



## Original Article

Goats singly heterozygous for *PRNP* S146 or K222 orally inoculated with classical scrapie at birth show no disease at ages well beyond 6 yearsM.U. Cinar<sup>a,b</sup>, D.A. Schneider<sup>a,c</sup>, D.F. Waldron<sup>d</sup>, K.I. O'Rourke<sup>a,c,1</sup>, S.N. White<sup>a,c,e,\*</sup><sup>a</sup> Department of Veterinary Microbiology and Pathology, Washington State University, 3003 ADBF, WSU, Pullman, WA 99164, USA<sup>b</sup> Erciyes University, Faculty of Agriculture, Department of Animal Science, Kayseri 38039, Turkey<sup>c</sup> United States Department of Agriculture, Agricultural Research Service, Animal Disease Research Unit, 3003 ADBF, WSU, Pullman, WA 99164, USA<sup>d</sup> Texas AgriLife Research, San Angelo, TX 76901, USA<sup>e</sup> Center for Reproductive Biology, Washington State University, 3003 ADBF, WSU, Pullman, WA 99164, USA

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## ABSTRACT

Scrapie is a transmissible spongiform encephalopathy of sheep and goats, and scrapie eradication programs in many parts of the world rely on strong genetic resistance to classical scrapie in sheep. However, the utility of putative resistance alleles in goats has been a focus of research because goats can transmit scrapie to sheep and may serve as a scrapie reservoir. Prior work showed that disease-free survival time was significantly extended in orally inoculated goats singly heterozygous for prion amino acid substitutions S146 or K222, but average durations were only around 3 years post-inoculation. The aim of this study was to investigate whether extended survival would exceed 6 years, which represents the productive lifetimes of most commercial goats.

While all control homozygotes were clinically affected by an average of <2 years, none of the NS146 or QK222 goats developed clinical scrapie or had PrP<sup>Sc</sup>-positive rectal biopsies. Several NS146 and QK222 goats developed other conditions unrelated to scrapie, but tissue accumulation of PrP<sup>Sc</sup> was not detected in any of these animals. The NS146 heterozygotes have remained disease-free for an average of 2734 days (approximately 7.5 years), the longest duration of any classical scrapie challenge experiment with any genotype to date. The QK222 heterozygotes have remained disease-free for an average of 2450 days (approximately 6.7 years), the longest reported average duration for QK222 goats challenged with classical scrapie. This research is ongoing, but the current results demonstrate S146 and K222 confer strong resistance to classical scrapie in goats.

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## Introduction

Transmissible spongiform encephalopathies (TSEs) are slowly progressive, fatal neurodegenerative diseases which affect a variety of mammalian species, and include bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, variant Creutzfeldt–Jakob disease (vCJD) in humans, and classical scrapie in sheep and goats (Curcio et al., 2016). In addition to causing neurodegeneration, TSEs are characterized by formation and accumulation of protease-resistant prion protein (PrP<sup>Sc</sup>) mainly in the central nervous system (DeArmond and Prusiner,

2003). This is a post-translational process that involves refolding (conversion) of the host-encoded prion protein (PrP<sup>C</sup>) into PrP<sup>Sc</sup>. In sheep and goats with classical scrapie, accumulation of PrP<sup>Sc</sup> is usually first detected in certain peripheral tissues, especially lymphoid follicles associated with the alimentary tract, before accumulation or neurodegeneration can be detected within the brain (Aguilar-Calvo et al., 2016).

The development of scrapie in sheep is closely linked to polymorphisms in the host prion protein gene (*PRNP*). In the case of sheep bearing the R171 substitution (the so-called ARR haplotype consisting of alanine at position 136, arginine at position 154, and arginine at position 171), the resistance to classical scrapie infection is so strong that scrapie eradication programs underway in many parts of the world include selective breeding to increase the frequency of this ovine prion protein haplotype (Curcio et al., 2016). Goats are also susceptible to classical scrapie disease and the milk and placenta of infected goats may serve as sources of infection to sheep (Schneider et al., 2015; Konold et al., 2016). In

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contrast to sheep, however, the genetic basis of susceptibility to scrapie in goats is not well understood.

While no complete resistance against classical scrapie has been identified in goats, several studies have shown associations between amino acid substitutions in the caprine prion protein and the probability of scrapie under natural exposure (Vaccari et al., 2009). Two caprine *PRNP* alleles appear to confer strong resistance: S146 and K222 (Curcio et al., 2016). Many studies have shown significant associations of the S146 and K222 alleles with protection from scrapie (Acutis et al., 2006; Bouzalas et al., 2010; Fragkiadaki et al., 2011; Papasavva-Stylianou et al., 2011, 2007; Ortiz-Pelaez et al., 2015; Georgiadou et al., 2017). Further, worldwide there are only a few confirmed cases of classical scrapie in goats bearing either allele (Barillet et al., 2009; Fragkiadaki et al., 2011; Papasavva-Stylianou et al., 2011; Corbiere et al., 2013). Within the context of the large studies in which these few cases were reported, the odds ratios for these two alleles were of 20 or more for improved resistance to classical scrapie (Barillet et al., 2009; Papasavva-Stylianou et al., 2011). Thus, the data to date suggest the S146 and K222 alleles each confer strong, if not complete, resistance to classical scrapie infection.

A major limitation of epidemiologic studies such as those conducted in goats, is that the exposure to infectious sources is not controlled; thus, exposure levels and the actual duration of survival post-exposure are not known. Experimental inoculation studies address these issues. In an oral challenge study of newborn goats (White et al., 2012), we demonstrated that disease-free incubations are extended at least 300 days in goats singly heterozygous for *PRNP* S146 or 600–660 days in goats singly heterozygous for K222. At the time of this initial finding, however, the mean age of surviving S146 and K222 heterozygotes were <3 and 4 years of age, respectively. Thus, this finding does not address whether a producer could reasonably expect protection from scrapie disease to extend beyond the average productive lifetimes of commercial goats. In this update, we are now able to test the hypothesis that the average duration of disease-free survival time in goats singly heterozygous for S146 or K222 has extended beyond 6 years, an age that represents or exceeds the average productive life of commercial goats (Acutis et al., 2012; Castañeda-Bustos et al., 2014).

## Materials and methods

### Animals and genotypes

We used a total of 21 Toggenburg, Alpine-Boer, and Spanish-Boer goats in three genotype groups with defined *PRNP* haplotypes. One control genotype group and two experimental genotype groups (S146 and K222) were compared to the common control as previously described (White et al., 2012). Briefly, the control group consisted of five homozygotes for P240, encoded on the most common goat *PRNP* haplotype in the U.S. (White et al., 2008). Each experimental genotype consisted of a single copy of the common P240 haplotype paired with a single copy of the allele of interest: either S146 or K222 (White et al., 2008). The experimental unit for this study was a single goat, and the comparison of five control goats vs. eight goats with an experimental genotype yielded 97% power to detect resistance using Fisher's exact test if presence or absence is the standard. Kids were genotyped as previously described (White et al., 2012).

### Ethics statement and animal care

All animal care and use procedures were reviewed and approved by the Washington State University Institutional Animal Care and Use Committee (Protocol Nos. 1649 and 3819 with respective approval dates of 11 July 2007 and 31 July 2008). All measures were taken to ensure the well-being of the animals. Goat kids in the experiment were born in 2008–2009 and were given an oral inoculation of classical scrapie homogenate within 24 h of birth, orally administered as a frozen lozenge which was immediately followed by brief milk feeding to minimize potential losses. The oral inoculum was prepared from a single, naturally infected 3–4 year old female Nubian goat (G3684) that had clinical scrapie with disorientation, weakness, and gait changes and which was euthanased a few months after parturition. Each dose of frozen homogenate included 1 g of brain and 5 g of

placenta cotyledons. The goat kids were separated from their dams at the time of inoculation and raised in small groups at a scrapie quarantine facility. Kids were initially raised on fresh cow's milk and then transitioned using a creep feed and grass hay until weaning at approximately 6–8 months of age. Thereafter, recipients were fed a mix of grass and alfalfa hay and maintained in small groups in adjacent paddocks separated by chain-link fencing with nose-to-nose contact between sections. Groups were defined by convenience within this overall experiment, and they varied over many years and were not always defined by genotype. As a consequence, older animals may have been housed with scrapie-positive control goats in the same enclosures or in adjacent enclosures with nose-to-nose contact, which could have provided additional scrapie exposure beyond the original inoculum. Each paddock provided indoor sheltering and free-choice access to water and mineral supplements. The goats routinely received multi-valent clostridial vaccinations and treatment with anthelmintics.

Veterinary inspections were conducted monthly by a boarded large animal internist (DAS), which included group observation before handling and individual examinations to identify progressive changes in behaviour, body condition, and movement, as well as presence of a scratch response. While no particular attention was paid to genotype during health inspections, the inspector was not masked to genotype since a central database contained this information. Animals were euthanased at predetermined endpoints since the clinical signs of scrapie are not pathognomonic but are very slowly progressive and ultimately fatal. The predetermined endpoints were at clinical recognition of two signs of scrapie or with the development of other conditions not readily amenable to treatment. In addition, two goats were withdrawn from the study: one which was replaced following its unexpected death at only 373 days of age; the other after it was discovered to bear a rare genotype (R101) that had never before been observed in goats outside of Morocco (White et al., 2012) (see Appendix A: Supplementary material). Scrapie infection status was monitored ante-mortem by periodic rectal biopsy sampling. The brain and lymphoid tissues were collected post-mortem and preserved for analysis from all goats.

### Tissue sampling and scrapie immunohistochemistry (IHC)

All tissue samples were subjected to automated scrapie immunohistochemistry (IHC) using the Discovery XT biomarker platform (Ventana Medical Systems) and previously described in-house and United States Department of Agriculture (USDA) National Veterinary Services laboratory (NVSL) protocols (Madsen-Bouterse et al., 2015). The in-house protocol utilized anti-prion monoclonal antibody (mAb) F99/97.6.1 either alone or in combination with anti-prion mAb F89/160.1.5 (Schneider et al., 2015). The NVSL protocol (SOP-PPE-0046) utilized kit-supplied F99 antibody (Anti-Prion [99] Research Kit, RTU, Cat# 760-231; Ventana Medical Systems). Scrapie infection status was determined in all animals by scrapie IHC applied to antemortem biopsy samples of the rectal mucosa (González et al., 2008) and to postmortem samples of the obex hindbrain, retropharyngeal lymph nodes (RPLNs), tonsils, nictitating membrane, and the ileocecal junction and lymph nodes of animals euthanased as clinical scrapie suspects or as necessitated by development or extent of other conditions (see Appendix A: Supplementary material). Each staining run included control slides bearing both scrapie-positive and scrapie-negative thin sections of goat obex hindbrain and of goat RPLN and tonsil.

### Statistical analysis

Survival time was recorded in days post-inoculation without clinical scrapie and analyzed using the *t* test procedure in SAS 9.2 (SAS Institute). Since both genotypic comparisons with controls failed initial tests for equal variance ( $P < 0.05$ ), reported results are from two-sample *t* tests with Satterthwaite adjustments for unequal variance (Satterthwaite, 1946).

## Results

### Donor source of the inoculum

Scrapie infection in the inoculum donor goat was confirmed by standard scrapie immunohistochemistry of obex hindbrain and lymph nodes (Fig. 1, G3684). The doe was homozygous SS240 with no other amino acid substitutions and its singleton kid was heterozygous PS240, also with no other amino acid substitutions.

### Scrapie status of recipients

A summary of the current status of recipient goats are provided in Table 1. In all wildtype recipient goats, accumulation of PrP<sup>Sc</sup> in rectoanal mucosa-associated lymphoid follicles (RAMALT) was detected as early as 363 days–779 days post-inoculation. Among these, 4/5 were detected antemortem and the remaining animal

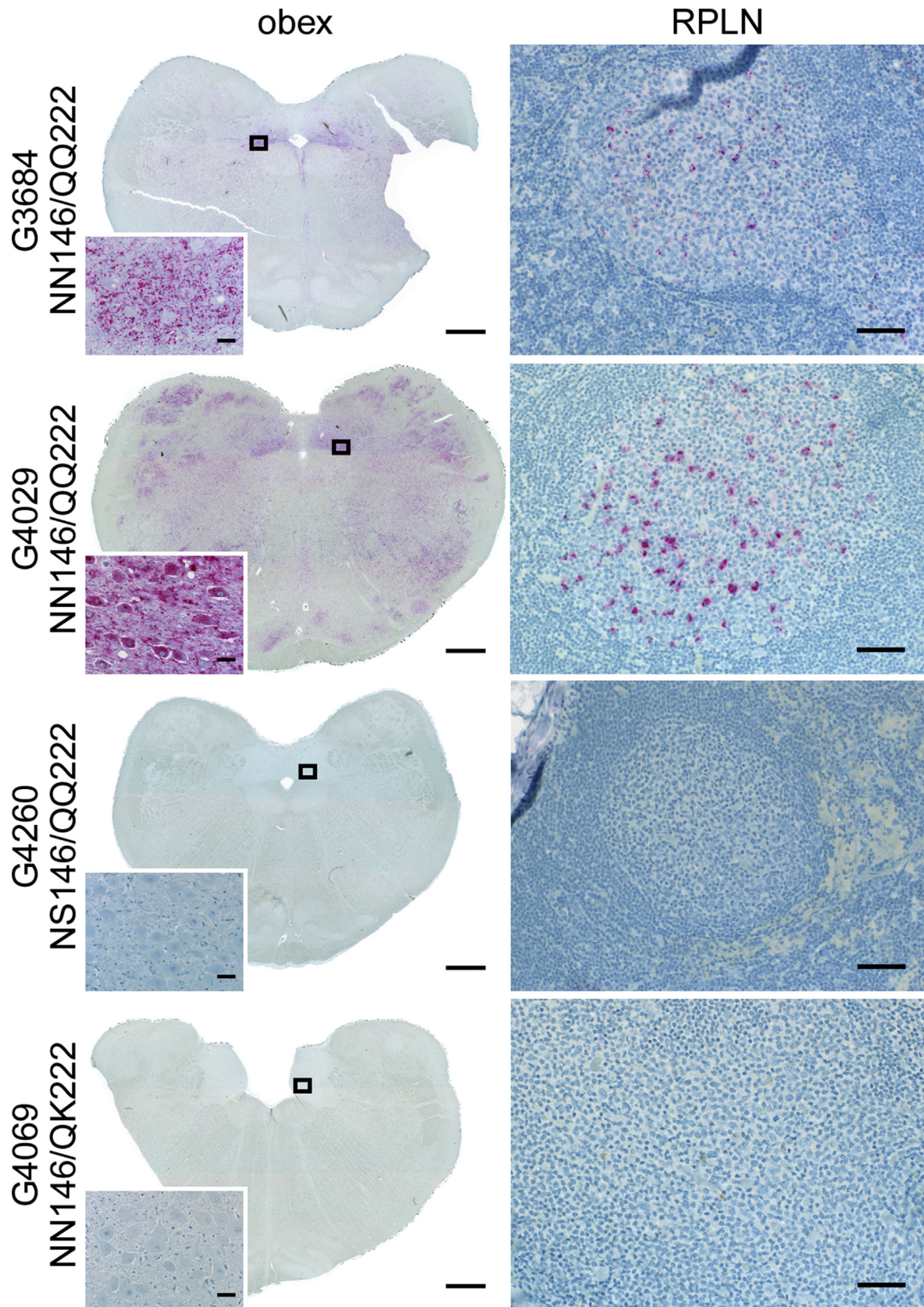


**Fig. 1.** Accumulation of PrP<sup>Sc</sup> in PRNP wildtype (NN146/QQ222) goats but not in NS146 or QK222 goats. Examples of scrapie immunochemistry performed on obex hindbrain and retropharyngeal lymph node (RPLN) from selected study goats. Note the accumulation of PrP<sup>Sc</sup> (red chromogen) in tissues of the naturally infected, 4-year-old donor goat G3684 and a clinically affected, 18-month-old recipient goat G4029; both goats were PRNP homozygous wildtype including at codons 146 (i.e., NN146) and 222 (QQ222). Accumulation of PrP<sup>Sc</sup> is not observed in recipient goats heterozygous either at codon 146 (G4260; NS146/QQ222) or at codon 222 (G4269; NN146/QK222). High magnification of the dorsal motor nucleus of the vagus nerve (DMNV) is shown as an inset of each low magnification of obex hindbrain; the approximate borders of the inset are indicated by a black box. All tissues counterstained with hematoxylin. Scale bars: obex hindbrain = 2000  $\mu$ m, inset of DMNV = 50  $\mu$ m, RPLN = 50  $\mu$ m.

had a positive postmortem sample. All wildtype recipients went on to develop clinical signs consistent with scrapie infection, were euthanased between 720 days and 1020 days post-inoculation and subsequently confirmed scrapie-positive with widespread accumulation of PrP<sup>Sc</sup> in the obex hindbrain as well as in the RPLNs and tonsils. Accumulations of PrP<sup>Sc</sup> in obex hindbrain and RPLN of the wildtype recipient with the shortest survival time are shown in Fig. 1 (G4029).

In contrast, the progressive clinical signs commonly associated with scrapie have not been observed in any recipient goats of the NS146 and QK222 genotype groups, nor have PrP<sup>Sc</sup> accumulations been detected in ante-mortem biopsy samples of the rectal mucosa. Comparison of disease-free survival time for the control group vs. the NS146 group was highly significant ( $P = 1.0 \times 10^{-5}$ ). The QK group comparison was also highly significant ( $P = 8.0 \times 10^{-4}$ ). Biopsy evaluations include those last obtained between 2643 days to 2811 days post-inoculation in NS146 recipient goats still alive ( $n = 5$ ) and at 3071 days post-inoculation in two QK222 recipient goats still alive. Furthermore, accumulation of PrP<sup>Sc</sup> has not been detected in postmortem tissue samples obtained from three NS146 and six QK222 recipient goats euthanased due to development of other conditions. These conditions included multiple cases of caseous lymphadenitis, a cementoma associated with a broken incisor tooth, chronic sinusitis, abomasal emptying disorder, an unexplained sudden death and probable sequelae associated with chronic infection with caprine arthritis encephalitis virus (lameness, pneumonitis, demodicosis). Individual outcome data is provided in Appendix A: Supplementary material. Postmortem scrapie IHC results are shown in Fig. 1 for the longest surviving recipient NS146 genotype goat (G4260) and longest surviving recipient QK222 goat (G4069).

## Discussion

In the current study, we used oral scrapie challenge of newborns to analyze disease-free duration for goats heterozygous for either S146 or K222 caprine alleles. The donor goat (G3684) had clinical scrapie, detectable PrP<sup>Sc</sup> in the obex and RPLN (Fig. 1), and had placental PrP<sup>Sc</sup> accumulation as previously described (O'Rourke et al., 2011). Donor G3684 was a homozygote SS240 and her offspring was a heterozygote PS240. The fact that all common haplotype homozygous control goats became clinically scrapie-positive by an average of approximately 720 days post-inoculation demonstrated that the North American goat scrapie isolate used as inoculum was both infectious and consistent. In contrast to the controls, none of the S146 or K222 heterozygotes have clinical scrapie or evidence for PrP<sup>Sc</sup> accumulation at mean post-inoculation times of 7.5 years for S146 heterozygotes and 6.7 years for K222 heterozygotes (Table 1; Fig. 1), which confirms and expands the definition of strong resistance to classical scrapie conferred by these alleles. In particular, not only do they confer underrepresentation among natural scrapie cases, they also confer long disease-free survival time comparable to commercial goat productive lifetimes following oral scrapie inoculation. Below, we

discuss our results for S146 and K222 in context of the literature, and then the overall implications for breeding goats.

Our data on NS146 heterozygotes confirm strong resistance to classical scrapie conferred by the S146 allele and indicate a mean disease-free survival of 2733.8 days (approximately 7.5 years), with a range of 1493–3149 days in NS146 heterozygotes (Table 1). To our knowledge, this is the longest mean extended survival time reported for any genotype in a goat classical scrapie challenge study to date. Two recent studies of natural scrapie cases in older goats provided more context for how long S146-bearing goats can remain scrapie-free (Georgiadou et al., 2017; Papasavva-Stylianou et al., 2017; Ricci et al., 2017). However, they inoculated older goats (average 125 days). Also, the survival times of the animals without clinical scrapie in our study were longer than previously reported orally challenged NS146 goats (Papasavva-Stylianou et al., 2017). In the study by Georgiadou et al. (2017), 556 goats from scrapie-positive flocks aged at least 6 years were tested for PrP<sup>Sc</sup>; none of the 103 goats with at least one copy of the S146 allele had evidence of scrapie infection, but scrapie was confirmed in 117 NN146 flock-mates (Georgiadou et al., 2017). That study used natural scrapie and the report gave little indication of the ages at which goats became scrapie-exposed. Nonetheless, those results are consistent with the long disease-free survival time observed here.

Additional sources of data for strong resistance conferred by S146 include cell-free conversion assays and an interesting case of placental PrP<sup>Sc</sup> accumulation by offspring genotype. Using a cell-free conversion assay, Eiden et al. (2011) observed that classical scrapie-seeded conversion of S146 bearing PrP substrate to PrP<sup>Sc</sup> was very inefficient and comparable to the conversion inefficiency observed for substrate bearing the sheep ARR haplotype. This finding suggests strong host resistance to classical scrapie and is consistent with the similarly small numbers of classical scrapie cases that have been observed in S146-bearing goats and sheep heterozygous for the ARR haplotype (Baylis and Goldmann, 2004). Interestingly, a study of placental PrP<sup>Sc</sup> accumulation included a scrapie-positive NN146 doe that had twins where one twin was homozygous NN146 and the other was heterozygous NS146 (O'Rourke et al., 2011). In that instance, PrP<sup>Sc</sup> accumulation was detected in the part of the placenta associated with the homozygous NN146 twin but was not detected in the part associated with the heterozygous NS146 twin. An analogous finding of disparate placenta accumulation of PrP<sup>Sc</sup> has been made in sheep bearing dizygotic twins, one ARQ/ARQ and the other ARQ/ARR (Alverson et al., 2006). Thus, several sources of data support a role of S146 providing protection from classical scrapie, and our data presented here which demonstrates such protection extends throughout and beyond the productive lifetimes of commercial goats.

Our data on QK222 heterozygotes confirm strong resistance to classical scrapie conferred by the K222 allele and indicate a mean disease-free survival time of 2450.4 days post-inoculation (approximately 6.7 years), with a range of 908–3409 days in QK222 heterozygotes (Table 1). To our knowledge, this is the longest mean extended survival time for QK222 heterozygous

**Table 1**  
Current scrapie status of goats by genotype.

| Genotype        | <i>n</i> | Biopsy positive | Clinical scrapie | Alive today | Mean survival time without clinical scrapie (days) | Age range (days)        |
|-----------------|----------|-----------------|------------------|-------------|----------------------------------------------------|-------------------------|
| Control (NN/QQ) | 5        | 4               | 5                | 0           | 719.0                                              | 607–810                 |
| NS146           | 8        | 0               | 0                | 5           | 2733.8 <sup>a</sup>                                | 1493 <sup>b</sup> –3149 |
| QK222           | 8        | 0               | 0                | 2           | 2450.4 <sup>a</sup>                                | 908 <sup>b</sup> –3409  |

<sup>a</sup> Significantly extended survival time without clinical scrapie compared to control ( $P < 0.001$ ).

<sup>b</sup> Nine animals have been euthanased for other conditions, but all remained scrapie negative by immunohistochemistry. Those animals were included in the statistical test for extended survival time.

goats in a classical scrapie challenge study to date. Other experimental challenge studies have also examined K222-bearing goats. After intracerebral challenge, Acutis et al. (2012) showed that all QQ222 goats had died of scrapie after a mean of 570 days post-inoculation but QK222 goats remained alive and disease-free up to the end of the study at 4.5 years post-inoculation. In an oral challenge experiment, Lacroux et al. (2014) observed no disease in five QK222 heterozygous goats up to one death due to another condition at 1815 days post-inoculation and up to approximately 2500 days in living animals at the time of the report; however, the mean duration of survival was not reported. Maestrale et al. (2015) showed that K222 protects goats not only against goat scrapie but also against ovine scrapie isolates. However, in their experiments goats were inoculated at 60 and 70 days of age and euthanased after showing severely impaired locomotor capacity at day 1854; years before the goats in our study. However, it is not clear whether the difference in disease-free survival time was due to the sheep scrapie source, other scrapie strain differences, or other factors. Overall, our results are consistent with these prior studies and extend those findings by demonstrating a definite, longer mean disease-free survival time and by using a North American goat scrapie isolate for challenge.

Additional data supporting the scrapie protective properties of the K222 allele come from transgenic mouse studies that formally demonstrated causation for scrapie protection in the live animal setting. An intracerebral inoculation study revealed that transgenic mice that carried the K222 allele (K<sub>222</sub>-Tg516) were resistant to all goat scrapie and also cattle BSE isolates unlike mice that carried the wild-type Q222 (Q<sub>222</sub>-Tg501) allele (Aguilar-Calvo et al., 2014). An interesting observation arose out of intracerebral challenge in the natural goat host in that 2 out of 5 QK222 and 1 out of 5 KK222 goats showed PrP<sup>Sc</sup> accumulation at advanced ages (1900 and 2174 days for QK222, and 2,101 days for KK222) which represented survival times that were 4–5 times longer than the wild-type QQ222 genotype (Lacroux et al., 2014). However, the unnatural conditions of intracerebral challenge bypass barriers present under more natural conditions of transmission, and to our knowledge there have been no reports of KK222 homozygous goats with scrapie from natural or oral challenge conditions. Furthermore, every report of scrapie susceptibility for K222-bearing goats also confirmed the overall strong resistance to classical scrapie provided by this allele.

## Conclusions

Our results confirm the strong resistance to classical scrapie provided by the S146 and K222 alleles in goats. Heterozygotes for each allele showed no scrapie disease or detectable PrP<sup>Sc</sup> accumulation over mean survival times of 7.5 years for NS146 goats and 6.7 years for QK222 goats (Table 1). In each case, the results suggest goats with these genotypes will not show clinical signs of scrapie during the productive lifetimes of most commercial goats. The S146 results have great value for breeding goats with strong resistance in breeds like Boer, and places like the U.S. where S146 is very common in many breeds (Papasavva-Stylianou et al., 2007, 2011; White et al., 2008; Goldmann et al., 2011, 2016; Ortiz-Pelaez et al., 2015; Georgiadou et al., 2017). However, some dairy breeds have higher frequencies of the K222 allele (Curcio et al., 2016), so breeding for strong resistance using the K222 allele may be more rapid and require less introgression in such circumstances. This experiment is still ongoing and many animals are still alive without any clinical signs of scrapie at the time of writing (Table 1). While the lack of clinical signs does not completely rule out accumulation of PrP<sup>Sc</sup> in the entire body, lack of clinical signs combined with lack of detection in live and deceased animals does suggest limited rates of accumulation in most key tissues. Since

there are situations in which goats can reach advanced ages (Rhône et al., 2016), future work will explore: (1) how long the survival times can become following oral scrapie challenge; (2) whether infection may have occurred despite the absence of detectable PrP<sup>Sc</sup> by using more sensitive methods; and (3) assess potential transmission from animals bearing S146 or K222.

## Conflict of interest statement

The funding source (USDA-Agricultural Research Service) had no role in study design; in the collection, analysis, and interpretation of data; in writing the report; or in the decision to submit the article for publication. None of the authors of this paper has a financial or personal relationship with other people or organisations that could inappropriately influence or bias the content of the paper.

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## Appendix A: Supplementary data

Supplementary data associated with this article can be found in the online version at <https://doi.org/10.1016/j.tvjl.2017.12.019>.

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