Temporal patterns of chronic wasting disease prion excretion in three cervid species

Ian H. Plummer, Scott D. Wright, Chad J. Johnson, Joel A. Pedersen and Michael D. Samuel

Abstract
Chronic wasting disease (CWD) is the only naturally occurring transmissible spongiform encephalopathy affecting free-ranging wildlife populations. Transmission of CWD occurs by direct contact or through contaminated environments; however, little is known about the temporal patterns of CWD prion excretion and shedding in wild cervids. We tested the urine and faeces of three species of captive cervids (elk, mule and white-tailed deer) at 6, 12, 18 and 24 months after oral inoculation to evaluate the temporal, species- and genotype-specific factors affecting the excretion of CWD prions. Although none of the animals exhibited clinical signs of CWD during the study, we determined that all three cervid species were excreting CWD prions by 6 months post-inoculation. Faecal samples were consistently positive for CWD prions for all three cervid species (88%), and were more likely to be positive than urine samples (28%). Cervids with genotypes encoding for the prion protein (PRNP) that were considered to be more susceptible to CWD were more likely to excrete CWD prions (94%) than cervids with genotypes considered to be less susceptible (64%). All cervids with CWD prions in their urine also had positive faeces (n=5), but the converse was not true. Our study is the first to demonstrate CWD prion excretion in urine by asymptomatic elk and mule deer. Our results indicate that the excretion of CWD prions in faeces and, to a lesser extent, urine may provide an important avenue for depositing prions in the environment.

INTRODUCTION
Chronic wasting disease (CWD) was first observed in captive mule deer (Odocoileus hemionus) in a Colorado research facility in 1967 [1]. Since then CWD has been found in free-ranging mule deer, white-tailed deer (O. virginianus), Rocky Mountain elk (Cervus canadensis nelsoni), moose (Alces alces) and reindeer (Rangifer tarandus) in 24 US states, two Canadian provinces, South Korea and Norway [2–6]. CWD is the only transmissible spongiform encephalopathy (TSE), a class of invariably fatal neurodegenerative mammalian diseases associated with a misfolded cellular prion protein [1, 7, 8], found in free-ranging animals. Transmissible spongiform encephalopathies have long species- and genotype-specific incubation periods, during which hosts shed infectious prions prior to the appearance of clinical disease [9]. During the asymptomatic incubation period CWD prions can be transmitted from the excreta and secretions (urine, faeces, saliva) of infected animals to susceptible hosts, either directly or via contamination of the environment [10–12].

Transmissible spongiform encephalopathies are caused by misfolded prion proteins (denoted PrP\text{TSE}; we use PrP\text{CWD} to refer to forms that cause cervid CWD) that is highly resistant to degradation [8, 13–16]. In cervids PrP\text{CWD} catalyzes conversion of the normal prion protein (PrP\text{C}) to the misfolded CWD form, which aggregates into amyloid plaques [17, 18]. PrP\text{CWD} has been detected in the saliva, urine and faeces of preclinical and clinically affected white-tailed and mule deer, but the time from initial infection to shedding has not been adequately determined [11, 19–22]. Differences in tissue infection patterns and levels of PrP\text{CWD} accumulation among cervid species suggest that species differences in excretion patterns of PrP\text{CWD} exist [23]. In addition, variability in probability of infection,...
incubation time and/or disease progression has been linked to differences in the gene encoding the prion protein (PRNP) in the host genome, suggesting variability in prion shedding patterns among genotypes, but this has not been thoroughly evaluated [24, 25].

The long-term impacts of CWD on cervid populations are not well established, but some epidemiological models suggest host extinction as one possibility [26, 27]. Other studies indicate that population impacts depend on the level of CWD prevalence, environmental contamination, disease management strategies and CWD-driven genetic selection [25, 28–31]. While reports of CWD spillover to livestock or humans are lacking, conflicting experimental results indicate the risk of cross-species transmission is not zero and warrants further investigation [32–34].

Reliable ante mortem detection of CWD infection has remained challenging. Prion concentrations are highest in lymphoid and neuronal tissues, which are difficult to sample effectively in live, free-ranging animals [35, 36]. Prions also circulate in other tissues, secretions and excreta during the pre-clinical stages of disease, but usually in concentrations that are too low to detect with mouse bioassays, which are expensive, require transgenic mice and can take up to 2 years [36]. To enhance the detection of prions, Saborio et al. [37] developed a novel prion amplification method known as protein misfolding cyclic amplification (PMCA), which is conceptually analogous to polymerase chain reaction amplification of genetic material. PMCA requires as little as 10 days to complete and can detect much lower prion concentrations than transgenic mouse bioassay [38, 39]. The development of effective methods to extract PrP\textsuperscript{CWD} from tissues and excreta has further facilitated the application of PMCA to a wide variety of sample types [20, 38, 40]. Thus, PMCA facilitates highly sensitive ante mortem prion detection in easily obtainable secretions and excretions from free-living and captive cervids, such as faeces, urine and saliva [41, 42]. In addition, previous research has shown that PrP\textsuperscript{TSE} generated by PMCA is infectious [43] and retains the strain properties of the original PrP\textsuperscript{TSE} seed [44]. Adaptation of the agent into a new species using PMCA also faithfully mimics adaptation in vivo [45]. This suite of findings reduces the need for bioassay following PMCA detection to demonstrate the presence of infectious PrP\textsuperscript{TSE}.

Determining species- and genotype-specific patterns of PrP\textsuperscript{CWD} excretion in saliva, urine and faeces helps to determine when ante mortem testing of cervid tissues/excreta is useful for determining the CWD infection status of individual deer and deer herds. Finally, accurately testing live animals for CWD infection can facilitate the early detection of infected herds and management of both wild and captive populations.

To evaluate temporal PrP\textsuperscript{CWD} excretion patterns we tested the urine and faeces from three species (elk, mule and white-tailed deer) of orally inoculated captive cervids. Because CWD prevalence is typically lower in elk than sympatric deer populations [12], we suspected elk would excrete lower amounts of PrP\textsuperscript{CWD} in urine and faeces than either deer species. Additionally, because prion loads within an infected cervid host increase with time, we predicted that prion detection in both excretions would also increase with time post-infection [24, 46, 47]. Although all cervid genotypes are susceptible to CWD infection, differences in infection rate, incubation period, disease progression, or CWD prion tissue deposition for species-specific PRNP genotypes led us to predict higher levels of excretion by animals with more susceptible genotypes compared to those with less susceptible genotypes [25]. For our study, urine and faeces from animals euthanized at 6-month intervals over 24 months post-inoculation (PI) were tested for PrP\textsuperscript{CWD} by PMCA. We evaluated whether the probability of excretion in either urine or faeces was related to time PI, cervid species, or relative susceptibility of PRNP genotypes.

RESULTS

We used PMCA with beads (PMCAb) developed by Johnson et al. [38] to test the urine and faeces of 12 elk, 10 white-tailed deer and 10 mule deer to document patterns of PrP\textsuperscript{CWD} excretion over 24 months PI. The animals used in this study were evaluated for CWD infection at necropsy using immunohistochemistry (IHC) of collected tissues. All (24/24) cervids tested by IHC at 12, 18 and 24 months PI exhibited PrP\textsuperscript{CWD} deposition in at least 1 of the 3 tissues (retropharyngeal lymph node, tonsil, brainstem) that typically contain the highest prion concentrations and are commonly used to determine infection status (Table 1) – indicating that these animals became infected with CWD. Most of these animals displayed PrP\textsuperscript{CWD} deposition in multiple tissues, and all but one elk at 12 months PI showed PrP\textsuperscript{CWD} deposition in the brainstem at the obex. At 6 months PI, 3/3 elk and 1/2 white-tailed deer, but 0/2 mule deer exhibited PrP\textsuperscript{CWD} deposition in at least 1 of these 3 tissues. However, the single white-tailed deer and two mule deer that were retropharyngeal lymph node+, tonsil- and brainstem-negative at 6 months PI displayed PrP\textsuperscript{CWD} deposition in either their cerebellum or spleen, potentially indicating early stages of CWD infection. Faeces from two of these three deer were positive for PrP\textsuperscript{CWD}, as were three of the four deer with IHC-positive retropharyngeal lymph node, tonsil, or brainstem tissues. Overall, 23/25 (92 %) of the cervids with either IHC-positive brainstem or retropharyngeal lymph nodes also had PrP\textsuperscript{CWD} positive faeces.
Of the 18 urine samples collected during the 4 sample periods, 5 were positive for PrP<sub>CWD</sub>. Only one animal (an elk at 18 months PI) had PrP<sub>CWD</sub> detected prior to the fourth round of PMCAb amplification. Urine from 1/5 elk and 1/5 mule deer tested positive, both collected at 18 months PI. Urine from 3/8 white-tailed deer tested positive, 1 in each of the 6-, 18- and 24-month samples. Most detections of PrP<sub>CWD</sub> in urine were from the 18-month PI group (3/5) compared to the 6-, 12- and 24-month PI groups, with 1/4, 0/3 and 1/6, respectively (Table 2). Logistic regression of PrP<sub>CWD</sub> excretion indicated a constant probability of excreting PrP<sub>CWD</sub> in urine for all cervid species (model 9; AICc=23.5; Table 3), and this was the best model in comparison to the models using time PI, species and genotype susceptibility. The estimated probability of infected cervids excreting PrP<sub>CWD</sub> in urine was 28\% (95\% CI: 12–52\%). Model 8 (PRNP genotype susceptibility) provided a similar fit (ΔAICc=1.9) to the data, but the coefficient for genotype was not significant (P>0.42).

Ten of the 12 elk faecal samples, 7/9 white-tailed deer and 7/8 mule deer were PrP<sub>CWD</sub> positive (Table 4). Faecal samples were consistently PrP<sub>CWD</sub> positive for all 3 cervid species beginning at 6 months PI; 5/7 at 6 months, 5/6 at 12 months, 7/7 at 18 months and 7/9 at 24 months. Most detections of PrP<sub>CWD</sub> (16/25) were made after only the third round of
Cervids were orally inoculated with PrP<sub>CWD</sub> and euthanized at 6, 12, 18 and 24 months post-inoculation. Urine samples, if present, were collected during the necropsy that immediately followed euthanasia. PrP<sub>CWD</sub> was detected using PMCAb. The numberer is the number of positive detections and the denominator is the number of animals from which urine samples were tested. NA, not available.

<table>
<thead>
<tr>
<th>Species</th>
<th>Months post-inoculation</th>
<th>Total</th>
<th>6</th>
<th>12</th>
<th>18</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elk</td>
<td>0/1</td>
<td>0/2</td>
<td>1/2</td>
<td>0/2</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>1/2</td>
<td>0/2</td>
<td>1/2</td>
<td>1/2</td>
<td>3/8</td>
<td></td>
</tr>
<tr>
<td>Mule deer</td>
<td>0/1</td>
<td>0/1</td>
<td>1/1</td>
<td>0/2</td>
<td>1/5</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>1/4</td>
<td>0/3</td>
<td>3/5</td>
<td>1/6</td>
<td>5/18</td>
<td></td>
</tr>
</tbody>
</table>

PMCAb amplification. Faecal samples were >10 times more likely to be PrP<sub>CWD</sub>-positive than urine (5/18 urine versus 24/29 faeces) across all 3 species and 4 intervals PI (two-sided Fisher’s exact OR=11.6, 95 % CI=2.6–65.4, df=1, P=0.001). Model 8 (PRNP genotype susceptibility) and model 9 (constant probability of excretion) had a similar fit to the faeces data (Table 3; ΔAICc=0.8); however, genotype was not significant (P>0.10), so we concluded that a constant probability of excreting PrP<sub>CWD</sub> (intercept only, P=0.001) was the most parsimonious representation of the data.

Our a priori hypothesis that deer with relatively susceptible PRNP genotypes were more likely to be PrP<sub>CWD</sub>-positive, along with the indication that PRNP genotype models for urine and faeces provided a similar fit to the constant models, led us to further analyse the connection between PRNP genotype and CWD prion excretion (Table 5). For the 30 animals with PrP<sub>CWD</sub> results for faeces or urine, we found that they divided almost evenly between less and more susceptible PRNP genotypes; 9/14 (64 %) less susceptible animals were positive compared to 15/16 (94 %) more susceptible animals (Table 6). A one-sided Fisher’s exact test indicated that relatively susceptible cervid PRNP genotypes were more likely to be excreting PrP<sub>CWD</sub> than less susceptible genotypes (odds ratio=7.77, 95 % CI: 0.93 ≤ OR ≤ ∞, df=1, P=0.06).

We also evaluated the likelihood of animals simultaneously excreting PrP<sub>CWD</sub> in both urine and faeces using paired urine and faecal samples from 17 animals. All cervids with positive urine also had positive faeces (n=5), but the converse was not true; nine animals had PrP<sub>CWD</sub>-positive faeces and PrP<sub>CWD</sub>-negative urine. Three cervids with a negative faecal test were also negative in urine (Table 7).

### DISCUSSION

Cervid species with subclinical CWD infections secrete or excrete PrP<sub>CWD</sub> in saliva, faeces and urine [19–22, 42, 48]. Previous studies indicated that saliva contains the highest concentration of PrP<sub>CWD</sub> followed by faeces and then urine [10, 21]. Saliva, urine, and faeces may play an important role in the environmental transmission of CWD [10–12], but the timing of PrP<sub>CWD</sub> shedding into the environment has received only limited investigation. Tangüiny et al. [22], using a transgenic mouse bioassay, found that 14/15 infected mule deer consistently excreted PrP<sub>CWD</sub> in faeces, starting at 9 months PI, long before clinical signs occurred. Using more sensitive PMCA methods, Pulford et al. [42] reported PrP<sub>CWD</sub> in the faeces of pre-clinical wild elk, with corresponding evidence of CWD infection by lymphoid tissue biopsy; however, the time PI was unknown for these animals. Although detection of PrP<sub>CWD</sub> in cervid urine has been more challenging, Haley et al. [20] reported PrP<sub>CWD</sub> in clinical-stage mule deer by both transgenic mouse bioassay and PMCA. Henderson et al. [21], using the real-time quaking-induced conversion

### Table 3. Comparison of nine alternative logistic regression models for urine and faeces shedding of CWD agent (PrP<sub>CWD</sub>) by infected elk, mule and white-tailed deer

Predictor variables included time post-inoculation (T), cervid species (S), susceptible versus resistant PRNP genotypes (G) and two-way interactions (*). Cervids were orally inoculated with PrP<sub>CWD</sub> and euthanized at 6, 12, 18 and 24 months post-inoculation. Urine and faeces, if available, were collected at time of euthanasia. PrP<sub>CWD</sub> was detected using PMCAb. The lowest P-value of the predictor variables within each model is listed.

<table>
<thead>
<tr>
<th>Model</th>
<th>Predictors</th>
<th>K‡</th>
<th>Urine AICc</th>
<th>P values for urine model coefficients</th>
<th>faeces AICc</th>
<th>P values for faeces model coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full</td>
<td>T+S+G+T<em>S+T</em>G</td>
<td>8</td>
<td>48.5</td>
<td>&gt;0.64</td>
<td>38.5</td>
<td>&gt;0.09</td>
</tr>
<tr>
<td>Model 1</td>
<td>T+S+G+T*S</td>
<td>7</td>
<td>41.7</td>
<td>&gt;0.64</td>
<td>41.0</td>
<td>&gt;0.30</td>
</tr>
<tr>
<td>Model 2</td>
<td>T+S+G+T*G</td>
<td>6</td>
<td>36.3</td>
<td>&gt;0.80</td>
<td>34.0</td>
<td>&gt;0.26</td>
</tr>
<tr>
<td>Model 3</td>
<td>T+S+G</td>
<td>5</td>
<td>31.7</td>
<td>&gt;0.80</td>
<td>35.6</td>
<td>&gt;0.09</td>
</tr>
<tr>
<td>Model 4</td>
<td>T+S</td>
<td>4</td>
<td>31.6</td>
<td>&gt;0.74</td>
<td>35.8</td>
<td>&gt;0.64</td>
</tr>
<tr>
<td>Model 5</td>
<td>T+G</td>
<td>3</td>
<td>28.3</td>
<td>&gt;0.43</td>
<td>30.4</td>
<td>&gt;0.12</td>
</tr>
<tr>
<td>Model 6</td>
<td>T</td>
<td>2</td>
<td>26.0</td>
<td>0.86</td>
<td>30.9</td>
<td>0.64</td>
</tr>
<tr>
<td>Model 7</td>
<td>S</td>
<td>3</td>
<td>28.3</td>
<td>&gt;0.51</td>
<td>33.3</td>
<td>&gt;0.75</td>
</tr>
<tr>
<td>Model 8</td>
<td>G</td>
<td>2</td>
<td>25.4</td>
<td>0.42</td>
<td>28.0</td>
<td>0.11</td>
</tr>
<tr>
<td>Model 9</td>
<td>Intercept only</td>
<td>1</td>
<td>23.5</td>
<td>0.07</td>
<td>28.8</td>
<td>0.001</td>
</tr>
</tbody>
</table>

‡Number of model parameters including an intercept.
Our study is the first to demonstrate PrP\textsubscript{CWD} excretion of PrP\textsubscript{CWD} in saliva and urine of infected white-tailed deer as early as 3 months PI. Using an enhanced RT-QuIC assay, Cheng et al. [48] reported the excretion of PrP\textsubscript{CWD} in the faeces of infected elk within 1 month PI. In contrast to these previous studies, we investigated the temporal patterns of PrP\textsubscript{CWD} excretion in both urine and faeces from the three species of cervids commonly affected by CWD.

We detected PrP\textsubscript{CWD} in 28\% of the urine samples from asymptomatic cervids, with no substantial differences in the frequency of PrP\textsubscript{CWD} excretion for species, genotype, or time PI. The frequency of PrP\textsubscript{CWD} excretion in faeces was much higher (88\%), but we still identified no significant differences among species or for time PI. Although our sample size was limited, the consistently high frequency of PrP\textsubscript{CWD} excretion in faeces corresponds with that reported by Tamgüney et al. [22]. However, we found that all three cervid species were excreting PrP\textsubscript{CWD} by 6 months PI, 3 months earlier than reported for mule deer by Tamgüney et al. [22].

Our study is the first to demonstrate PrP\textsubscript{CWD} excretion in urine by asymptomatic elk and mule deer. We found that the frequency of PrP\textsubscript{CWD} excretion was much lower in urine than in faeces, but we detected PrP\textsubscript{CWD} in urine as early as 6 months PI in white-tailed deer and 18 months PI in mule deer and elk. The low probability of detecting PrP\textsubscript{CWD} in urine and the higher number of rounds of PMCAb amplification required for detection compared to faeces suggests that the amount of PrP\textsubscript{CWD} is also lower in urine than in faeces, in agreement with previous research [42, 49]. In addition, we found that PrP\textsubscript{CWD} in urine was a strong predictor of faecal excretion; all animals with positive urine had positive faeces, but most animals with positive faeces had negative urine.

The secretion and excretion of PrP\textsubscript{CWD} in saliva, urine and faeces, in combination with the long-term persistence of prions in the environment [12, 50–52], probably contribute to the creation of environmental reservoirs of PrP\textsubscript{CWD}, particularly in areas where cervids concentrate, such as minerallicks and supplemental feeding areas. Our results correspond with other studies [20, 21, 53, 54] indicating that urine from infected cervids may play a smaller role than faeces in direct or environmental transmission of CWD. Overall, urine appears to have less consistent and likely lower concentration of PrP\textsubscript{CWD} than faeces. Tamgüney et al. [22] estimated that a similar amount of prions would be excreted in faeces during a 10 month period as would be present in the brain of a terminally sick mule deer. Henderson et al. [21] found that white-tailed deer shed PrP\textsubscript{CWD} as early as 3 months PI and that infected deer would excrete thousands of infectious doses during the course of the disease. Cheng et al. [48] found that elk excrete prions in faeces <1 month after infection. Our results also indicate that all three cervids likely excrete PrP\textsubscript{CWD} within 6 months of infection, indicating that total prion excretion during the course of infection may be higher than previously estimated. In addition, a large amount of prions would likely be deposited in the environment from the saliva and urine of infected cervids [21]. Further research is needed to determine where, when and how much PrP\textsubscript{CWD} is deposited into the environment.

### Table 4. Positive detections of CWD agent (PrP\textsubscript{CWD}) in cervid faeces using PMCAb.

Cervids were orally inoculated with PrP\textsubscript{CWD} and euthanized at 6, 12, 18 and 24 months post-inoculation.

Faecal samples, if present, were collected during the necropsy that immediately followed euthanasia. PrP\textsubscript{CWD} was detected using PMCAb. The numerator is the number of positive detections and the denominator is the number of animals from which faecal samples were tested.

<table>
<thead>
<tr>
<th>Species</th>
<th>Months post-inoculation</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>6</td>
<td>12</td>
</tr>
<tr>
<td>Elk</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>2/2</td>
<td>1/2</td>
</tr>
<tr>
<td>Mule deer</td>
<td>1/2</td>
<td>1/1</td>
</tr>
<tr>
<td>Total</td>
<td>5/7</td>
<td>5/6</td>
</tr>
</tbody>
</table>

(RT-QuIC) assay, reported PrP\textsubscript{CWD} in the saliva and urine of infected white-tailed deer as early as 3 months PI. Using an enhanced RT-QuIC assay, Cheng et al. [48] reported the excretion of PrP\textsubscript{CWD} in the faeces of infected elk within 1 month PI. In contrast to these previous studies, we investigated the temporal patterns of PrP\textsubscript{CWD} excretion in both urine and faeces from the three species of cervids commonly affected by CWD.

We detected PrP\textsubscript{CWD} in 28\% of the urine samples from asymptomatic cervids, with no substantial differences in the frequency of PrP\textsubscript{CWD} excretion for species, genotype, or time PI. The frequency of PrP\textsubscript{CWD} excretion in faeces was much higher (88\%), but we still identified no significant differences among species or for time PI. Although our sample size was limited, the consistently high frequency of PrP\textsubscript{CWD} excretion in faeces corresponds with that reported by Tamgüney et al. [22]. However, we found that all three cervid species were excreting PrP\textsubscript{CWD} by 6 months PI, 3 months earlier than reported for mule deer by Tamgüney et al. [22].

Our study is the first to demonstrate PrP\textsubscript{CWD} excretion in urine by asymptomatic elk and mule deer. We found that the frequency of PrP\textsubscript{CWD} excretion was much lower in urine than in faeces, but we detected PrP\textsubscript{CWD} in urine as early as 6 months PI in white-tailed deer and 18 months PI in mule deer and elk. The low probability of detecting PrP\textsubscript{CWD} in urine and the higher number of rounds of PMCAb amplification required for detection compared to faeces suggests that the amount of PrP\textsubscript{CWD} is also lower in urine than in faeces, in agreement with previous research [42, 49]. In addition, we found that PrP\textsubscript{CWD} in urine was a strong predictor of faecal excretion; all animals with positive urine had positive faeces, but most animals with positive faeces had negative urine.

The secretion and excretion of PrP\textsubscript{CWD} in saliva, urine and faeces, in combination with the long-term persistence of prions in the environment [12, 50–52], probably contribute to the creation of environmental reservoirs of PrP\textsubscript{CWD}, particularly in areas where cervids concentrate, such as mineral licks and supplemental feeding areas. Our results correspond with other studies [20, 21, 53, 54] indicating that urine from infected cervids may play a smaller role than faeces in direct or environmental transmission of CWD. Overall, urine appears to have less consistent and likely lower concentration of PrP\textsubscript{CWD} than faeces. Tamgüney et al. [22] estimated that a similar amount of prions would be excreted in faeces during a 10 month period as would be present in the brain of a terminally sick mule deer. Henderson et al. [21] found that white-tailed deer shed PrP\textsubscript{CWD} as early as 3 months PI and that infected deer would excrete thousands of infectious doses during the course of the disease. Cheng et al. [48] found that elk excrete prions in faeces <1 month after infection. Our results also indicate that all three cervids likely excrete PrP\textsubscript{CWD} within 6 months of infection, indicating that total prion excretion during the course of infection may be higher than previously estimated. In addition, a large amount of prions would likely be deposited in the environment from the saliva and urine of infected cervids [21]. Further research is needed to determine where, when and how much PrP\textsubscript{CWD} is deposited into the environment.

### Table 5. PRNP genotype and species of cervids orally inoculated with PrP\textsubscript{CWD} and euthanized at 6, 12, 18 and 24 months post-inoculation

<table>
<thead>
<tr>
<th>Species</th>
<th>More susceptible(^*)</th>
<th>Less susceptible(^†)</th>
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</thead>
<tbody>
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<td></td>
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</tr>
<tr>
<td>Elk</td>
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<td>12</td>
</tr>
<tr>
<td>Mule deer</td>
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<td>2</td>
<td>8</td>
</tr>
<tr>
<td>White-tailed deer</td>
<td>1</td>
<td>9</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>14</td>
<td>30</td>
</tr>
</tbody>
</table>

\(^*\)More susceptible: elk (132MM), mule deer (225SS), white-tailed deer (96GG).

\(^†\)Less susceptible: elk (three elk with 132ML), mule deer (two deer with 225SF), white-tailed deer (five deer with 96GS, four deer with 96SS).

### Table 6. Frequency of PRNP genetic susceptibility and detection of CWD agent (PrP\textsubscript{CWD}) in three species of cervids orally inoculated with PrP\textsubscript{CWD} and euthanized at 6, 12, 18 and 24 months post-inoculation

<table>
<thead>
<tr>
<th>Detection</th>
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<tbody>
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<td>More susceptible</td>
<td>Less susceptible</td>
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<td>Positive</td>
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<td>9</td>
</tr>
<tr>
<td>Negative</td>
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<td>5</td>
</tr>
<tr>
<td>Total</td>
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<td>14</td>
</tr>
</tbody>
</table>

### Table 7. Frequency of CWD agent (PrP\textsubscript{CWD}) in positive or negative faecal samples tested by PMCAb in paired samples for cervid urine and faeces.

Cervids were orally inoculated with PrP\textsubscript{CWD} and euthanized at 6, 12, 18 and 24 months post-inoculation.

<table>
<thead>
<tr>
<th>Faeces +</th>
<th>Faeces –</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine +</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>Urine –</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>14</td>
<td>3</td>
</tr>
</tbody>
</table>
by infected cervids, while it will also be necessary to study
the ingestion of PrP\textsuperscript{CWD} in the environment by animals and
the relative importance of direct and indirect routes of
infection during the course of a CWD epidemic in free-
ranging cervids.

Recent studies have predicted significant impacts of CWD
on cervid populations [29, 31, 55, 56]. Genetic selection
favouring less susceptible PRNP genotypes [25, 31] suggests
cervid populations may be able to persist with enzootic
CWD, but whether the less susceptible genotypes may shed
more prions during their longer incubation period is
unknown [25, 31]. In addition, less susceptible genotypes
may have other phenotypic effects that impact negatively on
survival and/or recruitment [57]. We found evidence for a
lower frequency of PrP\textsuperscript{CWD} in the faeces and urine of less
susceptible (64 %) compared to more susceptible (94 %)
PRNP genotypes, despite the small number of animals in
our study. Our results indicate that less susceptible PRNP
genotypes may excrete lower concentrations of prions, shed
prions for a shorter period of time, or both. We caution that
our results may in part reflect differences among cervid
species, as most of our less susceptible animals were white-
tailed deer, and most of the relatively susceptible animals
were elk and mule deer (Table 5). In addition, our results
differ from those of Henderson et al. [21], who were unable
to document genotypic differences in shedding for white-
tailed deer. However, our findings are similar to those for
infected elk, where the relatively resistant genotype
appeared to excrete prions later than the relatively suscepti-
ble genotype [48]. As a result, we urge that further research
be conducted to compare the PrP\textsuperscript{CWD} excretion patterns for
all cervids, and the survival and recruitment between geneti-
cally different susceptible PRNP genotypes, and also to
determine the total amount of prions shed during the life-
time of different genotypes and the consequences for CWD
transmission.

We found a low rate of prion detection in urine, suggesting
that urinary screening of individual cervids is not a robust
method for \textit{ante mortem} detection of infection. However,
testing environmental samples that multiple cervids have
urinated upon, such as soil from scrapes, wallows, mineral
licks, or confined pens may prove useful as a surveillance
technique or in identifying potential environmental reser-
voirs of PrP\textsuperscript{CWD}. Conversely, a high rate of prion detection
in faeces indicates that faecal screening is a promising \textit{ante mortem}
diagnostic and surveillance approach at both the
individual animal and population levels. We believe faecal
testing using PMCA\textsubscript{b} could be further developed as a com-
plement to the current \textit{ante mortem} tissue tests using tonsil and
rectal biopsy. PMCA\textsubscript{b} analysis of faeces combined with
tissue biopsy may also be useful for screening less suscepti-
ble genotypes, which have proven to be difficult to reliably
test using biopsy [57]. Recent work by Wyckoff et al. [58]
indicated that PMCA of brainstem tissue from pre-clinical
elk is also a more sensitive detection method than immuno-
histochemistry of brainstem or retropharyngeal lymph node
tissues. These results further validate the PMCA methodol-
ogy and suggest that continued development for potential
analysis of excreta, secretions and tissues sampled by non-
invasive methods be conducted. Along these lines, we stress
that further evaluation of PrP\textsuperscript{CWD} screening of urine, faeces
and environmental samples from captive and free-ranging
cervid populations with PMCA is necessary before these
methods can be integrated into the management decision-
making process.

Studies on CWD in captive cervids are accompanied by
numerous challenges and uncertainties, and our opportu-
nistic study is no exception. Some of the deer in our study
may have been infected prior to capture, which means we
may have underestimated the time PI for these animals.
However, we note that no cervids exhibited clinical signs
during study, suggesting that deer were probably not in an
advanced stage of CWD progression at capture. In addition,
animals were housed in a highly PrP\textsuperscript{CWD} contaminated
environment. Thus, measures of PrP\textsuperscript{CWD} excretion may
reflect the ingestion and passage of PrP\textsuperscript{CWD} rather than
shedding, which suggests that the detection of PrP\textsuperscript{CWD}
in faeces is useful for the detection of environmental exposure
do cervids to PrP\textsuperscript{CWD}. In addition, this conclusion would
suggest that cervids are a means of moving CWD around
the landscape. However, if faecal excretion primarily repre-
sented ingestion it seems unlikely that we would find a
strong association between IHC-positive brainstem or ret-
ropharyngeal lymph nodes and PrP\textsuperscript{CWD} detection, or differ-
ences among PRNP genotypes. We found only two deer at
6 months PI that had PrP\textsuperscript{CWD} in their faeces and were
IHC-negative in all three of the major tissues commonly
used to identify CWD infection; both of these deer were
IHC-positive in other organs. Whether these deer were
shedding PrP\textsuperscript{CWD} or their results indicate ingestion and
pass-through of PrP\textsuperscript{CWD} is uncertain. We note that the
Tamgüney et al. [22] study, like ours, was conducted using
captive deer within a highly contaminated facility; thus, the
relative importance of shedding versus ingestion and pass-
through in their results has similar uncertainties. Therefore,
we believe that more research is needed to determine the
excretion resulting from infection versus that resulting from
ingestion from contaminated environments. Finally,
we used white-tailed deer normal brain homogenate (NBH)
as the reaction substrate for all tests, which has been dem-
onstrated to successfully convert PrP\textsuperscript{CWD} from all three
species [59].

\textbf{Conclusions}

The general constancy of PrP\textsuperscript{CWD} excretion in faeces and
urine from 6 to 24 months PI suggests that infected cervids
present a risk of transmission to other animals, regardless of
the time PI. The high rate of PrP\textsuperscript{CWD} detection in faeces and
our inability to detect temporal or species differences in
the faecal excretion of prions suggests that the amount of
PrP\textsuperscript{CWD} excreted in faeces is higher than in urine and
remains relatively consistent during pre-clinical infection.
Thus, exposure to faeces from an infected animal may
present a higher risk of potential CWD infection than exposure to its urine. Likewise, infected animals probably continuously contribute to environmental reservoirs of PrP\textsuperscript{CWD}, beginning at least 6 months PI. Cervid concentrations at sites such as mineral licks, supplemental feeding areas, or winter congregation sites likely increases contact between animals and contact with faeces or urine that may contain PrP\textsuperscript{CWD}, both of which could play a role in CWD transmission. While white-tailed deer seem to avoid consumption of faeces at supplemental feeding sites, they may not be capable of completely avoiding it [60]. Further understanding of environmental transmission of CWD would benefit from testing for PrP\textsuperscript{CWD} in areas of the landscape where cervids congregate and may deposit PrP\textsuperscript{CWD} by either urination or faecal deposition (e.g., mineral licks, and supplemental food sites). We suggest focusing on sites that are used year-round and where cervids defecate, rather than seasonally used areas where urination is the primary means of depositing PrP\textsuperscript{CWD} into the environment (e.g., scrapes).

METHODS

Animals and sample collection

The US Geological Survey National Wildlife Health Center (NWHC), the Wyoming Game and Fish Department, the University of Wyoming – Department of Veterinary Sciences and the Wyoming Cooperative Fish and Wildlife Research Unit initiated a project to create an archive of CWD-positive tissue in 2004. Twelve elk (5 female, 7 male), 10 mule deer (6 female, 4 male) and 10 white-tailed deer (4 female, 5 male, 1 unknown) were captured in the spring of 2004 by the Wyoming Game and Fish Department. The elk were captured near Jackson, Wyoming, where CWD had not been detected in free-ranging cervids. The deer were captured in Converse and Platte County, Wyoming, where CWD had previously been detected in hunter-harvested, road-killed and sick animals that displayed signs of CWD infection. The estimated CWD prevalence in mule and white-tailed deer from the hunt areas encompassing Converse and Platte Counties between 2003 and 2005 was 27.3 % (152/556) and 32.4 % (34/105), respectively.

Following capture, animals were transferred to the Tom Thorne/Beth Williams Wildlife Research Unit at Sybille in southeastern Wyoming. Each species was housed in separate pens under similar conditions. The animals were orally gavaged with 5 g of homogenized pooled brain material from infected conspecific wild cervids [34]. Staff periodically monitored animals and no animals displayed classic clinical signs of CWD during the 2-year study. A subset of each species was immobilized with carfentanil and euthanized with potassium chloride at 6, 12, and 18 months post-inoculation; the remaining animals were euthanized at 24 months. (Please refer to AVMA guidelines for current recommendations regarding euthanasia methods.)

Necropsy was begun within minutes of death. Urine was collected directly from the urinary bladder via sterile syringe and needle and placed into sterile centrifuge vials. Faecal pellets were collected directly from the distal colon at necropsy. The colon was transected within a few cm of the rectum. The open end of the colon was placed untouched into an unused freshly opened Whirlpack bag. Once the open end of the colon was inside the bag, the faecal contents were extruded into the bag via hand pressure applied on the visceral surface of the colon above the bag. The faecal pellets were not touched in this process. Urine and faeces collected during necropsy were frozen at −18°C, transferred to the NWHC in Madison, WI and stored at −70°C.

The other tissues collected at necropsy were placed in 10% neutral buffered formalin (NBF) at a tissue-to-formalin ratio of 1:10. Tissue fixation was continued for a minimum of 5 days before further processing. Tissues were processed, embedded in paraffin, sectioned at 5 μm, mounted on positively charged glass slides and dehydrated in preparation for immunohistochemical staining [61–63]. Immunolabelling was completed using an automated immunostainer, and an anti-PrP murine monoclonal antibody F99/97.6.1, a biotinylated secondary antibody, an alkaline phosphatase-streptavidin conjugate, a substrate chromogen (Fast Red A, naphthol, Fast Red B), and a hematoxylin and bluing counterstain [61]. A positive control slide was included with each batch of tissues processed. Tissues were considered positive if they contained the hallmark bright red, coarse, granular staining against a light blue non-stained tissue [61]. Positive immuno-staining was readily visible at 10x power under a light microscope.

Genotyping

Animals were PRNP genotyped following capture, or from post-mortem tissue samples, and classified as more or less susceptible to CWD (Table 5) based on a species-specific genotype (reviewed in [64]). CWD is known to affect most North American cervid species and is transmissible among species and genotypes [64]. Although all deer are susceptible to CWD infection, the PRNP genotype influences the relative species-specific ‘susceptibility’ [25, 64], and the different genotypes are associated with differences in disease progression [25], or the accumulation of differential amounts of PrP\textsuperscript{CWD} in tissues. Elk that are homozygous for methionine at codon 132 of the PRNP gene are more susceptible, while the leucine/methionine heterozygote and the leucine homozygote are less susceptible [65–67]. We classified white-tailed deer with codon 96 homozygous for glycine as more susceptible, and those heterozygous for glycine/serine and homozygous for serine as less susceptible [25, 68, 69]. Mule deer were classified as more susceptible if they were homozygous for serine at the PRNP 225 codon, and as less susceptible if they were heterozygous for phenylalanine/serine or homozygous phenylalanine [70].

Laboratory tests for chronic wasting disease

Preparation of faecal/urine Samples

The extraction protocols for ovine faecal bound scrapie prion of Terry et al. [71] were adapted for cervid faeces and PrP\textsuperscript{CWD} as follows. We added 100 mg of faeces to 2 mL
tubes containing 2 mm glass beads and 1.6 mm silicon carbide particles (MP Biomedical #116916100). The faeces were diluted 1:9 (w/v) with ultrapure water containing Roche Complete ethylenediaminetetraacetic acid (EDTA) free protease inhibitor (Fisher Scientific #50-720-4069) before two 40 s homogenizations with a MP Biomedical FastPrep 24 (MP Biomedical #116004500). We added sodium dodecyl sulphate (SDS) to obtain a final concentration of 1 % (v/v) before three additional 45 s homogenizations. Samples were incubated and rotated for 1 h at room temperature. To separate the particulate matter, we centrifuged samples for 60 min at 10 °C and 15 000 g before transferring the supernatant to a new 1.5 mL Lo-Bind PCR tube, which was centrifuged for another 60 min under the same conditions. Each supernatant was transferred to a new 2.0 mL LoBind PCR tube and diluted 1:1 with phosphate buffered saline (PBS; 15 mM KH2PO4, 81 mM Na2HPO4, 137 mM NaCl and 3 mM KCl; pH 7.4) containing 4 % N-lauroylsarcosine sodium salt (Sarkosyl). We added 1 unit ml⁻¹ of Pierce Universal Nuclease for Cell Lysis (Thermo Scientific #88701) to each sample before heating at 50 °C for 30 min. Sodium phosphotungstic acid (NaPTA) was added to a final concentration of 0.57 % (v/v) before we concurrently incubated the samples at 37 °C and vortexed overnight. The next day, we centrifuged the samples (15 000g, 30 min, room temperature) and discarded the supernatant. We rinsed the pellet in 0.1 % Sarkosyl, 0.5 M EDTA, and centrifuged again (15 000g, 10 min, room temperature). The supernatant was discarded before the pellet was resuspended in 100 μL of PMCA buffer (PBS, additional 150 mM NaCl, 4 mM EDTA, pH 8.0, 1 % (v/v) polyethylene glycol ρ-(1,1,3,3-tetramethylbutyl)-phenyl ether (Triton X-100) and Roche Complete EDTA-free protease inhibitor). To ensure resuspension, we added one 2.38 mm polytetrafluoroethylene (PTFE; Amazon supply #BT-015-C) bead to the solution prior to two 10 s sonications in a cup sonicator. The solution was diluted 1:9 with NBH (preparation detailed below) in 0.2 ml tubes containing two 2.38 mm PTFE beads per tube. The samples were sonicated in a cup sonicator horn (detailed below).

We performed no extraction on the urine. Urine samples were prepared for sonication by diluting 1:9 with NBH in 0.2 ml thin-walled lo-bind PCR tubes containing two 2.38 mm PTFE beads per tube. At least two independent replicates were conducted for all urine and faecal samples.

Normal brain homogenate
The details for NBH preparation are covered in Johnson et al. [38]. Briefly, brains from transgenic cervid mice expressing the most common white-tailed deer PRNP allele (95QQ, 96GG, 226QQ) were homogenized on ice in PMCA conversion buffer (see [38]) to 10 % (w/v) brain homogenate. We clarified NBH by centrifugation (2000 g, 2.75 min, 4 °C). Homogenate was flash frozen in liquid nitrogen and stored at –80 °C until use.

Controls
We prepared positive controls for PMCAb from the brain tissue of an orally inoculated, clinically affected CWD-positive white-tailed deer of the predominant PRNP genotype listed above. A stock of 20 % (w/v) of infected brain homogenate in PBS was diluted 1:1 with the PMCA conversion buffer (see [38]) then serially diluted fivefold in NBH to generate a dilution series of infectious PrP(CWD) ranging from 2×10⁻² to 4.2×10⁻¹⁷ dilutions. We included the fourth and tenth fivefold dilutions in every experiment as positive controls. A 10 % (w/v) NBH was used as a negative control in every experiment. We ran a tube with no faecal material through the faecal extraction protocol to serve as a negative control on the faecal bound prion extraction process. Faeces from a CWD-negative white-tailed deer were used as a negative faecal control. Because no extraction was performed on the urine, tubes of 10 % (w/v) NBH served as negative controls for urine tests. In the infrequent event of a false-positive signal in the negative controls, results were discarded, lab protocols were evaluated and adjusted if necessary, new solutions were made, and the test was run again.

Protein misfolding cyclic amplification with beads
We placed the sample tubes in a rack, suspending them in the 325 ml ultrapure water (>18 MΩ·cm resistivity)-filled reservoir of a Misonex S-3000 or S-4000 microplate sonicator horn operated in an incubator at 37 °C. One round of PMCAb was defined as 96 cycles of sonication, with each cycle consisting of 20 s of sonication at a power setting of 35 % followed by 29 min·40 s of incubation. Between rounds, aliquots from each tube were diluted 1:9 (v/v) in a new tube of fresh NBH. Four rounds of PMCAb were conducted for all samples.

Immunoblotting
Following PMCAb amplification, we used immunoblotting to detect the presence of protease K (PK)-resistant prion protein (PrP(CWD)) (Fig. 1). We prepared samples for

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**Fig. 1.** An example of immunoblot confirmation of CWD agent (PrP(CWD)) in samples after amplification by protein misfolding amplification with beads (PMCAb). The two left lanes are normal brain homogenate (NBH) with no PrP(CWD) added, which serve as negative controls. The third lane from the left contains molecular weight markers. The next eight lanes are four samples, with two replicates each, containing PrP(CWD). The next lane contains molecular weight markers. Following that are two positive control lanes. The first positive control is the fourth fivefold dilution of 10 % brain homogenate of a clinically affected white-tailed deer diluted 1:9 in NBH. The second positive control is the tenth fivefold dilution of the same 10 % brain homogenate diluted 1:9 in NBH. The last lane is a sample of NBH that has not been treated with proteinase K (PK).
immunoblotting by transferring 20 μL from each PMCAb reaction to a 0.6 ml Lo-Bind PCR tube containing 3 μL of PK (2 μg μL⁻¹) and 7 μL of PK dilution buffer [0.1% (v/v) Triton X-100 and 4% (w/v) SDS in PBS]. The samples were incubated at 37°C and shaken at 600 r.p.m. for 40 min. After PK digestion, we added 10 μL of 4× LDS sample buffer (Invitrogen #NP0008) containing 200 mM 10× reducing agent (NuPAGE #NP0009) to each sample for 15 min of incubation at 100°C. We fractionated 16 μL samples and 4 μL no-PK controls on a 15-well 10% Bis-Tris gel (Invitrogen #NP0303BOX) and electrotransferred the protein to polyvinylidene fluoride (PVDF) membranes (Millipore #IPVH00010). The PVDF membrane was blocked for 1 h in 3% (w/v) powdered non-fat milk in Tris-buffered saline and Tween 20 (TBST). We applied Barr 224 (1:10 000 in 3% milk TBST; Cayman Chemicals #10009035) and mAb 8G8 (1:5000 in 3% milk TBST; Cayman Chemicals #189760) with 0.02% sodium azide overnight. The following day, the membranes were rinsed with TBST before horseradish peroxidase-conjugated goat anti-mouse IgG (1:20 000 dilution in 1% milk TBST; Bio-Rad #170-5047) was applied. We rinsed the membranes with TBST before Supersignal West chemiluminescent substrate (Thermo Scientific #34080) was applied to detect immunoreactivity.

**Statistical analysis**

After four rounds of PMCAb followed by immunoblot detection, we classified urine or faecal samples as positive if at least one of the replicate samples was positive. We classified samples as negative if all replicates were negative. We used separate logistic regression models to evaluate the importance of hypothesized predictor variables on positive/negative faeces and urine. The predictor variables were time (months PI), species (elk, mule deer, and white-tailed deer), genotype (more susceptible and less susceptible), the interaction between species and time, and the interaction between genotype and time. We expected that the proportion of animals with PrP<sub>CWD</sub> in both urine and faeces would increase with time PI. Further, because elk have lower CWD prevalence than sympatric deer populations, we predicted more deer would test positive than elk. We predicted that animals with a more susceptible PRNP genotype would have a higher probability of excreting PrP<sub>CWD</sub> than animals from the less susceptible genotypes. The full model included all of the predictor variables and interactions with time PI (Table 3). We used nine simplified models to test individual hypotheses about the association between PrP<sub>CWD</sub> detection and predictor variables. We compared models using the Akaike information criterion corrected for small sample size (AICc) [72–74]. We considered ΔAICc >2 to indicate a significant difference in the model fit to the data. We used a two-sided Fisher’s exact test to compare CWD detection probability between faeces and urine [75].

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

Mice were cared for in accordance with protocols approved by the Institutional Animal Care and Use Committee of the University of Wisconsin – Madison (assurance number A3464-01). Cervid species were cared for in accordance with the protocols approved in 2004 by the Wyoming Game and Fish Department Animal Care and Use Committee.

**References**

59. Hamir AN, Richt JA, Miller JM, Kunkle RA, Hall SM et al. Experimental transmission of chronic wasting disease (CWD) of elk (Cervus elaphus nelsoni), white-tailed deer (Odocoileus virginianus), and mule deer (Odocoileus hemionus hemionus) to white-tailed deer by intracerebral route. Vet Pathol 2008;45:297–306.


75. Fay MP. Confidence intervals that match Fisher’s exact or Blaker’s exact tests. Biostatistics 2010;11:373–374.