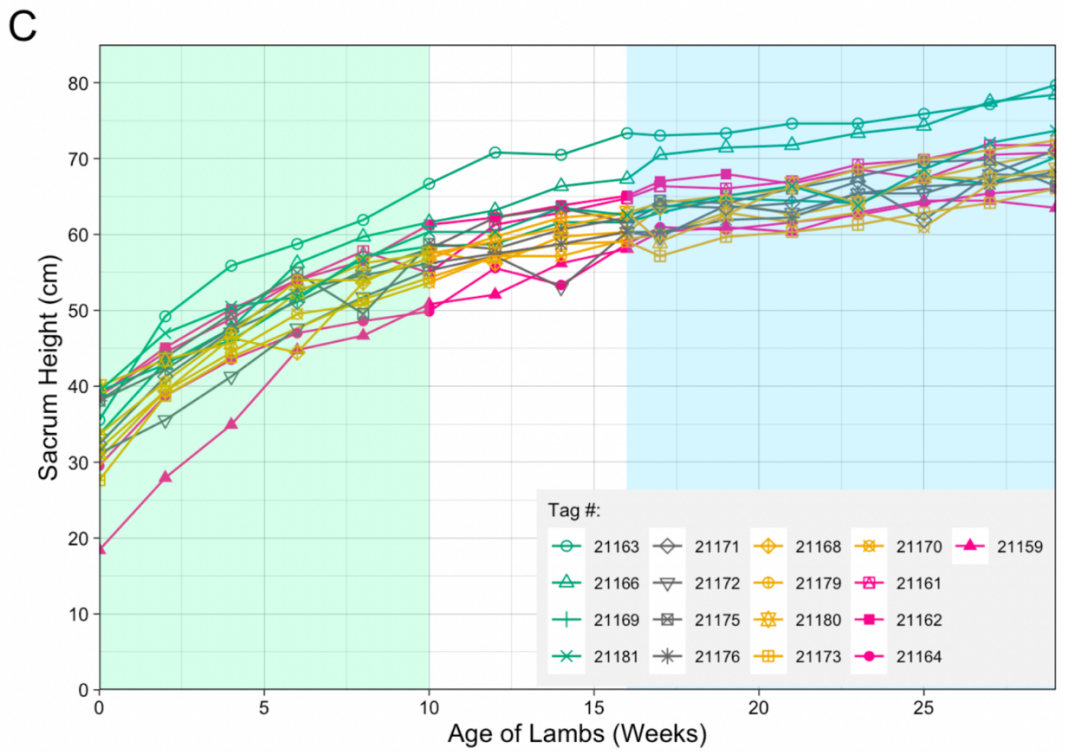
A


Weight (kg)
Age of Lambs (Weeks)
Tag #:
21163
21175
21170
21166
21176
21161
21169
21168
21162
21181
21179
21164
21171
21180
21159
21172
21173

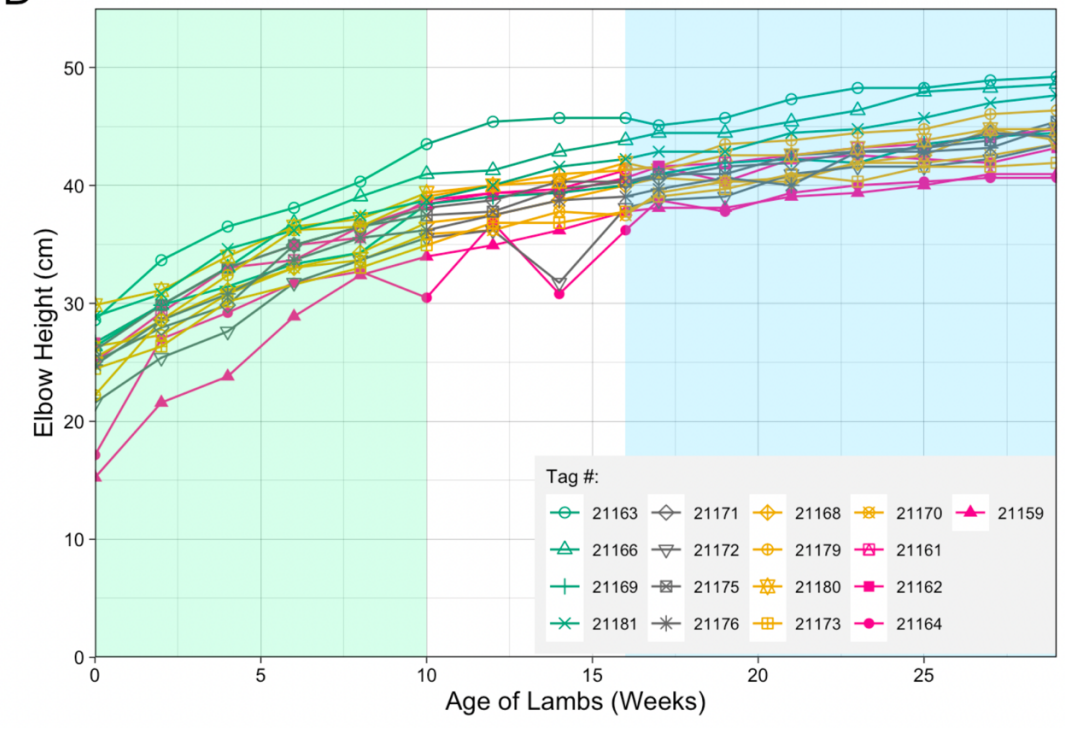
B


Withers Height (cm)
Age of Lambs (Weeks)
Tag #:
21163
21171
21168
21170
21159
21166
21172
21179
21161
21169
21175
21180
21162
21181
21176
21173
21164

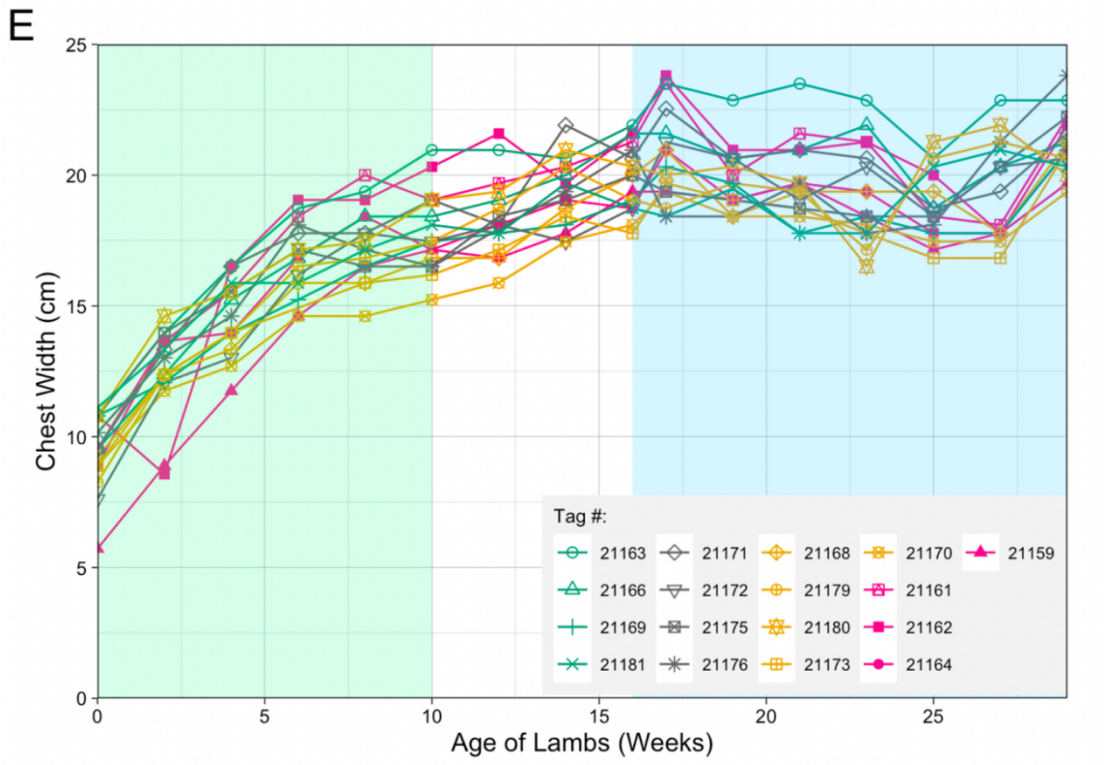
C


Sacrum Height (cm)
Age of Lambs (Weeks)
Tag #:
21163
21171
21168
21170
21159
21166
21172
21179
21161
21169
21175
21180
21162
21181
21176
21173
21164

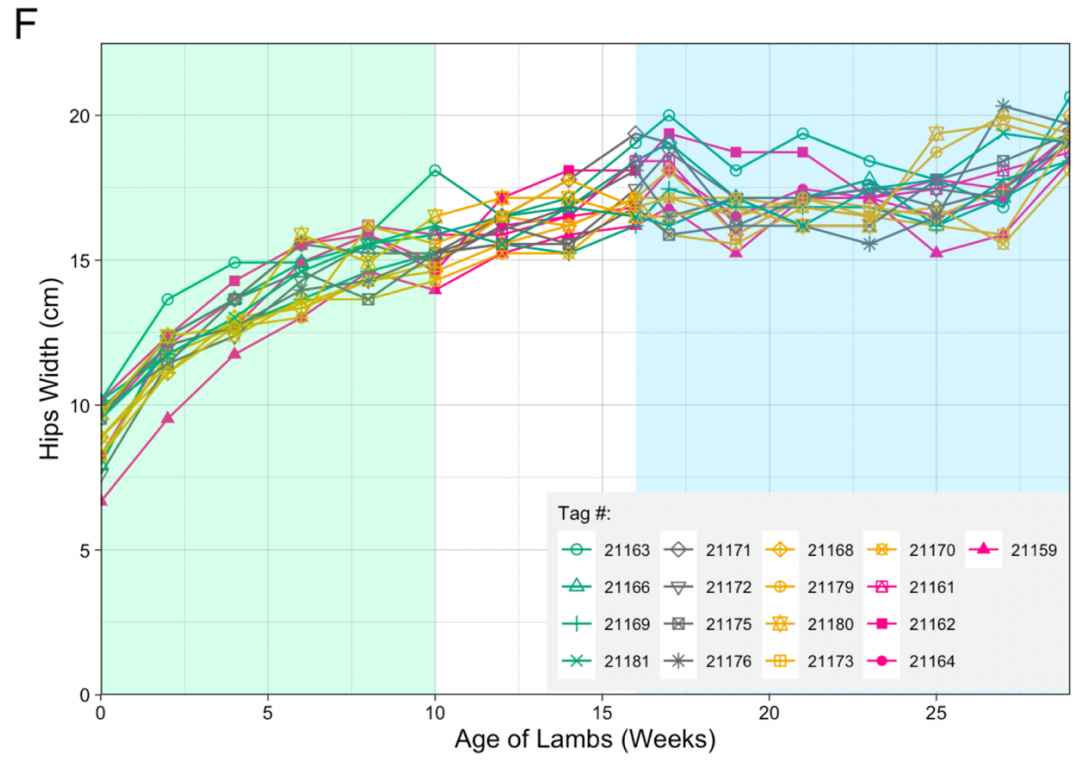
D


Elbow Height (cm)
Age of Lambs (Weeks)
Tag #:
21163
21171
21168
21170
21159
21166
21172
21179
21161
21169
21175
21180
21162
21181
21176
21173
21164

E


Chest Width (cm)
Age of Lambs (Weeks)
Tag #:
21163 21171 21168 21170 21159
21166 21172 21179 21161
21169 21175 21180 21162
21181 21176 21173 21164

F


Hips Width (cm)
Age of Lambs (Weeks)
Tag #:
21163 21171 21168 21170 21159
21166 21172 21179 21161
21169 21175 21180 21162
21181 21176 21173 21164

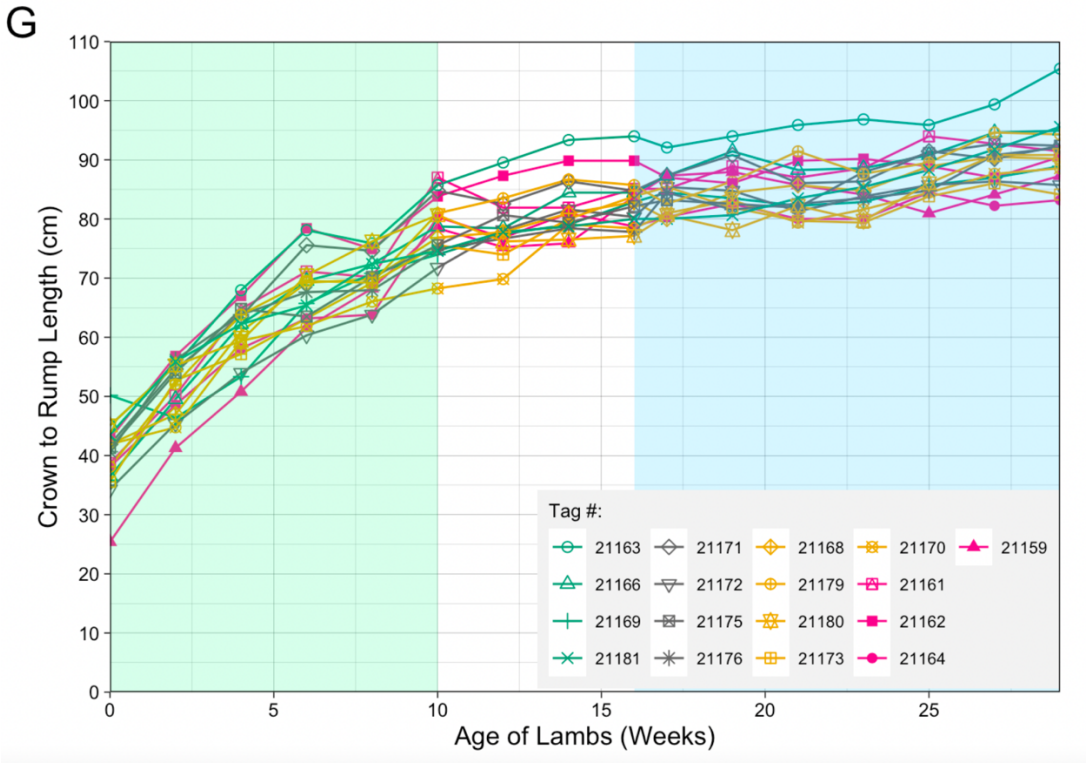


Figure 3.6A-G: Individual lamb measurements from birth to 30 weeks of age for weight and birth to 29 weeks of age for size. The green shaded region indicates the pre-weaning period from 0 to 10 weeks of age and the blue shaded region indicates the post-weaning period from 16 to 30 weeks of age. Heterozygous males, control males, heterozygous females, and control females are indicated by green, gray, yellow, and pink data points respectively. Charts are shown for weight (A), withers height (B), sacrum height (C), elbow height (D), chest width (E), hip width (F), and crown to rump length (G).

Table 3.12: Weekly and total individual feed efficiency data. "Het" indicates a heterozygous Socs2 KO genotype and "Con" indicates a control/wild type genotype. Finalized weekly feed intake data for sheep during the two week introductory period and 10 week feed efficiency starting at midnight on September 16th, 2021 to midnight on November 24th, 2021 and final measurements performed on November 25th, 2021. A disruption in feed data collection over seven days in weeks 17 and 18 was averaged over the remaining days from those two weeks. Individual visit data and outliers are available upon request. Weekly feed intake and average daily feed intake (ADFI) (A); weekly dry matter intake (DMI) and average daily DMI (B); post-weaning weight, average daily gains (ADG) and gain to DMI ratio (G:DMI) (C); Metabolic weights with metabolic mid weight (MetBW) bolded (D); Components used for calculating residual feed intake (RFI) (E).

A

		Weekly Feed Intake-Outliers (kgs)												
		Weeks Post-weaning												
Group	Tag#	7	8	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**ADFI**
Het Ram	21163	12.23	13.17	10.21	14.74	11.21	14.62	13.86	14.15	15.38	14.91	11.67	11.67	**1.89**
Het Ram	21166	19.66	15.74	16.77	15.31	11.99	14.33	16.86	18.29	22.85	24.60	16.65	16.65	**2.49**
Het Ram	21169	17.36	17.96	14.60	17.53	13.31	15.25	18.44	20.63	19.77	21.95	19.62	19.62	**2.58**
Het Ram	21181	12.86	21.97	15.27	17.51	15.17	23.19	17.91	21.01	22.25	19.36	20.62	20.62	**2.76**
Con Ram	21172	13.72	14.04	9.69	16.12	20.41	17.15	17.50	15.73	16.41	19.21	19.18	19.18	**2.44**
Con Ram	21171	15.59	10.13	9.86	16.96	15.83	16.73	19.94	20.01	20.55	23.96	18.41	18.41	**2.58**
Con Ram	21175	16.23	15.88	13.62	17.29	16.44	18.07	20.51	19.58	20.41	18.12	16.31	16.31	**2.52**
Con Ram	21176	16.38	10.56	9.42	15.32	11.91	16.32	18.07	21.64	20.60	23.18	16.92	16.92	**2.43**
Het Ewe	21173	11.88	13.91	11.99	12.13	9.16	15.29	13.46	13.62	14.93	18.18	12.78	12.78	**1.92**
Het Ewe	21168	11.53	19.49	13.31	14.66	9.10	14.75	15.84	17.14	18.23	18.81	15.32	15.32	**2.18**
Het Ewe	21170	10.08	17.02	15.07	14.45	9.49	13.55	16.85	16.70	18.14	18.35	16.91	16.91	**2.23**
Het Ewe	21179	11.39	17.14	15.19	14.38	13.41	18.44	18.50	17.29	19.37	20.00	16.25	16.25	**2.42**
Het Ewe	21180	15.14	20.04	13.68	14.51	17.02	18.86	16.68	19.42	19.24	23.87	17.48	17.48	**2.55**
Con Ewe	21159	10.01	15.37	11.66	11.31	7.72	15.66	13.66	11.63	15.16	14.30	13.93	13.93	**1.84**
Con Ewe	21161	15.26	18.43	15.90	15.12	12.48	21.28	18.15	21.26	19.13	17.67	18.45	18.45	**2.54**
Con Ewe	21162	2.20	14.51	9.38	4.47	6.66	14.31	15.02	13.84	9.72	18.17	17.13	17.13	**1.80**
Con Ewe	21164	9.41	13.98	11.49	12.76	9.24	12.15	11.76	15.89	16.19	17.21	13.82	13.82	**1.92**

B.

		Weekly DMI (kgs)												
		Weeks Post-weaning												
Group	Tag#	7	8	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**ADDMI**
Het Ram	21163	10.59	11.40	8.84	12.76	9.71	12.66	12.00	12.25	13.31	12.91	10.11	10.11	**1.64**
Het Ram	21166	17.02	13.63	14.52	13.26	10.38	12.41	14.60	15.84	19.78	21.30	14.41	14.41	**2.16**
Het Ram	21169	15.04	15.55	12.64	15.18	11.53	13.21	15.97	17.87	17.12	19.01	16.99	16.99	**2.24**
Het Ram	21181	11.13	19.02	13.22	15.16	13.14	20.08	15.51	18.19	19.27	16.76	17.86	17.86	**2.39**
Con Ram	21172	11.88	12.16	8.39	13.96	17.67	14.85	15.15	13.62	14.21	16.64	16.61	16.61	**2.11**
Con Ram	21171	13.50	8.77	8.54	14.69	13.71	14.49	17.27	17.33	17.80	20.75	15.94	15.94	**2.24**
Con Ram	21175	14.05	13.75	11.79	14.97	14.23	15.65	17.76	16.95	17.67	15.69	14.12	14.12	**2.19**
Con Ram	21176	14.18	9.14	8.16	13.26	10.31	14.13	15.64	18.74	17.84	20.07	14.65	14.65	**2.11**
Het Ewe	21173	10.29	12.04	10.38	10.50	7.93	13.24	11.65	11.79	12.93	15.74	11.06	11.06	**1.66**
Het Ewe	21168	9.98	16.88	11.52	12.69	7.88	12.77	13.72	14.84	15.79	16.28	13.27	13.27	**1.89**
Het Ewe	21170	8.73	14.74	13.05	12.51	8.22	11.74	14.59	14.46	15.71	15.89	14.64	14.64	**1.93**
Het Ewe	21179	9.86	14.84	13.15	12.45	11.61	15.97	16.02	14.97	16.77	17.32	14.07	14.07	**2.09**
Het Ewe	21180	13.11	17.35	11.84	12.56	14.74	16.33	14.44	16.81	16.66	20.67	15.14	15.14	**2.20**
Con Ewe	21159	8.66	13.31	10.10	9.80	6.69	13.56	11.83	10.07	13.13	12.38	12.06	12.06	**1.60**

Con Ewe	21161	13.21	15.96	13.77	13.09	10.81	18.42	15.72	18.40	16.57	15.30	15.97	15.97	**2.20**
Con Ewe	21162	1.90	12.57	8.12	3.87	5.77	12.39	13.01	11.99	8.41	15.73	14.83	14.83	**1.56**
Con Ewe	21164	8.15	12.11	9.95	11.05	8.00	10.52	10.18	13.76	14.02	14.90	11.97	11.97	**1.66**

C

		Weight (kg)														
		Weeks Post-weaning														
Group	Tag#	7	8	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**ADG (kg)**	**G:DMI**
Het Ram	21163	66.45	69.17	70.99	73.94	77.11	76.43	82.33	82.55	81.42	82.55	82.78	85.28	90.26	**0.2754**	**0.1681**
Het Ram	21166	55.11	58.97	59.65	62.14	64.86	65.54	71.67	71.67	70.08	73.94	76.20	77.79	82.10	**0.3208**	**0.1488**
Het Ram	21169	42.86	46.27	47.40	49.90	51.94	52.62	57.15	57.38	56.47	59.65	60.33	61.69	66.22	**0.2689**	**0.1203**
Het Ram	21181	35.38	38.78	41.50	42.86	46.49	46.72	52.84	52.62	52.84	55.11	58.29	57.38	62.82	**0.3046**	**0.1276**
Con Ram	21172	46.72	52.16	52.39	53.52	55.34	55.11	64.18	64.41	61.69	64.64	66.45	62.37	70.76	**0.2624**	**0.1244**
Con Ram	21171	52.16	55.11	52.62	56.70	60.55	61.92	64.86	65.54	64.18	69.40	70.31	70.31	78.47	**0.3694**	**0.1653**
Con Ram	21175	41.50	45.36	47.63	50.12	53.07	53.52	58.97	59.42	59.19	62.37	63.05	63.28	66.22	**0.2657**	**0.1216**
Con Ram	21176	39.24	43.09	46.04	48.99	51.48	52.16	57.61	61.23	59.19	60.33	63.96	61.69	69.17	**0.3305**	**0.1569**
Het Ewe	21173	38.33	40.37	41.73	43.32	45.13	43.77	46.95	47.85	45.36	47.17	47.17	46.27	49.90	**0.1166**	**0.0702**
Het Ewe	21168	43.54	45.59	47.40	49.90	51.03	53.52	57.15	54.43	53.30	53.98	54.43	53.75	60.33	**0.1847**	**0.0979**
Het Ewe	21170	37.19	39.01	41.28	42.18	44.00	43.54	47.40	47.17	46.95	48.53	46.49	48.08	54.43	**0.1879**	**0.0971**
Het Ewe	21179	43.09	44.91	45.81	50.35	52.39	53.75	57.15	59.87	59.87	61.46	59.19	60.78	66.90	**0.3013**	**0.1441**
Het Ewe	21180	39.24	41.96	43.32	46.04	47.17	47.17	51.94	51.71	50.80	52.16	52.16	53.30	58.97	**0.2236**	**0.1014**
Con Ewe	21159	38.56	41.50	41.96	45.36	46.04	44.91	48.53	48.31	47.40	48.08	47.40	49.67	53.98	**0.1717**	**0.1076**
Con Ewe	21161	51.26	54.20	54.88	57.38	60.33	60.33	64.18	64.64	63.50	67.13	64.41	66.45	73.48	**0.2657**	**0.1207**
Con Ewe	21162	49.44	52.16	54.66	54.20	52.16	51.71	55.79	54.43	55.34	54.43	55.79	58.51	64.64	**0.1426**	**0.0916**
Con Ewe	21164	42.18	44.00	43.54	46.72	47.85	46.95	52.16	50.12	51.03	52.39	51.26	53.30	58.06	**0.2074**	**0.1248**

D

		Metabolic Weight (kg)												
		Weeks Post-weaning												
Group	Tag#	7	8	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
Het Ram	21163	23.27	23.99	24.46	25.21	26.02	25.85	27.33	**27.39**	27.10	27.39	27.44	28.06	29.28
Het Ram	21166	20.23	21.28	21.46	22.13	22.86	23.04	24.63	**24.63**	24.22	25.21	25.79	26.19	27.27
Het Ram	21169	16.75	17.74	18.06	18.77	19.35	19.54	20.79	**20.85**	20.60	21.46	21.65	22.01	23.21
Het Ram	21181	14.51	15.54	16.35	16.75	17.80	17.87	19.60	**19.54**	19.60	20.23	21.09	20.85	22.31
Con Ram	21172	17.87	19.41	19.47	19.79	20.29	20.23	22.68	**22.74**	22.01	22.80	23.27	22.19	24.40
Con Ram	21171	19.41	20.23	19.54	20.66	21.71	22.07	22.86	**23.04**	22.68	24.04	24.28	24.28	26.37
Con Ram	21175	16.35	17.48	18.13	18.84	19.66	19.79	21.28	**21.40**	21.34	22.19	22.37	22.44	23.21
Con Ram	21176	15.68	16.82	17.67	18.52	19.22	19.41	20.91	**21.89**	21.34	21.65	22.62	22.01	23.99
Het Ewe	21173	15.40	16.02	16.42	16.89	17.41	17.02	17.94	**18.19**	17.48	18.00	18.00	17.74	18.77

Het Ewe	21168	16.95	17.54	18.06	18.77	19.09	19.79	20.79	**20.04**	19.73	19.91	20.04	19.85	21.65
Het Ewe	21170	15.06	15.61	16.28	16.55	17.08	16.95	18.06	**18.00**	17.94	18.39	17.80	18.26	20.04
Het Ewe	21179	16.82	17.35	17.61	18.90	19.47	19.85	20.79	**21.52**	21.52	21.95	21.34	21.77	23.39
Het Ewe	21180	15.68	16.49	16.89	17.67	18.00	18.00	19.35	**19.28**	19.03	19.41	19.41	19.73	21.28
Con Ewe	21159	15.47	16.35	16.49	17.48	17.67	17.35	18.39	**18.32**	18.06	18.26	18.06	18.71	19.91
Con Ewe	21161	19.16	19.98	20.16	20.85	21.65	21.65	22.68	**22.80**	22.50	23.45	22.74	23.27	25.10
Con Ewe	21162	18.65	19.41	20.10	19.98	19.41	19.28	20.41	**20.04**	20.29	20.04	20.41	21.16	22.80
Con Ewe	21164	16.55	17.08	16.95	17.87	18.19	17.94	19.41	**18.84**	19.09	19.47	19.16	19.73	21.03

E

		Components of RFI				
Group	Tag#	ADDMI	MetBW	ADG	Predicted DMI	**RFI**
Het Ram	21163	1.64	19.54	0.30	2.02	**-0.38**
Het Ram	21166	2.16	23.04	0.37	1.90	**0.26**
Het Ram	21169	2.24	21.89	0.33	1.94	**0.29**
Het Ram	21181	2.39	18.84	0.21	2.07	**0.32**
Con Ram	21168	1.89	21.40	0.27	1.98	**-0.09**
Con Ram	21170	1.93	18.19	0.12	2.11	**-0.18**
Con Ram	21173	1.66	21.52	0.30	1.96	**-0.30**
Con Ram	21179	2.09	22.80	0.27	1.94	**0.15**
Het Ewe	21180	2.20	20.04	0.14	2.05	**0.15**
Het Ewe	21171	2.24	20.04	0.18	2.04	**0.20**
Het Ewe	21172	2.11	18.00	0.19	2.10	**0.02**
Het Ewe	21175	2.19	19.28	0.22	2.05	**0.14**
Het Ewe	21176	2.11	18.32	0.17	2.09	**0.02**
Con Ewe	21159	1.60	27.39	0.28	1.81	**-0.21**
Con Ewe	21161	2.20	24.63	0.32	1.87	**0.33**
Con Ewe	21162	1.56	20.85	0.27	1.99	**-0.43**
Con Ewe	21164	1.66	22.74	0.26	1.94	**-0.28**

Table 3.13: Individual weekly feed intake amounts filtered from totals for outlying amounts and high feed intake rates (kilograms). "211" at the start of each tag # is removed.

	9/2-9/8	9/9-9/15	9/16-9/22	9/23-9/29	9/30-10/6	10/7-10/13	10/14-10/20	10/21-10/27	10/28-11/3	11/4-11/10	11/11,11/12, 11/20-11/24		
Tag#	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17-18 Average**		**Trial Period Total**
59	0.00	0.19	1.15	0.04	0.16	0.00	0.10	0.41	0.26	0.69	1.06	1.06	4.93
61	0.00	0.00	0.00	0.05	4.06	2.05	0.50	1.43	1.59	2.98	0.00	0.00	12.67
62	0.23	0.20	1.54	0.00	0.23	0.44	0.57	0.00	0.06	0.34	0.16	0.16	3.51
63	0.21	0.10	0.52	1.62	0.24	0.11	0.34	0.57	0.31	6.27	0.22	0.22	10.42
64	0.05	0.00	0.99	0.00	0.22	0.00	3.56	0.00	0.67	0.17	1.71	1.71	9.04
66	0.21	2.39	5.86	2.86	3.40	1.50	0.17	0.67	3.99	3.71	0.56	0.56	23.27
68	0.00	0.28	1.84	1.61	0.00	0.22	2.95	1.17	0.00	0.47	0.00	0.00	8.26
69	0.50	0.16	0.34	2.47	7.25	0.11	0.51	0.35	0.28	1.53	0.07	0.07	12.97
70	1.79	0.50	0.00	0.00	0.63	2.01	0.72	0.37	0.00	0.08	6.37	6.37	16.55
71	0.17	0.00	2.91	0.29	0.37	0.65	1.13	0.11	0.12	3.07	6.66	6.66	21.96
72	0.00	0.10	0.15	0.00	0.14	0.50	0.00	1.49	0.05	1.53	0.11	0.11	4.09
73	0.08	0.12	0.97	0.23	0.00	0.23	0.00	0.08	0.21	4.84	2.31	2.31	11.16
75	1.11	1.86	1.06	6.05	1.63	0.43	0.64	0.04	0.00	0.13	0.29	0.29	10.56
76	0.00	1.67	0.80	1.49	0.39	3.96	6.69	2.01	2.06	1.15	4.76	4.76	28.09
79	0.00	0.05	0.00	0.00	0.00	0.00	5.39	3.77	0.27	0.00	0.07	0.07	9.58
80	0.00	2.15	0.14	0.33	0.00	6.82	0.00	0.72	0.00	0.00	2.79	2.79	13.60
81	0.00	0.00	0.00	0.29	0.00	3.05	0.17	0.15	0.26	3.44	0.59	0.59	8.54

Table 3.14: Individual lamb birth measurements.

Lamb Tag#	Group	Litter Size	Weaned Litter Size	Weight (kg)	Withers Height (cm)	Sacrum Height (cm)	Elbow Height (cm)	Chest Width (cm)	Hip Width (cm)	Crown to Rump Length (cm)
21159	Control Ewe	1	1	0.23	18.733	18.415	15.24	5.715	6.6675	25.4
21161	Control Ewe	1	1	4.31	38.735	38.735	25.083	9.525	9.525	38.735
21162	Control Ewe	1	1	3.85	39.053	38.735	26.67	10.795	10.16	42.8625
21163	Heterozygous Ram	1	1	5.22	35.878	35.56	28.575	10.795	10.16	45.085
21164	Control Ewe	2	2	2.95	28.893	29.528	17.145	8.89	8.255	38.1
21172	Control Ram	2	2	2.72	31.433	31.115	21.59	7.62	7.62	34.29
21166	Heterozygous Ram	2	2	3.40	39.053	39.37	26.353	9.525	7.9375	36.5125
21173	Heterozygous Ewe	2	2	2.72	27.623	27.623	24.448	8.89	8.255	35.56
21169	Heterozygous Ram	3	3	4.99	33.655	33.655	26.67	10.795	10.16	50.165
21168	Heterozygous Ewe	3	3	2.72	32.385	30.48	25.4	9.2075	8.89	41.91
21170	Heterozygous Ewe	3	3	4.31	31.75	31.75	26.353	8.89	8.89	41.91
21171	Control Ram	1	1	3.85	34.608	32.385	25.083	9.525	9.525	41.91
21175	Control Ram	2	2	4.54	39.37	38.1	26.035	10.795	9.8425	41.275
21176	Control Ram	2	2	4.76	39.688	38.1	24.765	10.16	9.8425	40.64
21179	Heterozygous Ewe	2	1	2.95	34.925	33.655	22.225	8.255	8.255	38.1
21180	Heterozygous Ewe	2	2	4.08	40.005	40.005	29.845	10.795	9.7155	45.085
21181	Heterozygous Ram	2	2	4.76	40.64	39.37	28.893	11.113	9.525	43.4975
21174	Heterozygous Ram	1	0	5.22	39.053	38.735	27.94	9.525	9.8425	40.64
21177	Heterozygous Ewe	1	0	2.04	34.608	34.608	22.86	8.89	8.5725	33.655
21178	Heterozygous Ewe	2	1	1.59	37.148	28.575	20.955	7.62	7.3025	30.48

Table 3.15: Component analysis of lamb foreshank. Wilcoxon rank-sum test with continuity correction P values.

		Heterozygous		Control		
Component	**Sex**	**Mean**	**SE**	**Mean**	**SE**	**p-value**
Moisture	Ram	73.8	1.2	75.9	0.5	0.1143
	Ewe	74.4	0.7	74.4	0.7	0.8057
Protein	Ram	20.4	0.6	20.6	0.2	1
	Ewe	20.7	0.4	21.0	0.5	0.3252
Fat	Ram	3.1	0.6	1.9	0.4	0.2
	Ewe	3.2	0.3	3.7	0.9	0.9048
Ash	Ram	0.7	0.2	0.7	0.1	0.8687
	Ewe	0.7	0.1	0.5	0.2	0.8016
Carbohydrates	Ram	2.1	1.2	1.0	0.4	0.6857
	Ewe	1.1	0.3	0.4	0.1	0.1383
Calories	Ram	117.5	7.2	103.0	3.7	0.2
	Ewe	115.4	4.0	118.8	7.4	0.8057

Table 3.16: Pregnancy check ultrasound results

Knockout Ram 7182			
Tag #	**Bred/Open**	**Days Bred**	**Fetus Count**
7WF	Bred	70	2+
7041 (Red Tag)	Bred	28	Small Fluid
7091	Bred	60	1+
7090	Bred	70	2+
Blank Yellow	Bred	80	2+
7181 (Red Tag)	Bred	Small Fluid	Re Check
Knockout Ram 7183			
Tag #	**Bred/Open**	**Days Bred**	**Fetus Count**
8023	Bred	70	1+
8161	Bred	88	1+
7041 Yellow Tag	Bred	80	2+
3yrNT	Bred	85	1+
8153	Bred	70	1+
9199	Bred	75	1+
Control Ram 7068			
Tag #	**Bred/Open**	**Days Bred**	**Fetus Count**
8178	Bred	80	1+
6091	Bred	75	1+
8185	Bred	80	2 seen
4062	Bred	75	Re Check Low Fluid
8195	Bred	65	1 Seen
6096	Bred	88	1+

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