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Genetic investigation of the *AMH* and *AMHR2* genes in canine Persistent Müllerian Duct Syndrome

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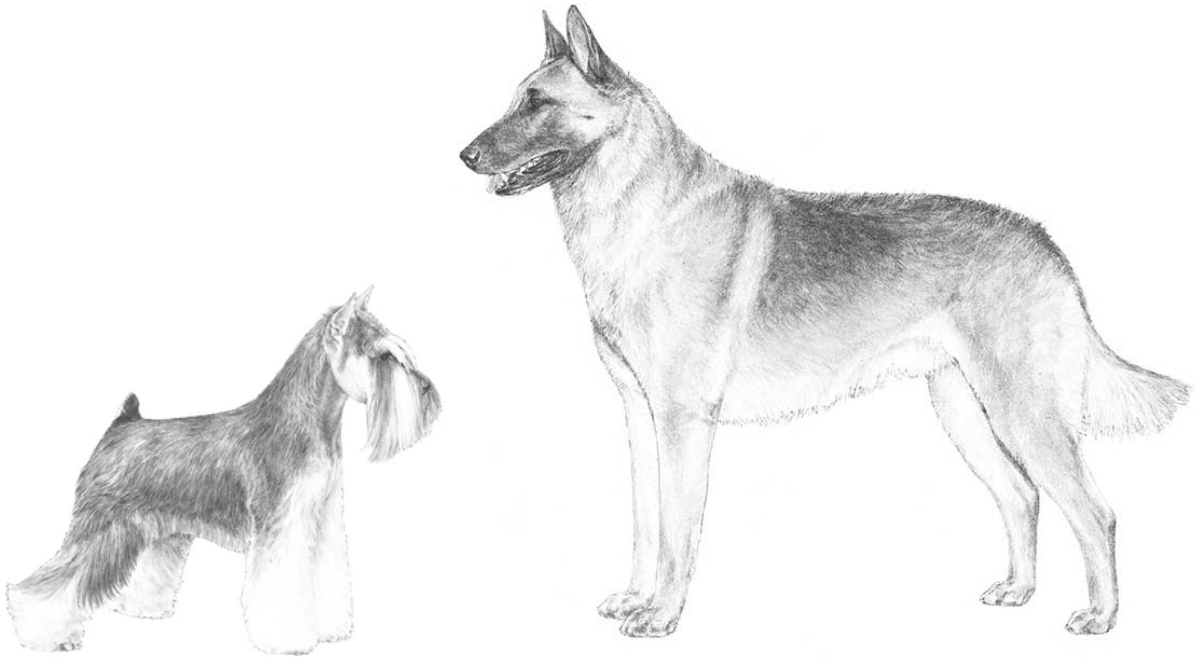

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Abstract

Persistent Müllerian duct syndrome (PMDS) is a sex-limited disorder in which male dogs develop portions of the female reproductive tract. Cryptorchidism and its sequelae of infertility and increased risk for testicular cancer are important consequences of PMDS. Anti-Müllerian hormone (AMH) and its receptor induce the regression of the Müllerian ducts in male embryos. The genetic basis for PMDS in Miniature Schnauzers (MS) is an autosomal recessive nonsense mutation in the AMH receptor gene, *AMHR2*. The objectives of this study were to determine the prevalence of the *AMHR2* mutation in MS and whether it was responsible for PMDS in a Belgian Malinois. Such information is crucial to determining the value of genetic testing in MS and would aid development of a genetic test for the Belgian Malinois breed. Allele-specific primers were designed to genotype dogs for the *AMHR2* mutation. Genomic DNA of 216 MS (containing one known male PMDS case) and 1 Belgian Malinois (a known male PMDS case) was tested. The MS cohort had a PMDS mutation allele frequency of 16% and a carrier genotypic frequency of 27%. Besides the known affected male MS dog, 1 male and 2 female MS dogs were homozygous for the mutation. These findings support a benefit to testing MS used for breeding for the AMHR2 mutation. The genetic basis for PMDS in the Belgian Malinois was not determined; no coding or splicing mutations were identified in *AMH* or *AMHR2*.

1. Introduction

Persistent Müllerian Duct Syndrome (PMDS) is a reproductive disorder in which male dogs develop parts of the female reproductive tract. In 1976, Brown, et al. described three cases of male pseudo-hermaphroditism and cryptorchidism in Miniature Schnauzers (Brown, Burek, & McEntee, 1976). This was the first report of PMDS in dogs. Over the next 40 years, multiple other case reports have been published describing clinical consequences of the disease. Affected males can have oviducts, a uterus, uterine body, cervix, and even a cranial vagina that enters into the prostate. (Kuiper, Wagner, Drögemüller, & Distl, 2004; Matsuu et al., 2009; Nickel, Ubbink, van der Gaag, & van Sluijs, 1992; Vegter, 2010) An important consequence of this disease is bilateral or unilateral cryptorchidism, which often occurs in PMDS-affected dogs, and causes infertility as well as increased risk for testicular tumors. (Meyers-Wallen, Donahoe, Ueno, Manganaro, & Patterson, 1989) PMDS as the underlying cause of cryptorchidism is often missed because external genitalia of affected dogs frequently look normal.

Anti-Müllerian hormone (AMH) and its receptor are involved in the regression of the Müllerian ducts in male embryos and are therefore essential in mammalian sex determination. During embryonic development, there is a sexually indifferent stage where both males and females have Müllerian ducts, the precursors of the female reproductive tract. Once testis differentiation occurs in males, there is a critical window where anti-Müllerian hormone is secreted by the testes, binds to the anti-Müllerian hormone type II receptor (coded by the gene *AMHR2*), and triggers a signaling pathway that results in regression of the Müllerian ducts. (Banco, Veronesi, Giudice, Rota, & Grieco, 2012; Meyers-Wallen et al., 1991; Pretzer, 2008) The genetic basis for PMDS in Miniature Schnauzers is a sex-limited autosomal recessive C to T transition (c.241C>T; p.R81*) nonsense mutation in the third exon of the *AMHR2* gene

(Wu et al., 2009). This mutation results in mRNA that, if translated, would result in a truncated protein; more likely, the mRNA undergoes nonsense mediated decay. As a result, *AMHR2* is not expressed, and the Müllerian ducts fail to regress in affected males. Females with the mutation are normal. Though a diagnostic test for this mutation has been available in the United States since 2009 (Pujar & Meyers-Wallen, 2009) there are no reports available that detail the prevalence of the mutation in the Miniature Schnauzer breed or in other dog breeds affected by PMDS. In addition, it is unknown if other breeds with PMDS cases have the same mutation as the Miniature Schnauzer or a different one. Therefore, a known Belgian Malinois PMDS case was also included in this study (Lim et al., 2015). Information about prevalence and occurrence of PMDS-associated mutations in other dog breeds is crucial to determining the value of genetic testing and would aid development of new breed-specific genetic tests. Accordingly, these genetic tests could guide future breeding decisions to decrease PMDS in affected dog breeds.

The aims of the present study were to 1) develop an allele-specific assay for the known Miniature Schnauzer mutation, 2) screen a large population of Miniature Schnauzers in order to establish the prevalence of this mutation in the breed, and 3) test DNA from a Belgian Malinois PMDS case for presence of the sex-determining region Y (SRY) gene and the Miniature Schnauzer *AMHR2* mutation; then, if normal, sequence coding exons of both *AMH* and *AMHR2* genes to identify a presumably new associated mutation.

2. Materials and Methods

2.1 Allele-specific mutation assay

Primers were designed to specifically target the known Miniature Schnauzer mutation site (241C>T) in exon 3 of *AMHR2*. To be allele-specific, two versions of the forward-primer were designed so that one would target only the normal allele and one the mutated allele, which was dependent on the last nucleotide of the 3'-end of the primer (shown in red text in Table 1). The same reverse primer was used for both forward primers with a product size of 153 basepairs.

Table 1: Primer sequences for allele-specific assay

Primer	Sequence
Normal allele forward primer	CCC ACC CTA TCA GGA TGC C
Mutant allele forward primer	CCC ACC CTA TCA GGA TGC T
Reverse primer	AGG CAG ATG GCT GTA ATT GG

Each dog's DNA was subjected to two PCR reactions, one with each forward primer. PCRs were carried out under standard conditions, with the following adaptations: equivalent amounts of forward and reverse primers were utilized from the allele-specific set, an additional set of internal control primers was included, which produced a differently-sized product of 390 basepairs, magnesium was added to boost amplification yield, and BSA (bovine serum albumin) was added to improve primer specificity. The PCR reaction conditions were: initial denaturation of 95 ºC for 2 minutes, followed by 35 cycles of denaturation (95 °C for 30 sec), annealing (62 °C for 30 sec), and extension (72 °C for 60 sec), with a final extension (72 ºC, 60 sec). After amplification, the products were confirmed (presence and size) by electrophoresis in a 2% agarose gel using 100-bp size standard ladder and visualized under UV light.

2.2 Allele-frequency in miniature Schnauzers

A total cohort of 216 Miniature Schnauzer (83 males and 133 females) genomic DNAs were randomly chosen from all banked Miniature Schnauzer samples available in the Canine Genetics Laboratory at the University of Minnesota. Blood samples were previously obtained at the University of Minnesota's Small Animal Hospital through patient recruitment for a different study, which was approved by the University of Minnesota's IACUC. All dog owners gave fully informed consent for participation. DNA was extracted via standard protocols. A known male PMDS case and his mother were Sanger sequenced for the previously described *AMH* mutation and served as 'case' and 'carrier' comparison PCR controls in this study. One additional Miniature Schnauzer, verified by Sanger sequencing to be clear of the disease allele, served as the 'normal' PCR comparison control. Each dog's DNA was tested separately with the normal allele forward primer and the disease allele forward primer and the reaction was verified with the internal control pair of primers. Each batch tested included DNA from the three comparison control dogs ('case', 'carrier' and 'normal') and one water control to check for contamination of the PCR mixture.

2.3 Belgian Malinois PMDS case

A confirmed PMDS case was observed in an 8 and a half year old intact male Belgian Malinois presenting at Purdue University's Small Animal Hospital for lethargy and inappetence (Lim et al., 2015). Subsequently, this dog was diagnosed with a small bowel perforation by a swallowed "popsicle" stick. Ultrasonography was performed in the course of treatment, and right-sided cryptorchidism as well as bilateral cylindrical uterine-like horns, diagnosed as uterus masculinus (persistent Müllerian duct), were observed. An exploratory laparotomy was performed, the uterus masculinus was removed, fixed in formalin and embedded in paraffin, and this tissue block was shared with the Canine Genetics laboratory at

the University of Minnesota. Genomic DNA was extracted using the Hemo-De version of the Qiagen Puregene DNA purification kit (for paraffin-embedded tissues), following manufacturer's instructions. This DNA was tested for the known Miniature Schnauzer mutation using the allele specific assay designed in the present study.

We additionally undertook sex verification to confirm that this dog was male. Its genomic DNA was tested for the presence of the sex-determining region Y (SRY-gene), an established method of sex determination. (Drobnic, 2006) The following primer pairs were used: forward 5'-GAACGCATTCTTGGTGTGGTCTC-3' and reverse 5'-GGCCATTTTTCGGCTTCTG-TAAG-3'. PCR reaction conditions were: initial denaturation of 95 ºC for 2 minutes, followed by 35 cycles of denaturation (95 °C for 30 sec), annealing (60 °C for 30 sec), and extension (72 °C for 60 sec), with a final extension (72 ºC, 60 sec). As positive and negative controls, DNA from three other male dogs and one female dog were included and tested under the same conditions. All products were confirmed and compared after electrophoresis in a 2% agarose gel, using 100-bp size standard ladder and visualization under UV light.

The Belgian Malinois DNA was subsequently sequenced for all coding exons and flanking intronic sequences of both *AMH* and *AMHR2*, reference sequences for which were obtained via Ensemble (www.ensembl.org) and compared with sequences from the University of California, Santa Cruz genome browser (https://genome.ucsc.edu). Primers were designed using Primer3 (http://bioinfo.ut.ee/primer3-0.4.0/), and any exons exceeding 300 base pairs were subdivided into more than one sequencing reaction. On review of protein alignments across multiple species (https://www.ncbi.nlm.nih.gov/homologene), it was discovered that the reference nucleotide sequence for exon 5 of *AMH* was incompletely annotated due to a gap in the canine reference genome (CanFam 3.1, chr20:56786329-56786911; the missing

nucleotides and correct annotation were determined by sequencing a control dog. The correct annotation has now been uploaded to GenBank (accession number KY769473). Since DNA quality from formalin-fixed paraffin-embedded tissues tends to be lower and because there were some GC-rich areas, amplification was performed using a 'slowdown' PCR program (Frey, Bachmann, Peters, & Siffert, 2008) with the only modification to the published protocol being that, rather than dropping the annealing temperature one degree Celsius every three cycles, the annealing temperature was dropped one third of a degree Celsius every cycle. Additionally, exon 2 of *AMHR2* and exon 5 of *AMH* required additives (dimethyl sulfoxide (DMSO) and betaine) to obtain products due to high GC content. After amplification, the products were confirmed (presence and size) by electrophoresis with 2% agarose gels, and visualized under UV light. The size of each product was estimated by comparison with a 100-bp molecular size standard ladder. Products with correct sizes were sent to the University of Minnesota Genomics Center (UMGC) for Sanger sequencing and results were viewed in the Sequencher program (v4.9, GeneCodes, Ann Arbor, MI) and compared with *Canis familiaris* reference sequence. (www.ensembl.org)

3. Results

3.1 Allele-Specific Assay

The allele specific assay designed for this study worked well as a definitive diagnostic test for the known MS mutation. Figure 1 demonstrates the assay with three known/control dogs, one for each genotype.

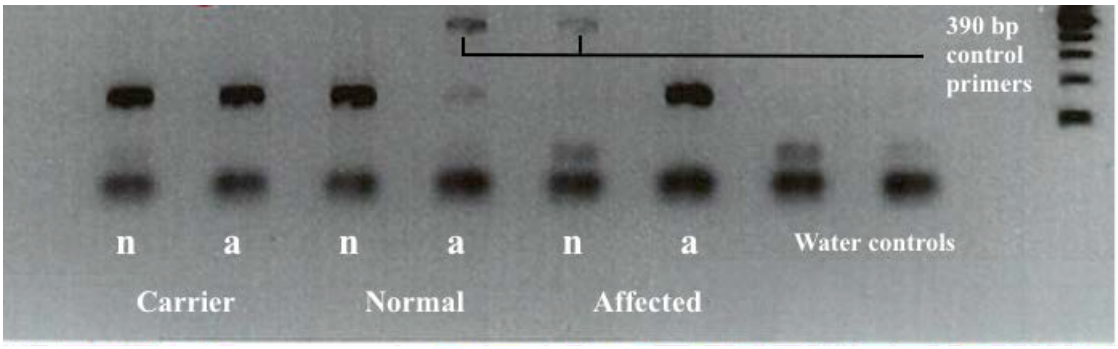

Figure 1. The *AMHR2* Allele Specific Assay. This gel image demonstrates the allele specific assay results with known genotype status dogs. Three dogs are shown, each with two columns, in which the normal and mutant *AMHR2* alleles are separately tested for their presence. The first (left) column for each dog contains primers for the normal allele (indicated with "n"), and the second (right) column for each dog contains primers for the mutant allele (indicated with "a"). A 100-bp standard ladder was used to confirm the product size of 153 basepairs for both mutant and the normal allele. From left to right, demonstrated are: a carrier dog (presence of both the normal and mutant allele, e.g., heterozygote), a normal/wild-type dog (homozygous for the normal allele), and an affected dog (homozygous for the mutant allele). Where no target allele was present, an additional primer for a 390 basepair product acted as an internal PCR control. The last two columns are water sample controls for detecting any PCR-mixture contamination.

3.2 Allele-frequency in Miniature Schnauzers

Besides the one known affected male dog, one additional male and two female dogs were found to be homozygous for the mutation (4 of 216 dogs, or 1.9%, Figure 2). The additionally identified male case was a neutered male dog with no reported history of PMDS. He had undergone abdominal imaging on multiple occasions, and reports were available for one set of

abdominal radiographs and four abdominal ultrasounds. There were no comments about a uterus masculinus or unidentified structure in the abdomen. Medical records were not available from the time of his neuter, and it is unknown whether or not he was cryptorchid. He went into cardiopulmonary arrest at 12 years of age after presenting for cluster seizures; a necropsy was not performed. In addition, 59 heterozygous dogs were observed (59 of 216 dogs, or 27.3%). We found 153 dogs to be homozygous for the normal allele (70.8%). Assuming Hardy-Weinberg equilibrium, the tested Miniature Schnauzer population had an *AMHR2* mutated allele frequency of 16%. Since PMDS is a sex-limited recessive trait, the predicted rate of affected males is low at 2.4% (2 affected males out of 83 total males). Affected males account for less than 1% of the entire tested MS population (2 out of 216 dogs).

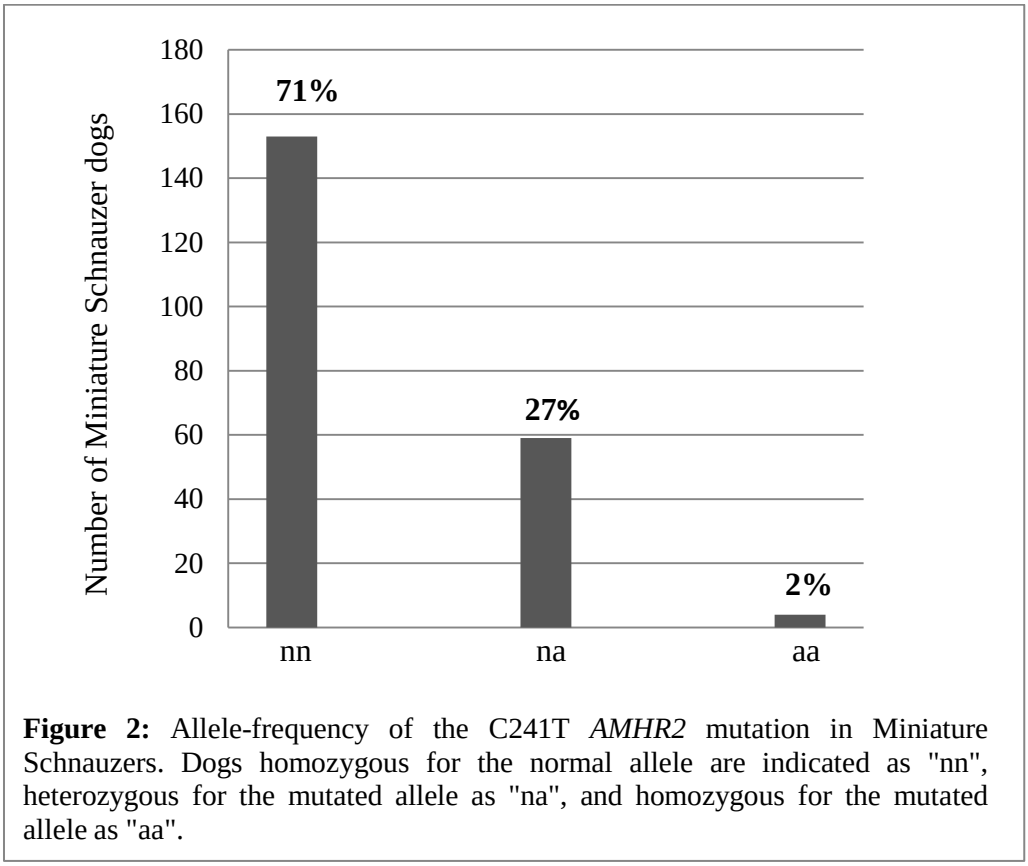

Figure 2: Allele-frequency of the C241T *AMHR2* mutation in Miniature Schnauzers. Dogs homozygous for the normal allele are indicated as "nn", heterozygous for the mutated allele as "na", and homozygous for the mutated allele as "aa".

3.3 Belgian Malinois PMDS case

The Belgian Malinois PMDS case DNA was first tested for the known Miniature Schnauzer mutation via Sanger sequencing; the allele-specific assay developed for this study was not used as this DNA was poorer quality, coming as it did from a formalin fixed paraffin-embedded block. This dog was homozygous normal for the Miniature Schnauzer mutation. The Belgian Malinois was then confirmed to have the SRY-gene, and was thus confirmed to be genetically male. Next, all exons and flanking intronic sequences of both *AMH* and *AMHR2* genes were successfully sequenced. After comparison with the reference sequence, no exonic mutations and no splicing mutations in the proximate flanking intronic sequence were found.

4. Discussion

4.1 Allele-specific mutation assay

Because PMDS may lead to infertility as well as increased risk for testicular cancer, genetic testing to diagnose young dogs would guide future breeding decisions and decrease PMDS in the Miniature Schnauzer. The first molecular diagnostic test developed was based on detecting the mutation via restriction fragment length polymorphism. (Pujar & Meyers-Wallen, 2009) Recently, another test was developed using Surveyor® nuclease, an enzyme which recognizes and cleaves the DNA fragment on mismatches due to single nucleotide polymorphisms, deletions or insertions (Kim et al., 2016). Both of these methods require PCR amplification of *AMHR2*'s third exon prior to the method of mutation identification. The method developed for this study combines both amplification of the specific mutational site on exon 3 of *AMHR2* and differentiation of the normal and mutated allele into one integrated

PCR reaction, and therefore gel electrophoresis is required only once. In addition, the combination of allele-specific primers and internal control primers ensure the accuracy of this method. The allele-specific method described in the present study serves as an accurate third technique of diagnosing PMDS in Miniature Schnauzers and could be considered as a model for developing diagnostic tests of PMDS in other dog breeds.

4.2 Allele-frequency in Miniature Schnauzers

The results of the present study indicate for the first time that the *AMHR2* mutation is common in Miniature Schnauzers, demonstrating that 2.4% of male MS are affected (homozygous for the mutated allele), that the mutated allele frequency in the population is 16%, and that the carrier/heterozygous genotype rate in the population is 27%. It is important to note that this cohort was recruited primarily from Minnesota and its surrounding states. As such it represents only a subset of the total Miniature Schnauzer population, and the true frequency in the entire breed could vary from the numbers observed. However, with 216 dogs (equating to 432 chromosomes tested), this is still expected to be an excellent representation of the breed.

Since male dogs homozygous for the mutation often have subclinical signs, and heterozygous dogs do not show any clinical signs, molecular diagnostic tools are essential for detection of both homozygous and heterozygous individuals (Lim et al., 2015). Both the prevalence of the mutated allele and the pathobiology of PMDS endorse the value of genetic testing and subsequently support the benefit of testing all Miniature Schnauzers used for breeding, those suffering from cryptorchidism, and any male dogs with imaging or gross findings suspicious for PMDS.

4.3 Belgian Malinois PMDS case

Since the Belgian Malinois PMDS case did not have the known MS *AMHR2* mutation and no coding mutations were identified in the total exonic *AMH* or *AMHR2* sequence analyzed, further research is needed. Regions that were not yet sequenced and are of interest include 5' and 3' untranslated regions, regulatory regions, and residual intronic sequences of both *AMH* and *AMHR2*. Despite the fact that the Belgian Malinois showed distinct features of PMDS and was confirmed to be male by the SRY-gene test, there is still a small chance the phenotype diagnosis could be incorrect, although this is unlikely. There are other genes which code for upstream or downstream products in the *AMH* transduction pathway, however, these are not likely candidates for the Belgian Malinois case, since the corresponding phenotypes are highly different. (Josso, Belville, di Clemente, & Picard, 2005; Matsushita, 2013) In humans, despite numerous known mutations in both genes, approximately 15% of described PMDS cases have no identified mutation explaining their phenotype, which demonstrates the complexity of identifying genetic causes for PMDS. (Nishi et al., 2012; Wongprasert, Somanunt, De Filippo, Picard, & Pitukcheewanont, 2013) Nevertheless, more research needs to be done in the Belgian Malinois and other breeds known to have PMDS cases, including the Papillion, Basset Hound, Leonberger, Doberman Pincher, and English Cocker Spaniel. (Lim et al., 2015; Nickel et al., 1992; Whyte et al., 2009)

4. Conclusions

In summary, this study introduces the allele-specific technique as an additional molecular diagnostic test for the *AMHR2* mutation in Miniature Schnauzers. We report for the first time the high frequency, at 16%, of the mutated *AMHR2* allele in the Miniature Schnauzer population using a cohort of 216 dogs. This substantial prevalence of the mutated allele endorses the value of genetic testing, and implies more MS dogs should be tested, particularly those used for breeding or those with clinical signs associated with PMDS. Finding casual variants in the Belgian Malinois and other dog breeds affected by PMDS should be the subject of further research, since this would provide the opportunity to develop more breed-specialized tests. For the Miniature Schnauzer, the Belgian Malinois, and all other breeds with PMDS cases, available genetic diagnostic testing would support better breeding decisions and thereby reduce the incidence of PMDS and its associated health problems.

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Looking back at the summer I spend in Minnesota, only warm memories fill my mind. I want to thank Kari, Katie, Eva, Mick and Ned for making me feel welcome in Minnesota and in the Canine Genetics lab. Working with them further increased my enthusiasm for genetic research and improved my laboratory techniques and English communication skills. I greatly appreciate them for teaching me, showing me around, taking me on trips and most of all for making this a great first experience in scientific research.

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Conflict of interest statement

Genetic testing for PMDS is offered through the University of Minnesota's Canine Genetics Laboratory, and proceeds from the test are used to fund ongoing research in the laboratory.

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