Soil, scat, and (*Ixodes*) *scapularis*: Assessing environmental factors in the chronic wasting disease system

by

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A wounded deer leaps the highest.

- Emily Dickinson

DEDICATION

To my husband and best friend, Marcel.
For always pushing me towards achieving my life's goals and keeping me grounded.
The hooved of the Swiss Alps await our shepherding.

And to all my family and friends,
For checking in with me weekly, showing interest in my passion for conservation,
And embracing my love for all things wild.

ABSTRACT

Soil, scat, and (*Ixodes*) *scapularis*: Assessing environmental factors in the chronic wasting disease system

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Infectious disease systems involving wildlife are complex. Chronic wasting disease (CWD) is a highly infectious, fatal neurodegenerative disease that affects wild and captive cervids. Despite management efforts in North America, CWD is now established in 30 states and 4 Canadian provinces and disease prevalence in endemic areas is increasing. While it is well accepted that direct transmission establishes CWD outbreak areas, indirect transmission through contaminated environments may facilitate maintenance of the disease. Infected hosts shed CWD prions (PrP^{CWD}) into the environment during a 1.5 to 2 year-long disease course, where they remain infectious for years to decades. The importance of PrP^{CWD} shedding, the subsequent accumulation and increasing bioavailability of PrP^{CWD} in the environment, and host behaviors that facilitate host-prion contacts are not well understood due to the challenges of detecting prions. In this dissertation, I leverage protein amplification technology, the real-time quaking-induced conversion (RT-QuIC) assay, in combination with field and additional laboratory-based approaches to help fill key knowledge gaps about ecologically- and management-relevant environmental sources of CWD

transmission. I examine and characterize the relative amounts and presence or absence of PrP^{CWD} in abiotic and biotic environmental samples.

In chapter 2 I assessed the potential risk of hematophagous ectoparasites as mechanical vectors of CWD. I hypothesized that ticks are an ecologically relevant community-level factor in the CWD system, with implications for CWD transmission. I revealed that deer ticks (*Ixodes scapularis*) assimilate and excrete PrP^{CWD} from inoculated blood meals using an artificial membrane feeding system and that deer ticks collected from hunter-harvested white-tailed deer (*Odocoileus virginianus*) contained transmission-relevant amounts of PrP^{CWD} and may pose a CWD risk to cervids.

In chapter 3 I assessed detection thresholds and relative quantification of PrP^{CWD} in carnivore feces. I hypothesized that carnivore excrement could be used as a non-invasive surveillance approach for CWD occurrence and help disentangle the role of carnivores in CWD spread. RT-QuIC was adaptable for detection of PrP^{CWD} from prion-spiked carnivore excrement and field samples showed that PrP^{CWD} is detectable from free-ranging carnivores. Scat-based surveillance could facilitate CWD detection in low-prevalence areas or areas without hunter-based surveillance.

In chapter 4, I assessed PrP^{CWD} accumulation dynamics at decommissioned artificial mineral licks in southwest Tennessee. I hypothesized that these sites would still serve of foci of PrP^{CWD} accumulation, and that site-specific management efforts, deer use, and soil characteristics would

influence PrP^{CWD} accumulation dynamics. I demonstrated that 32/49 (65%) mineral sites had PrP^{CWD} detected in soils. Detection of PrP^{CWD} from these artificial mineral sites was not dependent on site-specific management efforts or deer use intensity. Soil properties were very similar across sites and no correlation between PrP^{CWD} detection and soil physical properties was found.

Overall, these studies demonstrate the complex nature of CWD epidemiology and highlight the importance of teasing out details of fine-scale ecological factors and processes influencing the CWD processes. I showed that ticks readily assimilate PrP^{CWD} from infected blood meals and have the potential to act as mechanical vectors of CWD. In addition, I furnish a way to begin understanding the relationship of cervid consumers in structuring environmental PrP^{CWD} deposition by assessing the utility of RT-QuIC for examination of carnivore excrement. I advance the current understanding of PrP^{CWD} accumulation dynamics at attractant sites resulting from historic foci of deer aggregation due to prior attractant supplementation leading up to nascent CWD establishment in southwest Tennessee.

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I was never really given an opportunity to excel at anything during my childhood, of which I spent a large part growing up too fast to compensate for a lack of interest and involvement of my parents in mine and my brothers' lives. This inattentive upbringing was a result of having a mother who was/is mentally ill, emotionally abusive, and destructive, and a father that appeared to wish to distance himself. I divulge these personal details as an introduction to this "acknowledgement" section of this formal document not to whimper on and on about woe is me, but to inform about where my immense gratitude is rooted for those in my life who helped me build my self-confidence and belief in myself enough to think I was capable of achieving my personal and professional goals. So, in that way I am grateful for the lack of interest and uncaring nature of my parents because the void they created cultivated in me an intense sense of self-awareness, drive to succeed, and an immense appreciation for all the humans and non-humans in my life who bring positivity and balance.

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PREFACE

Chapter 2 is a complete and final draft of a manuscript that was accepted for publication by the journal *Scientific Reports*. It was included here in the form that it was submitted to the journal in March 2023, following requested revisions. H.N. Inzalaco is the first and corresponding author of the manuscript and it is coauthored by F. Bravo Risi, R. Morales, D. Walsh, D. Storm, J.A. Pedersen, W.C. Turner, and S.S. Lichtenberg. H.N. Inzalaco conceptualized the idea and approach, set up and conducted all experiments, analyzed and interpreted data, created all figures, and wrote the first draft of the manuscript. J.A. Pedersen, S.S. Lichtenberg, W.C. Turner, D. Storm, and D. Walsh aided in concept development. W.C. Turner, D. Walsh, and S.S. Lichtenberg guided me in data analysis methodology. F. Bravo Risi and R. Morales carried out all PMCA experiments and aided in the development of PMCA data figures. All coauthors reviewed and edited the manuscript.

Chapter 3 is a draft of a manuscript that has gone through several rounds of revisions. H.N. Inzalaco is the first and corresponding author of the manuscript and it is coauthored by E. E. Brandell, S. Wilson, M. Hunsaker, D. R. Stahler, K. Woelfe, D. P. Walsh, T. Nordeen, D. J. Storm, S. S. Lichtenberg, and W. C. Turner. H.N. Inzalaco conceptualized the idea and approach, set up and conducted all experiments, analyzed and interpreted data, created all figures, and wrote the first draft of the manuscript. W.C. Turner, S.S. Lichtenberg, D. Storm, D. Walsh, E.E. Brandell, D. Stahler, S. Wilson , and T. Nordeen aided in concept development. W.C. Tuner, D. Walsh, and S.S. Lichtenberg guided me in data analysis methodology. K. Woelfe, M. Hunsaker, E.E. Brandell,

D. Stahler, and S. Wilson coordinated sample acquisition. All coauthors reviewed and edited the manuscript.

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CHAPTER ONE: INTRODUCTION

Overview of infectious prion diseases

Transmissible spongiform encephalopathies (TSEs), also commonly referred to as prion diseases, are a family of subacute fatal neurodegenerative diseases affecting a wide variety of mammalian species. Infectious prion diseases include kuru or Creutzfeldt Jacob disease (CJD), variant Creutzfeldt Jacob disease (vCJD), bovine spongiform encephalopathy (BSE), transmissible mink encephalopathy (TME), chronic wasting disease (CWD), and scrapie. TSE's are capable of initiating epidemics of fatal disease in humans and non-human animals and have attracted global attention due to profound public health, agricultural, and ecological impacts of these diseases.

Although the most widely accepted identification and description of the causative agent of prion diseases was only determined comparatively recently, TSE's have been observed for hundreds of years. Scrapie, the prion disease that affects sheep and goats, has been observed and recorded by sheep farmers in Europe since the 1700's [1, 2]. Early characterization of a scrapie infection noted intense itching, ataxia, emaciation, and death in infected animals and reports of scrapie recognized that the affliction may be transmissible[1]. The transmissibility of scrapie was later confirmed in experiments carried out in 1936 [3, 4].

Kuru, the earliest known prion disease of humans, was first described in the Fore people of Papa New Guinea in 1951 by anthropologists Berndt and Berndt[5] and is thought to be the result of the

traditional ritualized endocannibalism during mortuary feasts[6-9]. Kuru is molecularly and neuropathologically indistinguishable from CJD, another human prion disease, which occurs as not only an infectious (iatrogenic) form, but also as sporadic and familial (i.e., genetic, hereditary) forms[10].

BSE is a prion disease of cattle that emerged in the United Kingdom (U.K.) in 1985[11]. While some debate exists regarding the origin of BSE, it is generally accepted that the disease likely originated from scrapie[12]. Prior to the BSE epidemic it was common practice for cattle and dairy cows to be fed rendered meat-and-bone meal prepared from the offal of sheep, cattle, pigs, and chickens. The emergence of BSE is thought to be the outcome of changes in the meat-and-bone meal feed rendering process, shifting from a solvent-based (wet, higher temperatures) to a pressure-based (dry, lower temperatures) method[13]. This new process produced feed containing a higher fat content, but consequently allowed scrapie-tainted biological material in the slurry to remain infectious[14, 15]. This trigger the BSE epidemic that was sustained artificially through subsequent oral exposure of cattle and dairy cows to rendered BSE-tainted meat-and-bone meal[16]. In 1997, following the BSE epidemic, heightened surveillance revealed the emergence of a new variant of CJD, termed vCJD[17], in multiple human patients in the U.K. This new variant was the result of zoonotic spillover of BSE to humans through ingestion of BSE-tainted meat products[18-21]. In addition, TME is a prion disease that was identified in farmed mink that emerged as a result of feeding mink downer cattle infected with an unrecognized BSE-like disease that was determined to be a variant of BSE, termed L-BSE[22-24].

CWD is a prion disease that affects captive and free-ranging cervid species that include white- and black-tailed deer (*Odocoileus virginianus* and *Odocoileus hemionus columbianus*, respectively), mule deer (*Odocoileus hemionus*), reindeer (*Rangifer tarandus*), red deer (*Cervus elaphus*), elk (*Cervus canadensis*), moose (*Alces alces*), sika (*Cervus nippon*), and muntjac (*Muntiacus muntjak*). CWD was first observed as a fatal wasting syndrome of mule deer in a captive deer facility in Colorado well before it was officially characterized as a prion disease in 1980[25, 26], and first appeared in free-ranging mule deer and elk in southeastern Wyoming and northeastern Colorado[25, 27, 28]. Although CWD can be transmitted vertically or horizontally[29-36], where it originated from still remains unknown[37]. It has long been hypothesized that CWD may have emerged as a spillover of sheep scrapie to cervids co-housed in pens or pastures with (scrapie) sheep in an animal research facility at Colorado State University's Foothills Campus[38, 39] and there is mounting evidence supporting the possible origin of CWD from scrapie[40, 41]. Currently, CWD is distributed across 30 states within the United States of America and four Canadian provinces[42], and has been identified in in Norway, Finland, Sweden[43, 44] and South Korea[45], and there is recent evidence that the strain present in Scandinavia is distinct from those found in North America[43, 46, 47]. Despite management efforts, CWD prevalence is still increasing endemically, and geographic spread continues to advance. Prevalence of CWD varies across North America but can reach 30% in free-ranging populations in endemic areas[48] and some instances in captive herds have reported rates up to 90%[49]. Areas with consistently high or increasing CWD prevalence have seen reductions in cervid populations[48, 50] and increases in prevalence in wild cervid herds is expected to affect recruitment[29] and herd viability[50].

Overview of infectious prion biology

In the early 20th century, prion diseases were identified, however the etiological agent responsible for this disease family has only recently been elucidated. Prior to the identification of the causative agent, prion diseases were initially thought to be caused by a slowly progressive viral infection and were described as "slow viruses"[51-54]. It is now widely accepted that the causative agent of prion diseases are misfolded prions (PrP^{TSE}), infectious protein-based particles that, unlike many conventional pathogens, are devoid of a nucleic acid genome and are composed exclusively of modified protein[16, 55, 56].

In infectious prion disease etiology, exogenous PrP^{TSE} causes disease by converting the host's endogenously expressed normal cell-surface prion proteins (PrP^{C}) to misfolded isoforms[57]. During this process the PrP^{TSE} isoform self-propagates by recruiting and inducing autocatalytic conformational conversion of α-helical regions of the PrP^{C} to β-pleated sheets, resulting in monomeric misfolded proteinaceous particles that readily form elongated amyloid aggregates with one another[58, 59]. These conformational changes cause dramatic alterations in the physiochemical and biological behavior of the PrP molecule. Newly formed insoluble PrP^{TSE} aggregates exhibit increased resistance to degradation by proteases, pH, heat, and several other environmental insults[60-64]. The self-propagating and stable PrP^{TSE} aggregates disseminate and accumulate in affected tissues, which leads to cellular dysfunction, neurodegeneration, gliosis, spongiosis and the subsequent appearance of neurological clinical symptoms such as ataxia, behavioral changes, lethargy, and ultimately host death[6, 65, 66].

Mammals express endogenous PrP^C which is coded by the *PRNP* gene. This gene is required for establishing prion infection, and the specific host prion protein gene variations can either allow or prevent prion transmission between hosts with different PrP genotypes[67]. Polymorphic differences in the host prion protein gene may be a source of prion 'strain'[68, 69] or 'ecotype'[70] variation, and by proxy for strain-specific conformational differences[68, 71-74]. Prion strains are defined as heritable strain-specific phenotypes of disease that have differing incubation periods, clinical signs of disease, CNS pathology, and tissue distribution patterns in the host[75, 76]. Differentiation of prion strains is based on characterization of distinct biochemical and biological properties that each produces in a susceptible host. These deterministic properties of PrP^{TSE} can include electrophoretic mobility, stability in the presence of protein denaturants, aggregate size distribution, and rate of formation[76-79]. Co-existence of more than one prion strain in a host can occur[80], and host polymorphisms may also influence which disease prion conformers prevail from the mixture of infectious prions possessing different conformations within a given host[81]. Variation of prion strains may also mediate interspecies transmission, as successful cross-species transmission of one form of a prion disease enables strain diversification, adaptation, which may enhance zoonotic potential [82-84]. Importantly, prion conformation appears to encode prion strain diversity and influence strain-specific cellular co-factors that may determine which tissues disease prions accumulate in[85], resulting in differences in temporal prion shedding patterns[86, 87].

The relevance of prion shedding and the CWD problem

Prion diseases encompass several forms that have been described as heritable, spontaneous, or acquired by various exposure routes. Although human prion diseases are largely heritable or spontaneous in etiology, many prion diseases of non-human animals mainly occur as infectious

disorders that are acquired peripherally, such as by oral exposure, lesions to the skin, or mucosal membranes (i.e., nasal cavity). These include natural sheep scrapie, CWD, and BSE. In addition, tissue tropism differences exist between these peripherally acquired prion diseases. For example, BSE prions (PrP^{BSE}) are demonstrated to be more neurotropic, with little to no peripheral tissue accumulation[88, 89], resulting in the lack of any observed PrP^{BSE} shedding by cattle[90-93]. This is contrast to the highly lymphotropic nature of scrapie prions (PrP^{SC}) and CWD prions (PrP^{CWD}), where once the specific prion agent is introduced, quickly disseminates (within hours) to lymphoid tissues and organs[94-96], resulting in the excretion or secretion of PrP^{TSE} [97-100]. The shedding of prions into the environment makes CWD particularly worrisome, since CWD is by far the most prevalent of all known prion diseases, is the only prion disease known to affect free-ranging wildlife, and the resistance of released prions to degradation and inactivation can result in establishment and accumulation of prions in the environment for years to decades[101].

At present, the transmission modes and factors that explain transmission efficiency of CWD in natural settings are not completely understood, but it is widely known that CWD infection can be transmitted horizontally or vertically. Vertical transmission (in utero) was initially thought to be rare and of reduced importance in CWD epidemiology due in part to high neonatal mortality in deer and elk and also considering the solitary nature of cervids during periods of parturition[102]. However, recent findings suggest that mother-to-offspring transmission may have been underestimated. For example, CWD is efficiently transmitted from sub-clinal and clinical doe to fawns, where 80% of fetuses from CWD-infected muntjac dams were CWD-positive and that maternal CWD infection may reduce rates of live-births[29]. In free-ranging elk in Colorado, natural

CWD transmission from cows to calves has also been reported to contribute to the CWD epidemic[30]. In addition, there have been several recent reports of PrP^{CWD} presence in parturient and fetal tissues and reproductive tissues of CWD-infected deer[31-33]. Early studies show that horizontal transmission of CWD is highly efficient, with reported incidence of disease in captive mule deer of 89%[35]. Horizontal transmission can be direct or indirect. Direct modes involve animal-to-animal contact of infected individuals with susceptible individuals. Indirect modes typically involve contact of susceptible animals with PrP^{CWD}-contaminated materials or environments, with natural oral exposure being the most likely route. For example, oral infection can occur via ingestion of PrP^{CWD}-contaminated plants and/or soil during foraging and dust inhalation[101]. Although direct animal-to-animal interactions contribute to initiating CWD epidemics[103], accumulation of PrP^{CWD} in the environment suggests a large role for indirect routes[104] in CWD epidemiology over time.

The time it takes for a clinical CWD infection to develop ranges from months to years. Following the period between exposure to PrP^{CWD} to development of symptomatic clinal disease in either naturally or experimentally infected cervids varies from 2 to 4 years[102]. These differences in the incubation times may be a result of infectious dose, the route of exposure, cervid host species, cervid host genotype, and PrP^{CWD} strain[87, 94, 105-108]. The maximum incubation period for free-ranging cervids has not been determined and, as mentioned above is influenced by host *PRNP* genotype and CWD strain[86, 87, 94, 109]. Most CWD cases are reported to occur in the 3 to 7-year-old age classes, with similar findings in captive cervids[25, 102, 110].

Clinal symptoms of CWD include weight loss, behavioral changes, ruminal stasis, polydipsia, polyuria, and excessive salivary defluxion in the later stages of disease[111]. Within ~42 days following the natural oral route of infection, modified enterocytes and microfold cells (M cells) facilitate the crossing of PrP^{CWD} through the intestinal epithelial barrier, where PrP^{CWD} then disseminates to lymphoid tissues associated with the alimentary tract, such as the gut-associated lymphoid tissue (GALT), tonsils, and retropharyngeal lymph nodes[94, 95, 112, 113]. Early reports demonstrated that during early CWD pathogenesis, PrP^{CWD} are transported by dendritic cells and/or macrophages to Peyer's Patches and mesenteric lymph nodes[95, 114]. Following establishment of PrP^{CWD} in secondary lymphoid organs (SLO) and GALT tissues, spread of PrP^{CWD} to other organs is facilitated through colonization of nerve endings of the Enteric Nervous System (ENS), draining lymph nodes and blood, and subsequent spread through sympathetic and parasympathetic nerves and eventual neuroinvasion via the dorsal motor nucleus vagus nerve (DMNV) [112, 115-117]. During the process of infection PrP^{CWD} also circulates and accumulates in nasal mucosa, salivary glands, bladder, pancreas, kidney, intestines, and reproductive components of deer[31, 32, 86, 94, 112, 118].

Accumulation of PrP^{CWD} in organs and tissues of infected hosts can result in PrP^{CWD} shedding during preclinical and clinical stages of the disease[118-123]. PrP^{CWD} is shed via urine[124], saliva[120], feces[125, 126], blood[121], antler velvet[127], and parturient materials[29, 32, 33], from infected individuals, or from decomposition of infected carcasses[101]. Extensive research over the last two decades has provided critical insights into the role of shedding in CWD epidemiology and the risk that various PrP^{CWD}-contaminated materials present. For example, oral inoculations of white-tailed deer

(WTD) with urine/feces from CWD-positive animals (50 mL and 50 g, respectively) resulted in disease[128]. An assessment of the risk that urine and feces pose in indirect transmission found that urine contains 1-5 infective doses (ID_{50}; the minimum inoculum required to induce a prion infection in 50% of the challenged population) per 10 mL[129], 30 µL of 10% fecal homogenates contain 0.03 and 1 ID_{50}[126], and that cumulative PrP^{CWD} shed in feces over the disease course is approximately equivalent to the relative quantity of PrP^{CWD} found in diseased brains[125, 126]. Saliva also represents a transmission-relevant source for environmentally deposited PrP^{CWD}, with oral exposure to three 10-mL doses sufficient to cause disease[130]. The tight association of PrP^{CWD} shedding from various secreta and excreta with establishing PrP^{CWD}-contaminated environments suggests an increasingly important role of host-environment interactions in CWD epidemiology.

Behavioral and ecological factors in CWD epidemiology

Behavior underpins inter- and intraspecific interactions as well as the interactions of a given species with the environment. $PrP^{TSE's}$ can remain stable and infectious in the environment for years to decades[131-133]. The influence of abiotic factors, heterogenous landscape compositions, and host movement, behavior, mortality events, and interactions with sympatric wildlife on the distribution and abundance of environmentally shed PrP^{CWD} in endemic areas is unknown.

Cervid interactions with soil and plants

Cervids tend to congregate in areas where pulsed resources or attractants may be present which may result in soils with high levels or "hotspots" of PrP^{CWD} contamination and risk for CWD transmission to visiting cervids. For example, annual acorn mast-fall alters deer home ranges and revisitations[134], which may result in natural PrP^{CWD} hotspots. Agricultural areas and attractants

such as backyard feeders, mineral licks, deer scrapes and baiting sites also result in congregation and revisitation of deer, potentially establishing foci for environmental PrP^{CWD} contamination where deer deliberately ingest soil[135-139]. Further, soil may serve both as an environmental reservoir and a facilitator of CWD prion transmission, where clay-rich soils in particular appear to bind prions, enhancing their infectiousness and transmission[140, 141]. How PrP^{TSE} interacts with soil may also influence the CWD disease system. Interaction of disease prions with soil could also alter prion conformation[142], resulting in changes that could potentially modify stability, bioavailability, and infectivity of disease prions[140, 143-146] depending on soil chemistry, textural class, and mineralogy. In addition, cervids are herbivorous mixed feeders that graze on herbaceous plants and browse woody shrubs and trees[147] and may come into contact with contaminated plant matter through foraging behaviors. A decade of research has led to a better understanding of how host browsing and grazing may facilitate risk of CWD transmission. Experimental work suggests that grass plants can bind, uptake, retain, and transport infectious prions[148]. Plants grown hydroponically and in solid state agar demonstrated uptake of PrP^{TSE} by plant roots, stems and leaves and subsequent infectivity of those plant tissues to mammalian hosts[149]. Additional reports also show that contact with prion-contaminated natural or man-made materials found in the environment such as rocks, wood, metals, and plastic debris may also serve as sources of disease transmission risk as PrP^{TSE} adsorbs to these materials while retaining infectivity[150].

Vertebrate carnivores, scavengers, and interspecific interactions

Interspecific encounters of wild cervids with sympatric wildlife include ecological interactions such as competition, predator-prey, parasitism, and mutualism. Among wildlife, foraging and scavenging behaviors are often correlated with an increase in exposure to infectious disease and contribution to disease dynamics[151-153]. Avian and mammalian scavengers and predators that frequent CWD-positive host carcass sites have the potential to influence CWD epidemiology[154, 155]. Consumption of CWD-positive carcasses may result in dispersal of PrP^{CWD} over a larger area, potentially resulting in less PrP^{CWD} deposited at carcass sites, but numerous smaller quantities of PrP^{CWD} dispersed elsewhere by predators and scavengers, by defecation after foraging at a carcass or translocating infected material during young provisioning. It is also reasonable to consider that some cervid consumer species may be removing or greatly reducing the magnitude of PrP^{CWD} from the landscape, if PrP^{CWD} is rendered inactivated or less infectious following consumption and digestion of infectious material. Cervid carcasses can persist for 18-101 days depending on season and geographic location, and are visited and revisited by species such as raccoons, opposums, coyotes, vultures, and crows [155]. While evaluation of these species suggests that a direct role in transmission is unlikely[156-158]; with the exception of swine[159], recent efforts indicate that some of these wildlife species mentioned above may have a more indirect role in the disease dynamics of TSEs[160, 161]. Coyotes (*Canis latrans*)[160], American crows (*Corvus brachyrhynchos*)[162], and cougars (*Puma concolor*)[163] shed misfolded prion following ingestion of PrP^{TSE}-contaminated material. Gut residency times appear to be fairly short, lasting $\leq$ 3 days in coyotes and cougars, to just several hours in crows. VanCauteren *et al.*[162] and Nichols *et al.*[160] suggest that consumers of PrP^{TSE}-contaminated cervid carrion could contribute to translocation and contamination of PrP^{CWD} in the

environment[160, 162], however Nichols *et al.*[160] also found a reduction of infectious PrP^{CWD} shed in coyote scat, implying scat-associated PrP^{CWD} deposits may be reduced compared to deposits associated with unconsumed contaminated carrion. Baune *et al.* found that most of the prions ingested by cougars are eliminated or sequestered, supporting the notion that predators may have a dilution effect on the CWD system[163]. Further, a modeling approach study determined that cougar and gray wolf (*Canis lupus*) predation pressure can independently decrease CWD outbreak size and delay prevalence increases of deer and elk, a cleansing effect amplified when predator selection for infected adults is greater than uninfected juveniles[164]. Predation by cougars may have slowed the increase in prevalence in an area of high CWD prevalence[165].

Following cervid mortality, avian and mammalian predators and scavengers ingest considerable amounts of contaminated material[155]. For example, cougar diets are composed of ~68% ungulate species[166], typically consuming up to three-quarters of an adult deer carcass in one or more feedings and carcasses of young deer are eaten almost in entirety at the first feeding[167, 168]. Although portions of cougar kills that are not immediately consumed are buried, guarded closely[167], and are typically eaten within 2-5 days[169-171], some level of kleptoparasitism of their cache is tolerated[172], and ~ 39% of their prey is either lost or abandoned to competitors and scavengers[173, 174]. This may allow increased opportunity for scavengers to consume contaminated carrion. Additionally, some scavenger species of cervid carrion, such as coyotes[175, 176], consume 10-12% of their body weight per day and up to two-thirds of edible portions of cervid carrion[176].

Certain avian species consume blood-feeding ectoparasites (i.e., deer keds, ticks) attached and feeding on cervids[177-179] and that both avian and small mammals (i.e., voles, shrews) may predate blood-feeding ectoparasites found in cervid bedding sites[177, 180, 181], some of which have been shown to harbor PrP^{CWD} [182, 183], suggesting that avian and small mammal species not typically associated with scavenging could also play a role in CWD epidemiology. Further consideration of other interspecific encounters or associations between birds and cervids or other mammals and cervids may be useful in better understanding the role of other wildlife in CWD epidemiology. Cleaning mutualism is a common interspecific encounter among birds and ungulates that often results in the removal of food by the bird from the larger mammal, presumably in the form of hematophagous ectoparasites that are easily accessible, such as ticks, deer keds, or flies. In North America, this particular kind of encounter has been observed between icterid [184], ardeid[185], as well as several corvid species, such as Western scrub-jay (*Aphelocoma californica*) [179, 186, 187], black-billed magpie (*Pica pica*)[181, 188, 189] and common raven (*Corvus corax*) [187] with cervids[181, 190] and other ungulates. Corvid necropsy evidence has demonstrated ingestion of engorged ticks following cleaning mutualism interactions[191, 192]. Cleaning mutualism has also been observed between Northern raccoons (*Procyon lotor*) and Key deer (*Odocoileus virginianus clavium*), where camera trap evidence demonstrates raccoons cleaning the face of the deer[193], and similar interactions between a Virginia opossum (*Didelphis virginiana*), an efficient predator of Ixodid ticks[194], and white-tailed deer (*Odocoileus virginianus*)[195]. More indirect forms of cleaning mutualism have been reported between insectivorous bats and WTD, where bats feast on hematophagous flies that swarm deer in high numbers during the summer season[196]. This consumption of ectoparasites further suggests a role for wildlife sympatric with cervids in CWD ecology.

Invertebrate species interactions

Wild and domestic ungulates are host to several hematophagous and sarcophagus ectoparasites such as ticks, deer keds, mites, and flies[191, 197]. Ticks, deer keds, and flies are commonly found feeding upon wild cervids[198], while mites and flies typically burden domestic sheep and cattle, although they are can become host to several species of ticks depending on location and level of shelter or care[199]. Sarcophagus flies that fed on scrapie infected brain material (*Sarcophaga carnaria*) have the capacity to act as mechanical vectors to orally exposed hamsters[200]. Homogenates of several species of mites gathered from scrapie infected sheep farms were also shown to be infectious in mice following intracerebral and intraperitoneal exposure routes[201, 202].

Of the various ectoparasites that parasitize cervids, ticks and deer keds are the most common[198]. Of these two, ticks arguably have the most potential to participate in the indirect transmission of CWD in wild and captive cervids due to several biological and behavioral traits. For example, ticks take a comparatively larger volume of bloodmeal than deer keds. An Ixodid tick bloodmeal can range from 0.5 mL to as high as 8.9 mL per female[203, 204], while a deer ked bloodmeal ranges from 1.5 µL to 3 µL[205, 206]. Another important difference is that deer keds feed periodically on different locations of the same host in 1-2 h bursts and completely digest their bloodmeal during each feeding[205, 206]. Ixodid ticks however remain attached to one bite location for as long as 13 days[207], during which there is a rapid engorgement phase in the last 24-36 h when blood is consumed and concentrated up to 300% due to reduced digestion and excretion of water and electrolytes[208]. This concentration of blood meal results in a fed body-weight increase in excess

of 100 times their unfed weight[204, 208] and is particularly pertinent since it may serve to concentrate PrP^{CWD}.

Though transmission of prion disease via parasitic feeding alone is unlikely due to lack of invertebrates expressing endogenous PrP^{C} [209, 210], cervids may encounter prion-infected blood through ingestion of ectoparasites during bouts of allogrooming, a known ectoparasite-defense mechanism[211-213] that involves grooming between individuals of the same species. This form of grooming is one of the most common nonaggressive interactions among females, females and young, as well as among males during the non-mating season of several cervid species such as white-tailed deer[214] and elk[213], during which ectoparasites may be consumed intentionally or unintentionally, as a result of grooming mechanics such as licking, or nibbling and chewing [214].

Blood from CWD-positive individuals represents a potent source of infectivity, as PrP^{CWD} is present during asymptomatic and symptomatic periods of the disease course[119-123], with concentrations higher than in urine[215] or feces[216]. Experimental evidence has demonstrated that CWD-positive whole blood has PrP^{CWD} loads ~ five orders of magnitude lower than that of CWD-positive brain, but exposure to blood may still result in transmission of CWD[217]. For example, whole blood from CWD-infected deer has an attack rate of up to 100% in cervids following peripheral exposure with 250 mL, and 22% in cervidized mice following oral exposure with 150 µL [218]. When considering the how to infer the risk that oral exposure to blood may present among cervids, it is important to understand the relative minimum amounts of PrP^{CWD} that result in oral

transmission of CWD. The relative minimum ID_{50} of PrP^{CWD} was assessed in a study where deer were orally exposed to increments of a CWD-positive brain homogenate derived from pooling several samples together and was found to be the equivalent to PrP^{CWD} loads present in ~300 ng of that pooled CWD-positive brain homogenate[130]. It is therefore reasonable to consider that an ~LD_{50} following oral exposure to CWD-positive blood may require amounts five orders of magnitude greater than that of brain (~30mg, or a volume of ~28.3 µL, based on volume-mass conversion of blood using the specific gravity of whole blood), which is well with the range of tick bloodmeal volumes.

These biological traits of ticks and the antiparasitic behaviors of cervids suggests that it may possible that an individual cervid could consume one or more partially or fully engorged ticks, orally exposing them to hematogenous transmission during a bout of allogrooming. Despite these compelling factors in support of ticks as potential vectors of CWD, their role in CWD epidemiology remains an underexplored area, with only two recent studies suggesting that nymphal ticks would be a poor vector for prion transmission, but that adults ticks may have the potential to take up PrP^{CWD} [183, 219].

It is also worth mentioning that other invertebrates (e.g., earthworms, slugs, beetles, etc.) that consume soil, sediment, and natural organic material (e.g., decaying plant matter, carcasses, feces, etc.) may interact with PrP^{CWD}-contaminated material. For example, earthworms are known decomposers of natural organic matter, including feces of white-tailed deer and other ungulates[220]. Experimental evidence has shown that recombinant prion proteins can survive digestion by earthworms[221] and that earthworms may play in horizontal transmission of infectious prions by

acting as passive carriers where PrP^{TSE} persists and can then be transported within soil environments[222]. Since endogenously expressed PrP^{C} is not conserved across invertebrate taxa[209, 210], it is unlikely that worms act as active carriers or biological vectors of PrP^{TSE} in the wild. However, due to their putative ability to transport PrP^{TSE} within the soil, they may serve as mechanical vectors through their direct interactions with environmental reservoirs such as soil and other natural organic materials. In addition, it is also possible that invertebrates interactions with PrP^{CWD}-contaminated material could also be a source of exposure risk for vermivorous and insectivorous wildlife, such as birds, rodents, foxes, and numerous invertebrates[223].

Prion detection methods for secreta, excreta, and environmental samples

Bioassays

Animal bioassay is still the gold standard for confirmation of TSE detection, despite the immense costs in both capital and labor. Environmental samples are viable in such assays, given proper treatment to ensure secondary infections or non-specific maladies do not endanger the subject animals. Within the subset of animal bioassays, an array of transgenic mouse models have been developed for the detection, confirmation, and study of TSEs in their competent hosts. PrP transgenic mouse lines for bovine[82, 224-230], sheep/goat[231-238], mink[239], and cervid[127, 240-245] have all been established as susceptible models for the corresponding TSEs. Other transgenic mouse lines expressing pig[246-248], rabbit[249], horse[250], and dog[251] have been developed to assess natural TSE disease resistance. Hamster[252-254], bank vole[255-257], and mouse[258-261] PrP have also been explored to compare and validate transmission studies or investigating the susceptibility of other species[160].

Additionally, studies with wild-type mice[162] and hamsters[106, 262, 263], guinea-pigs and gerbils[263], and voles[46, 262, 264-268] have also proven to be central in detection and transmission validation studies and understanding cross-species transmission of various TSEs and their associated strains.

Several recent developments in cell and organoid -based assays have been made that advance the study and detection of infectious prions. Various immortalized cells lines have proven useful in unraveling components of cellar mechanism TSE pathogenesis for various strains of BSE[269-272], scrapie[273-280], TME[281] and CWD[282-285]. Primary cell cultures are also useful in studying TSEs as they address several limitations of immortalized cell cultures, such as lacking the need for continuous and regular passage, are prepared from nervous system cell types[286], and have served to elucidate mechanisms of prion pathogenesis[287-290] and strain adaptation[291, 292]. Neurospheres, brain aggregates, organotypic slice cultures, and organoids are of the more advanced and relevant cellular based models being utilized to study transmission and therapeutic interventions of scrapie[293-295], CWD[296, 297], and BSE[294].

Immunoassays

Immunoassays have enabled the advancement of TSE diagnostics and offer rapid detection TSEs in numerous sample types. Immunoassays capitalize on the unique biochemical differences and degradation sensitivities between cellular prions and the various prion agents and strains. PrP^{C} has total protease sensitivity, while PrP^{TSE} typically has a protease-resistant core[16,298] with differing core lengths and subsequent proteolytic digestion profiles among the various prion agents and strains[46, 299]. Although immunoassays can provide rapid diagnostic results they are limited to

sensitive detection in samples that typically contain high levels of prion (e.g., brain or lymphatic tissues) and have been shown to of little use for analysis of environmental samples types that either contain prion levels that are too low for detection by these assays or contain substances that interfere with antibody-based detection[300-302].

Western blotting (immunoblotting) is an immunoassay that has been utilized for detection and characterization of TSEs since the early 1980's [225, 303]. This method of detection has been employed for various nervous tissues, as well as for environmental samples, such as soils[141, 304]. Western blotting can simultaneously distinguish between PrP^{C} and PrP^{TSE} while providing information about the unique biochemical properties that help to distinguish one prion agent or strain from another, such as the degree of proteolytic resistance, protease resistant core size, and the ratio of the un-, mono-, and diglycosylated PrP glycoforms when used in combination with a proteinase-K pre-treatment to digest any PrP^{C} content[305-308]. This feature of TSEs however is not without drawbacks, as prion agents can vary in protease sensitivity or are not as resistant to proteolysis during enzymatic pre-treatments, resulting in partial digestion and subsequent underestimations of TSE levels present in a given sample[309]. Another limiting aspect of Western blotting is the lack of sensitivity for biological materials that contain lower amounts of prion agent. To address the limits of detection by Western blotting, amplification assays, such as the protein misfolding cyclic amplification assay (PMCA), is often used to first amplify the prion agent within a given sample type, before the final product is evaluated by Western blot, as discussed in more detail in the next section. Enzyme immunoassays (EIA) are another antibody-antigen-based diagnostic tool applicable across all TSEs. As with Western blotting, these immunoassays typically utilize spinal

fluid[310], brain and lymphoid tissues[310-312], and have also proved useful in evaluating soil-TSE interactions[313-317].

Seeded amplification assays

Seeded amplification assays can detect ultra-low levels of PrP^{TSE} from a suite of sample types that include various secreta, excreta, tissues, and abiotic and biotic environmental samples not typically detectable by immunoassays. At their most elementary level, seeded amplification assays exploit the propensity of PrP^{TSE} to induce conformational conversion of a PrP^{C} substrate and allow for detection of femtogram to attogram amounts of PrP^{TSE}, making them as or more sensitive as mouse bioassay and far more sensitive than immunoassays[318, 319].

Protein misfolding cyclic amplification (PMCA) seeded amplification assay is an *in vitro* assay that utilizes repeated cycles of sonicating and incubating to facilitate cyclic amplification of protein misfolding[320, 321]. The reaction requires excess amounts of PrP^{C}, derived from susceptible mammalian hosts (typically transgenic or wild type mice), be incubated with small quantities test samples, which if containing PrP^{TSE} seeding material, then induces a conformational change of PrP^{C} to PrP^{TSE}. Repeated cycles of sonication facilitate newly formed PrP^{TSE} aggregates to fragment, which are then able to readily convert more PrP^{C} present in the reaction. Over the course of 48-96 hours, these repeated cycles of sonication and incubation produce an amplified product of the PrP^{Sc} seed that requires the use of protein solving gel-electrophoresis and Western blotting to visualize [322] .

PMCA has been used for detection studies of several TSEs and has utility for detection of prions in relevant environmental matrices, such as soil[138] and water[323]. Other relevant matrices that either serve as putative reservoirs of prions or are potential sources of prion accumulation within the environment; such as plants[150] and soil[133, 313, 317, 324-326], feces (from host and scavengers)[160, 327, 328], urine[328, 329], reproductive tissues and fluids[31], or invertebrates[222] have also been screened for prions using PMCA. This amplification assay has also been used to advance the ability to detect PrP^{TSE} in several other matrices where other assays have failed, including in skeletal muscle[330], blood[119, 122, 331, 332], and saliva[333, 334]. Although PMCA offer highly sensitive detection of prions in a wide array of sample types, further develop of an improved amplification tool is driven by the need for an assay that (1) can overcome assay-sample incompatibilities, ii) has improve sensitivity and specificity outcomes, (2) can detect all potential infectious prion conformers, including any protease sensitive prion conformers, (3) has high throughput capacity and requires shorter assay times, (4) can utilize alternative PrP^C conversion substrates without relying the continued use of animal hosts, and (5) furnish a practical way to interpret assay results for easier quantification.

Developed around the same timeframe as PMCA is the real-time quaking induced conversion (RT-QuIC) seeded amplification assay[335-337], which meets many of the amplification assay needs described above. Utilization of the RT-QuIC assay requires small volumes of test sample be combined with a reaction mix containing an appropriate recombinantly expressed PrP^C ($rPrP^C$) and thioflavin-T dye (ThT) in wells of either a 96 or 384-well plate. Plated samples are then subjected to cycles of shaking and resting under temperature control within a plate reader. Assay run times are typically 40-50 hours, during which time any amyloid fibrils that form presumably

intercalate with ThT[338], which once bound to fibrils, exhibits a different emission spectrum than when unbound, allowing for amyloid formation to be monitored in real-time. The RT-QuIC assay possesses distinct advantages over PMCA. For example, the RT-QuIC utilizes recombinant PrP^{C} ($rPrP^{C}$) that can be quickly and consistently produced from various bacterial expression systems (typically *E. coli*) designed to produce a number of cervid and non-cervid recombinant substrates, rather than relying on PrP^{C} derived from brain tissue of transgenic mice[337, 339]. Other advantages are that the RT-QuIC assay allows for amyloid amplification to be monitored in real time resulting in a simple fluorescence curve that allows for some level of quantification. Additionally, RT-QuIC assay run times are considerably shorter than PMCA.

In the last decade, RT-QuIC assays have been developed for detection of most types of PrP^{TSE} agents of human sporadic, heritable, and infectious prion disorders[340], as well as PrP^{TSE} strains affecting non-human animals such as CWD, scrapie, TME, and BSE[129, 217, 318, 341-350]. The adaptation of RT-QuIC for research, diagnostics, and surveillance of CWD in particular has led to critically needed advances in practical tools for understanding the implications of host PrP^{CWD} shedding and ecological factors of CWD epidemiology. Recent reports showcase the robust versatility of this assay for detection of PrP^{CWD} in lymphoid tissues and nasal brushings[351], saliva[341, 352], urine[352] and feces[216, 353], as well as from potentially ecologically relevant abiotic and biotic samples types such as soil[354], and black-legged ticks from white-tailed deer[182], winter ticks from North America elk[183], and excrement from predators and scavengers of cervids[163, 355]. RT-QuIC can also be applied to provide a semi-quantitative assessment of the relative amounts of PrP^{CWD} in a test sample, making it very useful in estimating risk of host interactions with PrP^{CWD}-

contaminated environments[129]. It is also important to note that, similar to PMCA, RT-QuIC has demonstrated limitations in diagnostic sensitivity (false negative results from samples known to be infectious) and specificity (false positive results for samples from disease-free sources)[344, 356] for some sample types. However, tuning of assay conditions and sample enrichment have proven useful in overcoming sensitivity and specificity limitations for various sample matrices[216, 357, 358], in addition to combined use of both assays to maximize the strengths of each for improve diagnostic sensitivity[334]. While the RT-QuIC assay clearly provides a practical way to detect PrP^{CWD} and other PrP^{TSE} strains, further improvements in quantitative precision and range of applications are still needed and would be helpful in continuing to assess unconventional (non-tissue) sample types.

Overview of dissertation chapters

CWD is a highly infectious and fatal neurodegenerative disease of cervids with a geographic range that is both expanding broadly and increasing endemically, despite state and federal management efforts. Prolonged host shedding and stability of PrP^{CWD} in the environment is thought to drive endemic prevalence increases and contribute to broad geographic spread of CWD, with the potential to promote cervid population declines. Interactions of hosts and sympatric wildlife with PrP^{CWD}-contaminated abiotic and biotic environmental factors may be important in mediating uncontrolled endemic increases in CWD and furthering geographic spread, however an assessment of these factors and their influence on CWD epidemiology remains incomplete or overlooked altogether. The aim of this thesis was to address this shortcoming through implementing a

semiquantitative approach with RT-QuIC to evaluate abiotic and biotic factors hypothesized to play some role in structuring CWD processes in the environment.

Chapter 2 describes the ability of ticks to assimilate and excrete PrP^{CWD} from CWD-positive bloodmeals and assess the potential risk of oral exposure of deer to ticks that have taken a bloodmeal from a CWD-positive animal, with implications for a common host behavior that my influence CWD exposure events. The utility of RT-QuIC for detection and relative quantification of PrP^{CWD} in spike and field collected excrement of cervid consumers was assessed in *chapter 3*. In *chapter 4*, a field and laboratory study were conducted for collection and RT-QuIC characterization of PrP^{CWD} accumulation dynamics in surface soil samples from decommissioned artificial mineral lick sites within the study area where CWD has recently established. Finally, I discuss my work and what the next steps could be to better understand ecological process underpinning transmission risk and geographic expansion of CWD in wild cervids.

This dissertation was driven by the question: What are the roles of abiotic and biotic factors in structuring how and where CWD transmission is occurring among free-ranging cervids? The lingering question was if we could provide a semiquantitative assessment of certain abiotic and biotic samples from the field. Having a way to assessing fine-scale environmental factors would advance our understanding of what is driving CWD transmission events and endemic increases in CWD prevalence and will aid in informing wildlife and disease management adaptation strategies for limiting further geographic expansion of CWD. This dissertation research was also driven by

a personal interest and passion for wildlife disease ecology and wildlife behavior. The varying behavioral traits of differing wildlife taxa can be tightly associated in epidemiology of infectious disease and are often underappreciated and underexplored in understanding drivers of infectious disease processes[359]. The CWD system is one example where we see the critical need to recognize these associations if we are to succeed in effectively managing this disease.

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CHAPTER TWO: Ticks harbor and excrete chronic wasting disease prions

ABSTRACT

Chronic wasting disease (CWD) is a fatal neurodegenerative disease caused by infectious prions (PrP^{CWD}) affecting cervids. Circulating PrP^{CWD} in blood may pose a risk for indirect transmission by way of hematophagous ectoparasites acting as mechanical vectors. Cervids can carry high tick infestations and exhibit allogrooming, a common tick defense strategy between conspecifics. Ingestion of ticks during allogrooming may expose naïve animals to CWD, if ticks harbor PrP^{CWD}. This study investigates whether ticks can harbor transmission-relevant quantities of PrP^{CWD} by combining experimental tick feeding trials and evaluation of ticks from free-ranging white-tailed deer (*Odocoileus virginianus*). Using the real-time quaking-induced conversion (RT-QuIC) assay, we show that black-legged ticks (*Ixodes scapularis*) fed PrP^{CWD}-spiked blood using artificial membranes ingest and excrete PrP^{CWD}. Combining results of RT-QuIC and protein misfolding cyclic amplification (PMCA), we detected seeding activity from 6 of 15 (40%) pooled tick samples collected from wild CWD-infected white-tailed deer. Seeding activities in ticks were analogous to 10 –1,000 ng of CWD-positive retropharyngeal lymph node collected from deer upon which they were feeding. Estimates revealed a median infectious dose range of 0.3 – 42.4 per tick, suggesting that ticks can take up transmission-relevant amounts of PrP^{CWD} and may pose a CWD risk to cervids.

INTRODUCTION

Chronic wasting disease (CWD) is an infectious, slowly progressing, and invariably fatal neurodegenerative disease afflicting wild and domestic cervids. Both direct and indirect routes of exposure and transmission have contributed to endemic increases and broad geographic spread of CWD [1]. However, there are significant gaps in our understanding of how CWD is transmitted among susceptible hosts. Proposed putative routes of exposure and transmission among susceptible cervid species include sexual contact [2], consumption of contaminated soil, water, and plants [3-5], mucosal contact with contaminated fomites [6], or antler cannibalism behavior [7]. With many unknowns about transmission pathways and their relative risks it is prudent to consider how host behavior and life-history traits facilitate CWD exposure events. Here we examine the potential for ticks to contribute to indirect transmission of CWD.

In the host, CWD presents with a broad distribution of disease-associated prions (PrP^{CWD}) in peripheral tissues and biological fluids prior to neuroinvasion. Blood of prion-infected animals harbors infectivity at the pre-symptomatic disease stage with relatively higher circulating amounts of PrP^{CWD} than those found in urine [8] or feces [9]. Animal challenge studies demonstrate that CWD-positive whole blood has a disease attack rate of up to 100% in cervids following intravenous exposure with 250 mL and 22% in cervidized mice following oral exposure with 150 µL [10].

Arthropods that interact with cervids, such as biting flies or blood-obligate ectoparasites, could play a role in prion transmission. Early investigations revealed that homogenates of several species of mites gathered from scrapie infected sheep farms harbored infectivity following intracerebral

(i.c.) and intraperitoneal (i.p.) exposure of mice [11]. Recent studies examining the role of ticks in transmission of transmissible spongiform encephalopathies (TSEs) suggest that nymphal ticks would be poor mechanical vectors for certain disease-causing prions, but that adults of at least one species of Ixodid tick may have the potential to take up PrP^{CWD} [12, 13]. Ticks possess several biological and behavioral traits that may implicate their involvement in indirect transmission. Ticks take a blood meal that can range in volume from 0.3 mL to as high as 8.9 mL per female [14]. Ixodid ticks remain attached to one bite location for as long as 14 days [14], during which time there is a rapid engorgement phase in the last 24-36 hours where consumed blood is concentrated due to reduced digestion and excretion of water and electrolytes [15]. This concentration of blood meal results in a fed body weight increase in excess of 100 times their unfed weight [16]. This tick feeding behavior may be pertinent to disease transmission since it may concentrate infectious prions.

Arthropods and other invertebrates do not express cellular prion (PrP^{C}), a prerequisite for establishing a prion infection [17], indicating that a more likely role for prion disease transmission by ticks would be as a mechanical vector rather than as a biological vector. Cervids may encounter ticks harboring prion infected blood during bouts of allogrooming, an ectoparasite-defense strategy used by social mammals [18] that involves grooming between members of the same species. This form of grooming is one of the most common nonaggressive interactions among females, females and young, as well as among males during the non-mating season of several cervid species [18, 19]. Higher ectoparasite infestations increase allogrooming behavior in cervids such as white-tailed deer (*Odocoileus virginianus*, WTD) [19] and elk (*Cervus canadensis*) [18], during which ectoparasites may be consumed intentionally or unintentionally, as a result of grooming mechanics such as

licking, or nibbling and chewing [19]. These host and parasite traits may make allogrooming a possible transmission pathway for CWD, if hosts consume partially or fully engorged ticks during bouts of allogrooming.

Using an ultrasensitive *in vitro* prion amplification assay, real-time quaking-induced conversion (RT-QuIC), we investigate this hypothesized pathway to i) determine experimentally if ticks can harbor prions taken up from infected blood meals and ii) survey ticks on CWD-positive WTD to determine if PrP^{CWD} can be detected in ticks collected from free-ranging deer in a CWD endemic region. Given that prion seeding activity was detected in these ticks, we further iii) estimated amounts of PrP^{CWD} found in ticks relative to amounts of PrP^{CWD} found in CWD-positive deer lymphatic tissues, and iv) estimated a per-tick infectious dose (ID_{50}) based on the pooled tick amyloid formation rates (AFR) (i.e., 1/time to threshold) equivalence to AFRs of retropharyngeal lymph node (RPLN) combined with an established minimum mass of CWD-positive brain sufficient to orally transmit CWD disease.

RESULTS

Our experimental spiking studies aimed to test recovery of PrP^{CWD} from different sample types, using a brain sample (from the obex region) from a late-stage CWD-positive WTD as the source material for spiking in all experiments. We were able to detect PrP^{CWD} using RT-QuIC from samples spiked with PrP^{CWD}, including blood, tick homogenates and homogenates of ticks fed blood spiked with PrP^{CWD}. Whole blood or tick homogenates spiked with 10-fold dilutions of

CWD-positive brain homogenates showed sensitivity of at least 10^{-6}, which corresponds with the sensitivity detection limit for the brain sample used for the spiking experiments (Fig. 1a, 1b, 1c, 1d). Blood and engorged tick homogenates spiked with 10^{-3} through 10^{-5} dilutions of CWD-positive brain showed PrP^{CWD} seeding activity (assay fluorescence from sample with PrP^{CWD} present) in 8/8 technical replicates (Fig. 1a, 1b, 1c). The blood and tick homogenates spiked with the 10^{-6} dilution of the CWD-positive brain showed PrP^{CWD} seeding activity in 7/8 and 4/8 replicates, respectively (Fig. 1a, 1b, 1c). Neither blood nor tick homogenate sample types produced any false seeding activity, constituting a specificity rate of 100% for each sample type using the RT-QuIC assay in these spiking experiments (Fig. 1a). AFR values for brain-spiked samples differed among sample types (F (2, 68) = 18.626, $p < 0.0001$), with mean AFR values higher for blood (mean ± standard error: 0.1 ± 0.003), compared with brain (0.09 ± 0.004), or tick homogenates (0.08 ± 0.003; Tukey honestly significant difference (HSD) post-hoc test blood versus brain: 0.01 ± 0.006, $p = 0.03$; blood versus tick: 0.2 ± 0.005 $p<0.001$). AFR values for all sample types decreased across the dilution series (F (3, 68) = 131.352, $p < 0.0001$), however, there was no statistically significant interaction between AFR values by sample type across the dilution series (F (6, 68) = 0.871, $p > 0.05$).

To evaluate whether ticks can take up and excrete prions, we used a previously established artificial tick membrane-feeding system [20] to feed *I. scapularis* a blood meal inoculated with a 10^{-3} dilution of CWD-positive brain (10^6 ng) (Fig. 1e, 1f,1g). Although, the relative amounts of PrP^{CWD} found in blood of CWD-infected deer is likely ~ 1000-fold less than the dilution used to inoculate blood meals [21], this 10^{-3} mg/mL dilution was chosen to demonstrate the potential for *I. scapularis* to assimilate prions from a blood meal rather than demonstrate natural uptake. Feeding assay

attachment rates reached 100% by 72 hours following placement of adult female and male ticks within blood-exposed feeding chambers for all treatment groups and individual engorgement occurred between day 9 and 14 across both treatment groups. Serial dilutions of tick homogenates from the 10^{-3} PrP^{CWD} membrane-fed exposure group demonstrated PrP^{CWD} seeding activity in 15/24, 24/24, 15/24, and 2/24 technical replicates for inoculum-based dilutions of 10^{-3} through 10^{-6}, respectively (Fig. 1h, 1j). Serial dilutions of tick frass collected from the 10^{-3} PrP^{CWD} treatment group demonstrated seeding activity in 24/24 replicates for inoculum-based dilutions of 10^{-3} through 10^{-5}, and 22/24 replicates for the 10^{-6} inoculum-based dilution (Fig. 1h, 1i). AFR values were significantly different based on sample type (F (2, 196) = 24.753, $p < 0.0001$). Mean AFRs were significantly higher for frass (mean ± standard error: 0.093 ± 0.0038), and brain (0.088 ± 0.0094), compared to tick homogenates (0.06 ± 0.004; Tukey HSD post-hoc test of frass versus tick: 0.04, ± 0.005, $p < 0.0001$; brain versus tick: 0.03, ± 0.010, $p = 0.005$). AFR values differed along the dilution series (F (3, 196) = 24.848, $p < 0.0001$), with a significant interaction between sample type and dilution (F (6, 196) = 5.759, $p < 0.0001$; significant Tukey HSD post-hoc test: frass and brain for the 10^{-3} dilution: -0.05, ± 0.02, $p = 0.036$; frass and tick at dilutions 10^{-4} to 10^{-6}: +0.05, ± 0.01, $p< 0.0001$; 0.07, ± 0.01, $p< 0.0001$; 0.05, ± 0.01, $p< 0.0001$, respectively).

After these proof-of-concept experiments, we examined ~2,000 Wisconsin hunter-harvested deer heads for ticks. Of the 2,000 heads examined, 174 were tick-infested. From the sample set of 174 heads infested with ticks that were evaluated for CWD in RPLN through enzyme-linked immunosorbent assay (ELISA), 15 tested positive (data not shown). CWD status in these heads were cross-confirmed by RT-QuIC, providing similar results. Then, we determined if prions could

be detected in ear tissue and in engorged ticks from these 15 CWD-positive WTD (See Supplementary Table S1 for county harvested in). As negative control, 15 additional pooled tick samples collected from CWD-negative WTD were included in this analysis but were analyzed without blinding in completely separate experiments (Supplemental Figures S1 and S2). The number of attached and partially or fully engorged ticks collected from each WTD head examined, regardless of CWD status, ranged from 1 to 30 (6.1 ±5.4). The number of attached ticks ranged from 2 to 8 (3.7 ±1.8) for the 15 CWD-positive deer heads and 3 to 16 (5.3 ±4.4) for the 15 CWD-negative deer heads.

No false seeding activity was observed for tick or ear tissue samples collected from CWD-negative WTD. However, detection of PrP^{CWD} in these peripheral samples (ticks and ear tissue) was limited compared to detection in RPLN for each of the 15 CWD-positive WTD (Fig. 2a, Supplementary Table S2). Comparing AFR values among sample types, ear samples were positively correlated with wild-fed tick samples (R^2 = 0.5, t = 3.62, p-value = 0.003, N=15; Fig. 2d), suggesting that ticks may perform as well as ear tissues in detecting prions. However, RPLN AFRs were not correlated with seeding activity in either ear tissues (R^2 = 0.15, t = 1.51, p-value = 0.155, N = 15; Fig. 2c) or tick samples (R^2 = 0.02, t = 0.5, p-value = 0.628, N = 15; Fig. 2e), indicating that these peripheral samples had reduced sensitivity for detecting prions compared to the RPLN tissue samples in our study using RT-QuIC. Most of the ear samples showed positive seeding activity to only a 10^{-2} dilution using RT-QuIC, however those that demonstrated seeding activity out to a 10^{-3}, 10^{-4}, 10^{-5} dilution also appeared to be reflective of higher AFRs from the corresponding tick samples that we determined to be CWD-positive (Sample IDs 1 (7/8), 4 (7/8), 11 (3/8) (p-values

0.0722, 0.0015, 0.0182, respectively, using Dunnett's Multiple Comparison Test) (Fig. 2b, 2a). These findings by RT-QuIC indicate a CWD prevalence of 20% (3/15) in *I. scapularis* based on this specific sample of CWD-positive WTD, and suggest that i) circulation of PrP^{CWD} in peripheral tissues is associated with detectable levels of prionemia, which is consistent with previous evaluations of peripheral levels of PrP^{CWD} during presymptomatic and symptomatic stages of the disease [22, 23] and ii) that ear tissue or attached and partially or fully engorged ticks may be a less sensitive sample source for CWD diagnostics compared to RPLN using RT-QuIC. Nevertheless, our data indicate that ticks may be considered as an *antemortem* detection method.

While it is possible that the variation observed in seeding activity from peripheral samples compared to RPLN may have been the result of differences in assay sensitivity for the different sample types, it may also be explained by other factors. The variation observed could have been influenced by the differences in total mass of tick per pooled tick sample; however, no correlation was found between higher or lower AFR values and higher or lower pooled tick samples mass ($R^2 = 0.02$, $t = 0.46$, p-value = 0.66, $N = 15$; Fig. 2f). Additionally, differences in polymorphisms of the prion protein encoding gene (*PRNP*) can directly influence the rate of disease progression and distribution of PrP^{CWD} in WTD [24-27]. The WTD sample size in this study was not large enough to make inferences on how genotype may explain the variable distribution of seeding activity from tick or ear tissue in relation to activity from RPLN. However, genotyping results did show that all ticks from CWD-positive 96G/96S or 96S/96S animals were negative by RT-QuIC (Supplementary Table S1). This is in agreement with the previously suggested delayed accumulation of prions in peripheral tissues by animals harboring 96S alleles [26, 27].

Because the pooled tick samples collected from free-ranging CWD-positive WTD appeared to contain relatively low levels of PrP^{CWD} detectable by RT-QuIC, we employed an additional protein amplification assay, the protein misfolding cyclic amplification (PMCA) technique, to further assess the presence of PrP^{CWD} in these samples. This assay is akin to RT-QuIC, but uses brain extract from healthy rodents as a source of PrP substrate and relies on cycles of sonication rather than shaking [28] for sensitive and specific detection of PrP^{CWD} (Fig. 2i). Relevant to this study, previous reports have shown that PMCA is able to amplify low levels of PrP^{CWD} from a wide variety of animal-derived and environmental samples [2, 4-6, 23, 29-33]. From the 30 pooled tick samples collected from CWD-positive and negative WTD previously analyzed by RT-QuIC, PMCA-seeding activity was identified in four samples (Fig. 2g, 2h). Sample IDs 7, 10, and 15, which were negative based on RT-QuIC seeding activity, showed positive PMCA detection in 1/2 technical replicates. The fourth sample to test positive by PMCA, sample 11 (positive PMCA detection in 2/2 replicates) (Fig. 2g, 2h), was the only pooled tick sample that was positive based on both assays (Fig. 2a, 2g, 2h). Interestingly, this sample also demonstrated the highest seeding activity across all 15 WTD RPLN tested by RT-QuIC and had some of the most sensitive ear tissue seeding activity (Fig. 2a, 2b). These PMCA results demonstrate a CWD prevalence of 26.7% in *I. scapularis* based on this specific sample of CWD-positive WTD. However, if we consider where the detection of PrP^{CWD} agreed between the two amplification assays, the prevalence was only 6.7%.

Because titers of PrP^{CWD} from a CWD-positive brain are similar to those found in CWD-positive RPLN [34], we were able to first extrapolate predicted values for the mass of seeding material present in each RT-QuIC, CWD-positive pooled tick sample (Fig. 3). Then, based on the predicted mass of seeding material present and the previously reported minimum oral ID_{50} of CWD-positive brain [35], we further estimated an ID_{50} for ticks from WTD samples 1, 4, and 11 to be 0.3, 42.4, and 6.9, respectively (equations (1) and (3) in Data Analysis, Supplementary Table S3). These estimations suggest that a single *I. scapularis* tick taking a blood meal (i.e., fully, or partially engorged) from a CWD-positive WTD poses a risk to naïve individuals if consumed during social interactions.

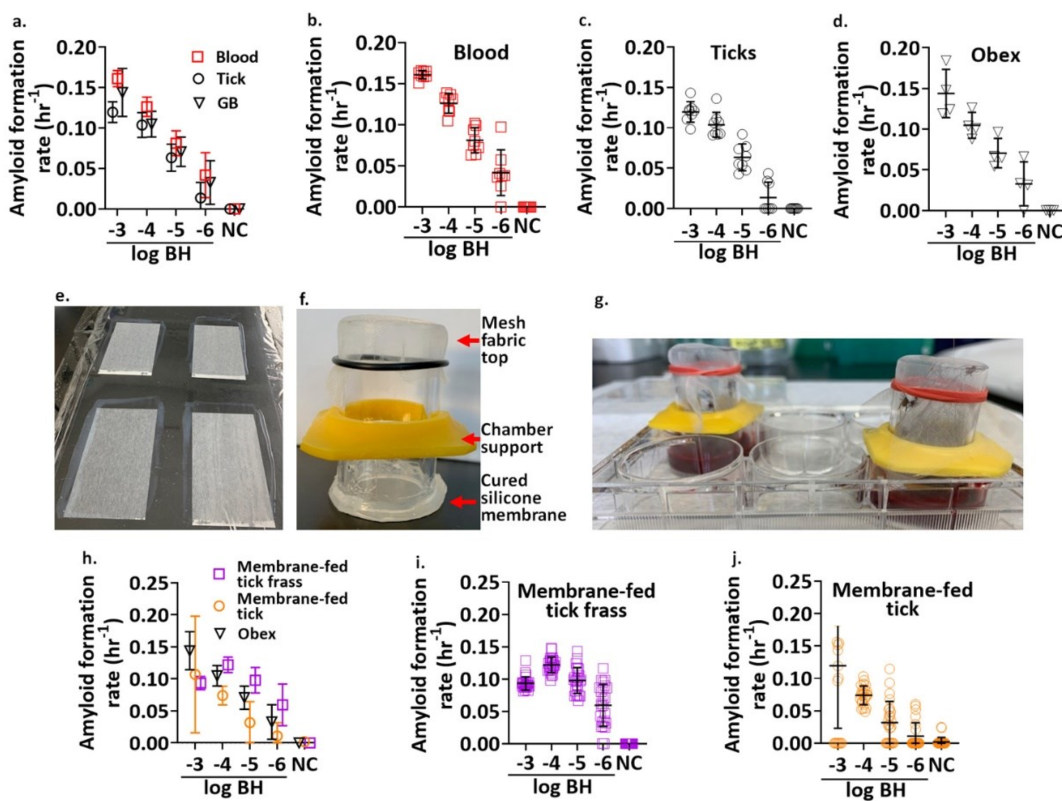


Figure 1. Recovery of chronic wasting disease (CWD) prions (PrP^{CWD}) from spiking and membrane feeding experiments for analysis using the real-time quaking-induced conversion (RT-QuIC) assay. Comparison of amyloid formation rates (AFR) by RT-QuIC of **(a-c)** defibrinated bovine whole blood spiked with a 10^{-3} dilution of the same 10% brain homogenate as used for the brain dilution series, and artificial membrane-fed tick homogenates spiked in the same manner as the blood ("b" and "c" depict AFRs of all 8 technical replicates for spiked whole blood or tick homogenates averaged in "a") or **(d)**10-fold dilutions of 10% brain homogenate (BH) (from the obex region) from a CWD-positive white-tailed deer. Membrane feeding units were constructed using **(e)** cured silicon membranes adhered to the base of **(f)** assembled feeding chambers. **(g)** Depiction of the assembled feeding unit with feeding chambers being held upright by the chamber supports. **(h-j)** Comparison of AFRs by RT-QuIC of homogenates from membrane-fed PrP^{CWD} exposed or negative control ticks and tick frass ("i "and "j" depict AFRs

for all 24 technical replicates (from 3 biological replicates run on 3 separate plates) for frass or membrane-fed tick homogenates averaged in "h"). Negative controls (NC) in each AFR plot are representative for the same sample type.

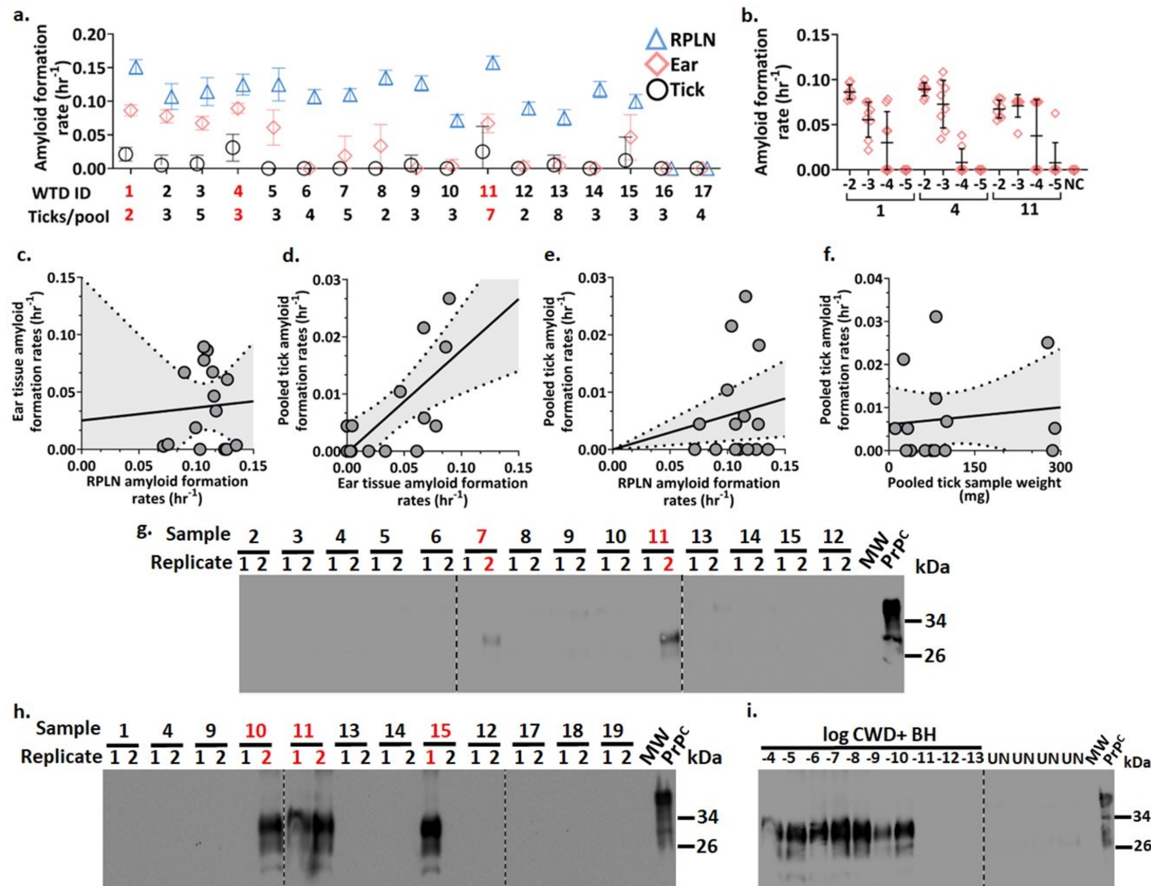


Figure 2. Presence of chronic wasting disease (CWD) prions (PrP^{CWD}) in tissues and *Ixodes scapularis* from hunter-harvested white-tailed deer (WTD) assessed by two protein amplification assays. Comparisons of real-time quaking-induced conversation (RT-QuIC) amyloid formation rates (AFR) for **(a)** deer retropharyngeal lymph node (RPLN), ear tissue (pinna), and pooled tick samples from 15 CWD-positive (ID 1-15) and two of the 15 CWD-negative (ID 16,17) WTD (all other negative sample results are shown in Supplementary Table S2, Supplementary Figure S1 and S2) and **(b)** 10-fold dilutions of ear tissue homogenates (ID 1,4,11). Samples are grouped by WTD IDs on the x-axis. Data points represent mean AFR ± standard deviation of 8 technical replicates. Negative controls (NC) represent the same sample

types. **(c-f)** Scatterplots with fitted linear regression line with 95% confidence intervals comparing AFR relationships between **(c)** RPLN to ear samples, **(d)** tick to ear samples, **(e)** RPLN to tick samples, or **(f)** pooled tick samples with pooled tick sample weight in milligrams (mg). Each point represents the mean AFR of 8 technical replicates from WTD ID 1-15. **(g-i)** Western blot analysis of PMCA products to assess the presence of prion seeding activity for; **(g)** 14 pooled tick samples (WTD IDs 2-15) stored in RT-QuIC sample buffer evaluated in "a"; **(h)** 10% tick homogenates (ID 17-19 are NCs); **(i)** serial dilutions of a CWD-positive (CWD+) brain homogenate (BH) (PMCA positive control), and unseeded (UN) or cellular prion (PrP^{C}) (PMCA NCs). (Original uncropped blots/gels are presented in Supplementary Figure S3) Samples analyzed in this figure were tested in duplicate and represent a third PMCA round. Numbers at the right of each panel represent molecular weight markers in kilodaltons (kDa). Red font indicates samples determined to contain PrP^{CWD} by either RT-QuIC or PMCA.

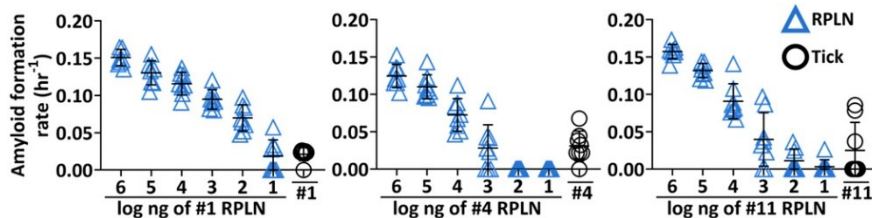


Figure 3. Comparison of chronic wasting disease (CWD) prion (PrP^{CWD}) loads by *Ixodes scapularis* following infected blood meal with retropharyngeal lymph node (RPLN) from free-ranging white-tailed deer (WTD). Comparisons of relative PrP^{CWD} loads present in three separate pooled tick homogenates (ID numbers 1, 4, 11) from Figure 2a, which tested CWD-positive by our parameters, against a 10-fold dilution series of RPLN from corresponding WTD. Data points for 8 technical replicates are depicted for each sample ± standard deviation.

DISCUSSION

Natural modes of indirect transmission of CWD among free-ranging cervids remain poorly examined and may perpetuate endemic increases and broad geographic spread of the disease [1]. The presence of PrP^{CWD} in blood may pose a risk for indirect transmission by way of hematophagous ectoparasites acting as mechanical vectors, as cervids can carry high tick infestations and exhibit allogrooming, a common tick defense strategy between conspecifics. However, the role of ticks as mechanical vectors of CWD remains unclear. Here, we demonstrate that a single adult *I. scapularis* found feeding on a CWD-infected WTD may contain approximately 0.3 - 42.4 ID_{50} per tick. These findings suggest that consumption of ticks by deer during bouts of allogrooming may facilitate oral exposure of PrP^{CWD} from ticks that consumed blood meals from CWD-infected deer. We demonstrate with artificial membrane feeding assays that i) *I. scapularis* has the capacity to ingest and excrete PrP^{CWD} and ii) sensitivity and specificity of PrP^{CWD}-exposed ticks with RT-QuIC to

validate the presence or absence of PrP^{CWD} in wild-fed ticks. Our results show that RT-QuIC seeding activities in wild-fed ticks were analogous to 10 to 1,000 ng of CWD-infected RPLN from each animal (Fig. 3) and peripheral samples were less sensitive using RT-QuIC compared to RPLN (Fig. 2a). Using both RT-QuIC and PMCA, we showed seeding activity in 6 (4 by PMCA, 3 by RT-QuIC, 1 by both) of 15 pooled tick samples removed from wild CWD-infected white-tailed deer (Fig. 2a, 2g, 2h). These results indicate CWD prevalence ranging from 7 - 40% in *I. scapularis* that had fed on CWD-infected WTD and suggest that i) the amount of PrP^{CWD} present in tick samples were near the detection threshold for each method and ii) that when the amount of PrP^{CWD} present in a given sample is relatively low, multiple methods increase the chance of detection in ticks.

Although the ultra-sensitive capabilities of both RT-QuIC and PMCA are well established for detection of misfolded prions in blood from experimental and naturally occurring prion disease [8, 21, 36] and lymph and skin tissues [37, 38], we observed variation between the two amplification assays in which pooled tick samples were determined CWD-positive (Fig. 2a, 2g, 2h). While both RT-QuIC and PMCA are clearly capable of detecting misfolded prions from blood samples, an understanding of how blood-engorged tick extracts behave in either assay is limited. Some sample types have highly sensitive and specific detection (e.g., RPLN), while others have lower sensitivity —often attributed to reaction inhibitors, depending on sample type and amplification assay, necessitating sample or assay optimization to improve sensitivity (e.g., saliva) [39]. Our optimization methods for both whole blood and blood-engorged ticks allowed for sensitive and specific detection of PrP^{CWD} from both sample types by RT-QuIC for the spiking experiments (Fig. 1a,

1h). However, whole blood contained within the engorged ticks may have influenced assay sensitivity, as whole blood components have been shown to inhibit detection of misfolded prion by RT-QuIC [40] and PMCA [41], which may explain the sensitivity differences between the two assays. The PMCA assay has been utilized to readily detect misfolded prions in blood seemingly without the need for extensive optimization to overcome inhibitors [23] and was therefore used in this study to cross analyze the pooled engorged tick samples tested by RT-QuIC. Although we were able to detect the presence of PrP^{CWD} in pooled engorged tick samples by PMCA as well as RT-QuIC, the samples only detected a single sample in common. This relative inconsistency in sample detection between assays could be due to low circulating levels of misfolded prions, which is known to result in lower and inconsistent assay sensitivity [42]. Therefore, it is possible that the differences in sensitivity between RT-QuIC and PMCA observed in this study for detecting PrP^{CWD} from wild-fed tick samples may have been the result of low circulating levels of PrP^{CWD} in blood, hence low amounts of PrP^{CWD} present in tick samples. Additional research in this area could determine the comparative power of these two techniques for this specific sample type.

We fully recognize that the 10^{-3} mg/ mL dilution used for the spiking blood meals for the membrane feeding assays is not typical of what is found in blood of early or late-stage CWD-infected deer and that based on results from McNulty *et al.* [21], the relative concentration of prions in blood is likely ~ 3 orders of magnitude lower than a 10^{-3} mg/ mL concentration of CWD-positive brain. However, considering results published by Shikiya *et al.* [12], which found no uptake of prions by nymphal Rocky Mountain wood ticks (*Dermacentor andersoni*) following an animal challenge study, we felt it necessary to use such high concentrations of CWD-positive spiking material to demonstrate the capacity of *I. scapularis* to assimilate prions from a blood meal rather than

demonstrate natural uptake under the most ideal controlled conditions. Future studies considering both—blood specimens spiked with more diluted prion titers (mimicking prion concentrations at different stages of the animal's disease course) and blood from actual deer—would be important to consider when evaluating assay sensitivity between PMCA and RT-QuIC.

The observation of inconsistences of seeding activity by ear or tick samples compared to seeding activity of RPLN from the 15 CWD-positive WTD may be the result of differences in the stage of CWD disease progression across the individuals sampled, as variation in disease stage could influence PrP^{CWD} distribution [43]. Although we cannot confirm disease stage for any of the sampled WTD included in this study, this sample set of WTD was not randomly selected, but rather based on those selectively harvested by hunters, making it unlikely that any of the sampled individuals were in the end-stages of CWD. Hence, there is an expectation that the ear tissue and pooled tick samples would contain relatively low PrP^{CWD} levels, given lower blood flow to ear tissue, the small sample volumes taken up by adult ticks, and that it is unlikely that a deer in the terminal stage of CWD would have been targeted for hunter harvest. Although PMCA and RT-QuIC are capable of detection of misfolded prions at femtogram levels—similar to the lower detection limit of bioassay [44, 45]—the inconsistencies in sensitivity between the two assays for our sample set may have resulted from having samples that contain PrP^{CWD} levels below detection thresholds for even these ultra-sensitive amplification assays. This possible detection limit may explain why WTD 11 was the only deer with a pooled tick sample that overlapped in positivity between the two assays (Fig. 2a, 2g, 2h). The elevated RPLN AFRs and more sensitive seeding of ear tissue from sample 11 compared to all other RPLN and ear tissue samples suggests that this individual had higher

circulating amounts of PrP^{CWD} levels that were at or above the detection threshold between the two assays (Fig. 2a, 2b). Future work aimed at better understanding how the range of PrP^{CWD} levels that ticks may harbor affects assay sensitivity and detection rates could incorporate a broader sample set that includes samples from deer that are in the end-stages of CWD. Additionally, this study only investigated ticks concentrated around the head and neck of deer. It is possible that ticks collected from other anatomical regions lacking a common vasculature with the head may exhibit different PrP^{CWD} detectability and prevalence. Further studies investigating engorged ticks across the entire anatomy of WTD are warranted.

While variation of seeding activity observed in pooled tick and ear tissue samples across the individual free-ranging WTD from this study may demonstrate limits of detection for both PMCA and RT-QuIC, the naturally occurring PrP^{CWD} loads from ticks and ear tissue collected from the 15 CWD-positive WTD may also be influenced by *PRNP* genotype [27]. Although the small sample size of CWD-positive animals in this study limited our ability to assess how genotype alters variation in seeding activities, we found that RT-QuIC tested pooled tick samples from CWD-positive 96G/96S or 96S/96S animals were negative and positive pooled tick samples came from CWD-positive 96G/96G. The four positive PCMA results for the same pooled tick samples showed detection of PrP^{CWD} from one CWD-positive 96G/96S, and three CWD-positive 96G/96G (See Supplementary Table S1 for genotype results and more information regarding genotypes). An additional consideration is that CWD strain variation may be another plausible explanation for the distribution in seeding activity across the sample types examined in this study, as peripheral and neural PrP^{CWD} distribution can differ across recognized CWD strains [46].

Indirect transmission routes of CWD likely play an important role in CWD disease dynamics [1] and are quite possibly a combination of various modes of exposure that may include consumption or inhalation of contaminated soil [47, 48], consumption of contaminated plant matter [4], or mucosal contact with contaminated fomites or other environmental materials [49]. Few studies have utilized RT-QuIC to evaluate the involvement of other species in the ecological community that may influence CWD exposure or transmission. We have identified a potential mechanical vector of CWD not previously evaluated for WTD, with implications for host behavior that may influence CWD exposure events. We recognize that experimentally-determined oral ID_{50} of our tick samples may vary significantly from our estimates, as it is quite evident different prion loads were present in each animal based on the RPLN dilution series (Fig. 3), and the prior study [35] used to estimate the ID_{50} utilized a pool of infectious material generated from laboratory-infected late-stage animals. As such, these can be considered preliminary estimates for ID_{50} in ticks; our sample size is small and experimentation in mouse models would establish a true ID_{50}. Nevertheless, our findings suggest that infection relevant loads of seeding material are indeed present in individual ticks, likely within an order of magnitude of 1 ID_{50}. These findings and implications may prove useful for CWD research and adaptive management efforts moving forward as we advance our understanding of ecologically relevant drivers of CWD disease dynamics. Future studies could clarify the prevalence of CWD in ticks for a single deer and explore the potential importance of the relationship between tick CWD prevalence and where on the host's body the tick attaches. Although the detection rate varied depending on the seeded amplification assay being used, this was not surprising as it is well known that biological and environmental specimens may carry

components affecting the PrP^{C} – PrP^{CWD} conversion process. Future studies could determine whether sample pre-treatments or modifications in the RT-QuIC or PMCA processes increase prion detection ratio in ticks and other parasites. Importantly, future research including bioassays, evaluating larger samples sizes of *I. scapularis* and other tick species collected from WTD, and additional wild cervid species across different regions of North America would expand our understanding of the role that blood obligate ectoparasites and associated biogeographical factors may have on CWD dynamics. For example, land use change and shifts in regional climate regimes may result in higher tick infestations on cervids and contemporary range expansion for different tick species [50], potentially increasing the likelihood of this type of exposure event among WTD and other cervids. Additionally, behavioral studies evaluating cervid allogrooming frequency, rate, and preferences for allogrooming across different cervid species could shed light on links between host behavior, disease status and conspecific exposure risk. For example, do end-stage CWD-infected cervids accumulate higher tick burdens (suggested in [51]) through altered grooming or habitat selection behaviors? Do healthy individuals continue to groom infected conspecifics or do they avoid allogrooming interactions with visibly sick individuals?). Future research efforts could help elucidate the impact of ticks and grooming behaviors on transmission of CWD in free-ranging cervids.

METHODS

Membrane fabrication and feeding chamber assembly. The tick artificial membrane-feeding method used was based on Oliver *et al.*[20], with some modifications. Briefly, silicone membranes were produced by infiltrating 50% rayon and 50% cellulose microscope lens paper (Matin) with a

two-component liquid silicone rubber with a shore hardness of 00-50 (Ecoflex Supersoft 0050, Smooth-On, PA, USA), mixed 1:1 and thinned by adding 1.5 mL hexane into 10 mL silicone mixture (Fig. 1e). The lens cleaning paper was taped to a flat, plastic wrap-lined surface and the silicone mixture scraped over the lens cleaning paper to obtain a membrane with a thickness of ~70-100 µm. Membrane thickness was determined by measuring 4-6 points on each cured membrane with a micrometer. Membranes were allowed to cure overnight before feeding chamber attachment using a 1:1 mixture of a two-part silicone glue with shore A hardness of 30 (Mold Star 30, Smooth-On) as previously described, resulting in 4-6 feeding chambers per cured membrane sheet (Fig.1i). Assembled feeding chambers were cured overnight, trimmed with a scalpel, and leak-tested by adding 5 mL of sterile water to each chamber for at least 1 hour, discarding any leaky membranes. To ensure feeding chambers remained upright and membranes were submerged into blood-containing wells, plastic graduated cylinder bumpers were placed around feeding units and glued into place with silicone glue (Gorilla Glue) (Fig. 1f, 1g).

Tick housing and feeding. Pathogen free *I. scapularis* male and female adults were acquired from the Oklahoma State Tick Rearing facility, Stillwater, Oklahoma, U.S.A., and housed in a humidity chamber (Durabilt, 64-quart clear storage tote with lid) at 24 °C with 97-99% relative humidity (Durac, hygrometer). Humidity was maintained by placing lidless, smaller separate plastic containers containing a saturated potassium sulfate solution (~0.5 kg K_2SO_4 (potassium sulfate) submerged in ~500 mL of sterile deionized water) within the larger humidity chamber. Ticks were acclimated to the humidity chamber for ~5 h prior to starting artificial membrane feeding. Sterile, mechanically defibrinated bovine blood (Hemostat Laboratories, Dixon, CA) was used. Three mL of blood supplemented with 4.5 µL of 3 mM ATP was prewarmed to 37 °C, then added to each

well of a six-well plate to stimulate feeding [52]. In total 10-14 female and 5-7 male *I. scapularis* were placed in each feeding chamber enclosed using a fine synthetic mesh fabric (Anteer Crystal Organza, China), fixed in place with a tight rubber band. If available from previous feedings, 10-15 granules of tick frass were added to each feeding chamber as a feeding stimulant. Feeding chambers were positioned in direct contact with the blood without trapping air bubbles between the membrane and the blood, submerging membranes by at least 2 cm. Each plate was placed afloat in a 37 °C water bath in a room that provided a 16:8 h photoperiod. Every 24 h, blood was replaced by placing 3 mL of prewarmed bovine blood as described above in a new six-well plate. To remove the build-up of blood residue from the previous day's feeding, the outer surface of each feeding chamber and membrane that were in contact with the blood were vigorously rinsed with sterile 1X phosphate-buffered saline (PBS) using a pipette.

Exposure of *I. scapularis* to CWD by membrane feeding. To determine tick uptake of PrP^{CWD} from inoculated blood meals, we inoculated 2.970 mL of defibrinated bovine blood with a 30 µL of a 10% (w/v) CWD-positive WTD brain homogenate (from the obex region; sourced from Wisconsin Department of Natural Resources (WDNR) tagged WTD #5219) prepared in 1X PBS to achieve a final concentration of 10^{-3} mg/mL of CWD-positive brain for the CWD-positive treatment group. For the negative control treatment group, 2.970 mL defibrinated blood was treated with 30 µL of 1X PBS. Separate six-well plates were used for each treatment group and each six-well plate with feeding chambers attached were placed afloat in a 37 °C water bath that was housed within a humidity chamber (24 °C with 97-99% relative humidity). Every 24 hours, feeding unit plates were replaced and refilled with fresh blood for each treatment group. The outer surfaces of the feeding chamber and membranes were washed as detailed above, washing the

negative control feeding units first and the CWD-positive treatment feeding chambers last to prevent any possibility for cross contamination. Ticks were allowed to feed to repletion for ~9 to 14 days, during which frass was also collected from each treatment group. Engorged, detached ticks and frass were stored at –20 °C prior to preparation for RT-QuIC or PMCA analyses.

Collection of ear tissue and ticks attached to hunter harvested, wild white-tailed deer. Through cooperation with the WIDNR CWD processing center, heads of voluntarily submitted hunter-harvested WTD from several Wisconsin counties were manually examined for ticks from October 2021 to December 2021. Examinations generally consisted of combing fingers through pelage for attached ticks on each WTD head while wearing nitrile gloves. When detected, attached ticks were removed, typically found on the outer ear, brow, chin, cheek, snout, or neck regions. Each head was contained within a leak proof disposable plastic bag and tagged with a WIDNR barcode for identification and linking to metadata for CWD testing. To prevent cross contamination of each deer head being examined, heads were kept within their respective bags, gloves were changed between each head examination, and a new sterile scalpel was used for removal of the entire left ear of each head. Each ear sample was placed in a sterile Whirl-Pak bag (Nasco, 48137), and any ticks collected from a given head were collectively placed in sterile 1.5 mL snap-cap centrifuge tubes. All samples were labeled with the respective barcodes for each head examined, and the number and species of tick collected from CWD-positive WTD were cataloged. Tissue and tick samples were stored at -20°C prior to preparation for use in the RT-QuIC or PMCA assays.

Tick, blood, and frass homogenate preparations for RT-QuIC. To prepare tick homogenates, a mix of partially and fully engorged *I. scapularis* female ticks equaling a total weight of ~200 mg (unless stated otherwise, equivalent to ~142 µL of blood meal), were placed in a ring-sealed 1.5 mL centrifuge tube with ~30-40 0.7 mm zirconia beads (BioSpec) with 1 mL of PBS and processed at room temperature in a bead mill homogenizer (Fisherbrand Bead Mill 24) on the highest setting (setting six) for 3 minutes. The mass of each tick sample collected from hunter-submitted deer heads varied and was therefore prepared using volumes of 1X PBS to result in a 10% (w/v) homogenate. Next, tick homogenates were mixed with chitinase (1 mg/ mL final concentration) (Sigma-Aldrich Cat. # C824) and allowed to digest in a thermomixer (1,400 rpm, 24 h, 45°C; Eppendorf ThermoMixer F1.5). Following digestion, Lipase AY30 (100 µg/mL final concentration; Acros Organics) was added, and homogenates were thermomixed for 1 h (37 °C, 1400 rpm; Eppendorf ThermoMixer F1.5), followed by centrifugation (25 °C, 15 min, 16,000 x*g*). Supernatants were collected, centrifuged again to ensure sample clarification (25 °C, 15 min, 16,000 x *g*), mixed 1:1 with 23.1 mM sodium phosphotungstate hydrate (Na-PTA) (Sigma-Aldrich, Cat. # 496626), incubated without agitation for 16 hours at 4°C. Samples were then centrifuged (4 °C, 30 min, 5000 x *g*), pellets were retained and washed with a 1:1 solution of 18 MΩ H_2O and 23.1 mM sodium phosphotungstate (Na-PTA) followed by centrifugation (4 °C, 30 min, 5,000 x *g*) and aspiration of the wash solution. Pellets were resuspended in 30 µL of RT-QuIC sample buffer (0.1% SDS in 1X PBS and N2 supplement (Gibco, 17502048)) using sonication (1 min, amplitude 36; Qsonica Q-700), and 2 µL was used to seed each reaction well of the 96 well-plate for the RT-QuIC assay. Eight technical replicates were used per biological replicate, unless stated otherwise.

For spiking experiments using tick homogenates for RT-QuIC optimization, the initial volume of the spiked sample consisted of 450 µL of negative control tick homogenate (as prepared above), 450 µL chitinase (1 mg/ mL final concentration), and 100 µL of a 10^{-3} dilution of either CWD-positive or CWD-negative WTD brain. Then the steps outlined above for preparing tick homogenates were performed, with Na-PTA pellets resuspended in 100 µL and ten-fold dilutions were prepared from the 10^{-3} spiked sample. Two µL of each dilution were used to seed each reaction well for eight technical replicates. Spiking experiments used to demonstrate recovery of PrP^{CWD} from defibrinated bovine blood were carried out using 200 µL of blood combined with 1 mL PBS, followed by the homogenization step with an additional 1 mL of PBS added. The initial volume of the spiked blood samples consisted of 450 µL blood homogenate, 450 µL PBS, and 100 µL 10^{-3} CWD-positive of CWD-negative WTD brain homogenate dilution. Samples were incubated in a thermomixer for 16 h (45 °C, 1400 rpm), then 1.5 µL of Lipase AY30 (100 µg/mL final concentration; Acros Organics) was added and the samples homogenized with Na-PTA pellets resuspended in 100 µL of RT-QuIC sample buffer and 10-fold dilutions were prepared from the 10^{-3} spiked sample, using 2 µL of each dilution to seed each reaction well for eight technical replicates per biological replicate. For RT-QuIC analysis of tick frass from feeding experiments, 70 mg of frass from each treatment group was added to 1 mL of 1X PBS, followed by homogenization. Lipase AY30 (100 µg/mL final concentration) was added, and samples were incubated in a thermomixer for 1 h (37° C, 1400 rpm), centrifuged (16,000 x *g*, 15 min, 25 °C), and supernatants were collected. Next, 500 µL of 1X PBS was added to 500 µL of the supernatant, then mixed 1:1 with 23.1 mM sodium phosphotungstate followed by incubated without agitation

for 16 h at 4 °C, Na-PTA pellets were centrifuged, washed, and centrifuged again, then resuspended in 30 µL with sonication. Then, 2 µL of each sample was used to seed each reaction well for 8 technical replicates.

Tissue homogenate preparation for RT-QuIC. Ear tissue was prepared as described in Burgener *et al*.[53]. Briefly, 100 mg of ear tissue collected from the central area of the pinna was placed in a digestion solution (1X PBS, 2 mM $CaCl_2$ (Dot Scientific DSC20010-1000), and collagenase A (2.5 mg/mL final concentration) (Sigma-Aldrich 10103586001)) were homogenized with a bead beater (1 min, 4 m/s; Fisherbrand Bead Mill 24) and 0.7 mm diameter zirconia beads (BioSpec). These samples were then further processed with a thermomixer (1,400 rpm, 24 h, 45 °C; Eppendorf ThermoMixer F1.5), after which they were centrifuged (2 min, 3,000 x *g*), and the supernatants retained. The supernatants were centrifuged again (3 min, 3,000 x *g*) to remove any small particulate matter, aliquoted, and frozen at –20 °C until use for RT-QuIC analysis.

Real-time Quaking-induced Conversion Assay. The RT-QuIC *in vitro* prion amplification assay was performed as described by Metrick *et al*. [54] with minor modifications. Briefly, 2 µL of sample extracts were added to a given well of a 96-well format optical-bottom black microplate (Fisher), each already containing 98 µL of RT-QuIC reaction mixture (0.1 $mg \cdot mL^{-1}$ 90-231 recombinant hamster prion protein (produced as previously described by Orru *et al*. [55], 300 mM sodium iodide, 20 mM sodium phosphate, 1.0 mM ethylenediaminetetraacetic acid, and 10 µM thioflavin T). Microplate-compatible spectrophotometers capable of heating, shaking, and fluorescence monitoring (BMG FLUOstar, Cary, NC) were used with the following instrument settings: 50 °C for spiked samples (unless described otherwise) double orbital pattern shaking at 700 rpm with 60-

s shake / 60-s rest cycles, fluorescent scans ($\lambda_{excitation}$ = 448 nm, $\lambda_{emission}$ = 482 nm) every 15 minutes, at a gain of 1,600, and a total run time of 48 h.

CWD Status by ELISA. Retropharyngeal lymph nodes collected from hunter-harvested deer were tested by enzyme-linked immunosorbent assay (ELISA) using the standard protocol approved by the U.S. Department of Agriculture (USDA) at the Wisconsin Veterinary Diagnostic Center, Madison, Wisconsin, U.S.A. The ELISA assay was conducted using a commercial Transmissible Spongiform Encephalopathy Antigen Test kit (Bio-Rad, Catalogue# 12004413) (bovine obex or mule deer/WTD/elk RPLN and obex), following manufacturer's instruction. Identification of the presence of CWD is based on an optical density (OD) value that is equal to or greater than the USDA cut-off value (0.035).

Protein misfolding cyclic amplification. The PMCA substrate was generated from a pool of brains from Tg(CerPrP)1536$^{+/+}$ [56] mice as described in Morales *et al.*, 2012 [28]. PMCA substrate was supplemented with digitonin (Invitrogen, Carlsbad, CA, USA) and EDTA (Promega, Madison, WI, USA) at final concentrations of 0.025% and 6 mM, respectively. Aliquots of 90 µL of PMCA substrate were transferred in 0.2 mL PCR tubes strips (Eppendorf, Enfield, CT, USA) containing PTFE beads (Engineering Laboratories, Inc., Oakland, NJ, USA) and mixed with 10 µL of tick-derived samples. Tick samples used for PMCA were prepared in either RT-QuIC sample buffer or as a clarified homogenate following treatment with chitinase, Lypase AY30, and centrifugation to further clarify the sample prior to adding Na-PTA as described above in the "Tick, blood, and frass homogenate preparations for RT-QuIC" section above. The PMCA reactions were submitted to a first round of 144 cycles of incubation/sonication. The resulting PMCA products (10 µL) were mixed with fresh PMCA substrate supplemented (90 µL) and subjected to two

additional PMCA rounds of 96 cycles each. Each PMCA cycle consisted of 29 min., and 40 s of incubation, and 20 s of sonication at 37 °C. Each PMCA reaction set included PMCA reactions spiked with serial dilutions of CWD-positive brain (10 µL) of known PMCA activity and 4 unseeded reactions as negative controls. PMCA products were mixed with proteinase K (PK, Sigma-Aldrich, Saint Louis, MO, USA) at final concentration of 100 µg/mL and incubated at 37°C for 90 min with shaking. The PK catalytic activity was stopped by adding NuPAGE LDS sample buffer (Invitrogen, Carlsbad, CA, USA) at final concentration of 1X and heated at 90°C for 10 min. PK-treated PMCA products were visualized by western blot using the Bar-224 antibody (Bertin Corp, Rockville, MD, USA) at 1:10,000 dilution. PMCA manipulators were blinded to the identity of the samples.

***PRNP* analysis.** Genomic DNA was extracted from ~100 mg of ear tissue from 17 out of 30 WTD included in this study using methods outlined in Green and Sambrook, 2012 for phenol-chloroform extraction and ethanol precipitation [57]. An approximately 750bp *PRNP* gene sequence was amplified by conventional PCR and sequenced at the University of Wisconsin Biotechnology Center (Madison, Wisconsin, U.S.A) using primer sequences developed by O'Rourke *et al.* [24]. PCR sequences were then aligned and evaluated using Unipro UGENE software version 42.0 (www.ugene.net). Specific single nucleotide polymorphisms at position 95 (glutamine [Q] or histidine [H]), 96 (glycine [G] or serine [S]) were identified and recorded. Although an updated set of primers which accounts for rare *PRNP* alleles was recently identified [58, 59] following the initial submission of our findings, primers used for this study have demonstrated consistent utility for the past decade [25, 60].

Data Analysis. Data were analyzed and visualized using Jmp Pro 15 (SAS Institute, Cary, NC) and Prism 8 (GraphPad, San Diego, CA). Thresholds used to determine AFRs were calculated by adding twenty times the standard deviation of the relative fluorescence unit (RFU) values from cycles 3-14 to the mean of RFU values from cycles 3-14 to account for baseline variation amongst samples and to apply a rigorous standard for distinguishing true positive samples from true negatives.

We first evaluated if we could recover and detect PrP^{CWD} from spiked tick homogenates or spiked blood as compared to the source material (CWD-positive brain tissue), and if the recovery rates differed by sample type. We used a two-way (factorial) analysis of variance (ANOVA) to compare AFR values among sample types (CWD-positive brain, spiked-blood or spiked tick homogenates). We included an interaction between sample type and sample dilution, to assess if detection/recovery in different sample types was sensitive to the sample concentration across the 10-fold dilution series.

After this proof of concept, we then evaluated whether ticks experimentally fed blood inoculated with CWD-positive BH could ingest and excrete prions. We used a two-way (factorial) ANOVA to assess differences in AFR values based on sample type (CWD-positive brain tissue, ticks fed prion-spiked blood, or frass from the experimentally-fed ticks) and the interaction between sample type and sample dilution across the 10-fold dilution series.

To analyze whether PrP^{CWD} was detectable in engorged ticks collected from free-ranging CWD-positive deer, we compared results generated by two protein amplification assays, RT-QuIC and PMCA. Pooled tick samples amplified by PMCA that demonstrated bands between 34-26 kDa were interpreted as being positive for having PrP^{CWD} present [61]. Pooled tick samples analyzed by

RT-QuIC were considered positive if a sample had at least 3 out of 8 technical replicates with seeding activity and also by statistical analysis, using Dunnett's multiple comparison test of AFR values to distinguish which pooled tick samples were significantly different from the negative control pooled tick samples collected from free-ranging CWD-negative WTD heads.

Since CWD testing in free-ranging cervids currently relies upon invasive sampling of RPLN, we explored if CWD status could be assessed through more readily accessible tissues (such as ear tissue or ectoparasites) which could provide support for performing less invasive *antemortem* CWD testing. Therefore, we collected RT-QuIC average AFR values for three sample types (lymph node, ear, or pooled tick samples) from individual deer to evaluate if AFR values were correlated (i.e., do deer with high AFR values in lymph nodes have higher AFR values in ear or tick samples than deer with lower AFR values?). Using linear regressions, we explored relationships between average AFRs for the three sample types collected from the CWD-positive deer in our sample set.

We then calculated if prion concentrations detected in ticks from free-ranging deer had the potential to be infectious, based on estimates of the amount of prion seeding material in our samples relative to an experimentally-determined ID_{50} for an equivalent amount of prion seeding material in brain [35]. To estimate a predicted per-tick ID_{50} (ID_{50P}) we need to know the ng of predicted seeding material per 1 mg of tick for a pooled sample (*S*), the average mass (mg) for a single tick from a pooled sample (*m*) from WTD ID 1, 4, and 11, and the actual ID_{50} of an equivalent mass of CWD-positive brain (300 ng = ID_{50A}):

$$ID_{50P} = (S \times m) \div ID_{50A} \quad (1)$$

Titers of PrP^{CWD} from a CWD-positive brain are similar to those found in CWD-positive RPLN [34], and a recent study has described a minimum mass of 300 ng of CWD-positive brain derived from a pool of 6 CWD-positive deer to be an effective oral ID_{50} for WTD [35]. As such, to calculate *S*, we first modeled, using a sum of exponential functions, the AFR values generated for the 10-fold dilution series using mass of RPLN tissue per 2 μL (the amount of sample used to seed each well) for each of the RPLN samples (1, 4, and 11) as the explanatory variable. Thus, our global model was:

$$AFR_i = a + b \times \exp(-d \times ng_i) + c \times \exp(-f \times ng_i), \qquad (2)$$

where AFR_i is the observed AFR for the ith observation, *a, b, c, d* and *f* are parameters that are estimated and ng_i is the mass of sample for the ith observation. Parameters for each model were estimated using a least squares loss function within the Nonlinear Fit Curve Personality of Jmp Pro 15 (SAS Institute, Cary, NC). For each dataset we examined a suite of 4 models, which were based on the global model described above with either 2, 3, 4, or 5 parameters. We used the Akaike information criterion (AIC_c) corrected for small sample size, to select which model from this suite of models provided the most parsimonious fit for each of the three RPLN data sets. (see Supplementary Table S4 and S5 for AIC_c values and associated suite of models examined) [62]. Based on AIC_c values, the global 5-parameter model was chosen for data for sample #1 and the 4-parameter model best fit data for samples #4 and 11. The fitted models were then used as calibration curves to predict the relative amount of seeding material present in 2 μL of each pooled tick sample (For full model equations see Supplementary Table S4). Therefore, if ng_p is the predicted mass of seeding material in a 30 μL volume (the total volume of prepared pooled tick sample in RT-QuIC sample buffer, see Supplemental Material Methods section), and M_t is the total

mass of (mg) of a pooled tick sample, then *S*, the ng of seeding material per 1 mg of tick for a pooled sample, is estimated as:

$$S = (ng_P) \div (M_t) \tag{3}$$

SUPPLEMENTARY MATERIAL

SUPPLEMENTARY TABLES AND FIGURES

Supplementary Table S1. Summary of genotype, age, sex, Wisconsin county harvested from, and chronic wasting disease (CWD) status by retropharyngeal lymph nodes (RPLN), ear, or pooled tick samples using real-time quaking-induced conversion (RT-QuIC) for 15 CWD-positive and two (out of 15) CWD-negative white-tailed deer (WTD) included in this study. Polymorphisms in prion protein gene (*PRNP*) can directly influence the rate of disease progression and distribution of CWD prions (PrP^{CWD}) in WTD [24-26]. White-tailed deer (WTD) homozygous for glycine at the 96th allele of the *PRNP* gene (96GG) differ in duration of disease progression compared to those that are hetero- or homozygous for serine (96GS/96SS) [26, 63, 64] and WTD expressing amino acid histidine (H) at the 95th allele have limited peripheral accumulation of PrP^{CWD} compared to those expressing glutamine (Q) (H95/Q95) [27].

WTD ID	Genotype	Age	Sex	Hunter harvest county	CWD status by RT-QuIC RPLN/ear/tick
1	GG	3	F	Richland	+/+/+
2	GG	1	M	Dane	+/+/-
3	GG	3	F	Iowa	+/+/-
4	GG	4	M	Dunn	+/+/+
5	GS	3	M	Columbia	+/+/-
6	GG	4	F	Iowa	+/-/-
7	GG	1	M	Dane	+/-/-
8	GG	3	M	Iowa	+/+/-
9	GG	3	F	Iowa	+/-/-
10	GG	3	M	Dane	+/-/-
11	GG	2	M	Sauk	+/+/+
12	SS	3	M	Columbia	+/-/-
13	GG	1	M	Iowa	+/-/-
14	GS	1	F	Dane	+/-/-
15	GS	2	M	Marquette	+/+/-
16	GG	2	F	Monroe	-/-/-
17	GS	3	F	Richland	-/-/-

Supplementary Table S2. Summary of results for pooled tick samples tested by real-time quaking-induced conversion (RT-QuIC) and protein misfolding cyclic amplification (PMCA) assays. NA indicates negative control samples that were evaluated by one assay but not the other.

Sample ID	Deer CWD status	Tick PMCA results		Tick RT-QuIC results	
		+/-	Technical replicates with seeding activity	+/-	Technical replicates with seeding activity
1	+	-	0/2	+	7/8
2	+	-	0/2	-	1/8
3	+	-	0/2	-	1/8
4	+	-	0/2	+	7/8
5	+	-	0/2	-	0/8
6	+	-	0/2	-	0/8
7	+	+	1/2	-	0/8
8	+	-	0/2	-	0/8
9	+	-	0/2	-	1/8
10	+	+	1/2	-	0/8
11	+	+	2/2	+	3/8
12	+	-	0/2	-	0/8
13	+	-	0/2	-	1/8
14	+	-	0/2	-	0/8
15	+	+	1/2	-	1/8
16	-	NA	NA	-	0/8
17	-	-	0/2	-	0/8
18	-	-	0/2	NA	NA
19	-	-	0/2	NA	NA
20	-	-	0/1	-	0/8
21	-	-	0/1	-	0/8
22	-	-	0/1	-	0/8
23	-	-	0/1	-	0/8
24	-	-	0/1	-	0/8
25	-	-	0/1	-	0/8
26	-	-	0/1	-	0/8
27	-	-	0/1	-	0/8
28	-	-	0/1	-	0/8
29	-	-	0/1	-	0/8
30	-	-	0/1	-	0/8
31	-	-	0/1	-	0/8
32	-	-	0/1	-	0/8

Supplementary Table S3. Derived values and the associated estimated individual tick ID_{50} based on the amyloid formation rate (AFR) prediction model with the lowest Akaike information criterion corrected (AIC*c*) value for 10-fold dilutions of retropharyngeal lymph node tissue from white-tailed deer (WTD) #1, 4, and 11. Parameter names in bold-italics indicate variable names for equations in text.

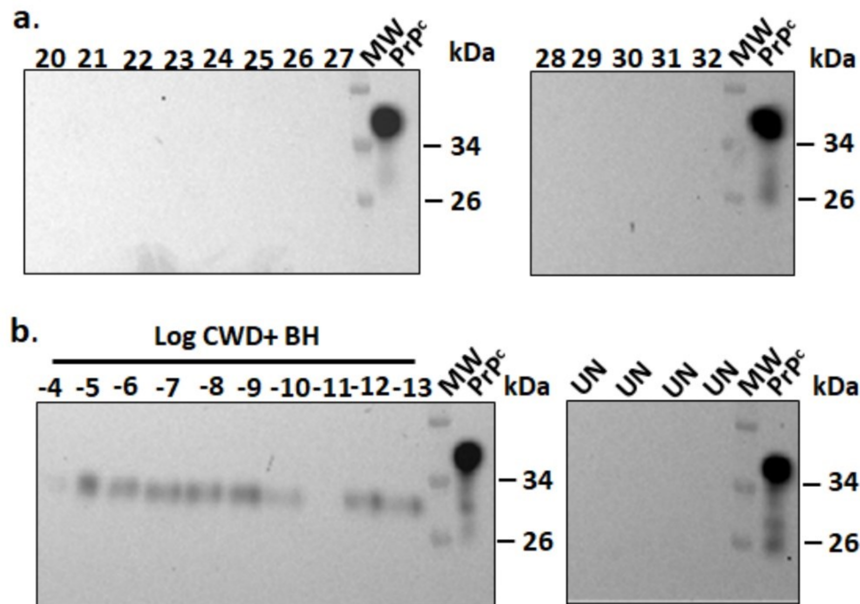
Estimated Tick ID_{50}								
Sample #	Predicted ng of seed in 2 µl	Predicted ng of seed per µl	Number of ticks in sample	Predicted ng of seed per 30 µl – ($\boldsymbol{ng_P}$)	Tick sample mass (mg) total – ($\boldsymbol{M_t}$)	ng of seed per mg of tick – ($\boldsymbol{S}$)	Average mass (mg) for a single tick – ($\boldsymbol{m}$)	Predicted ID_{50} per tick – ($\boldsymbol{ID_{50P}}$)
1	13.1	6.55	2	196.5	25.6	7.68	12.8	0.3
4	2545	1272.5	3	38,175	82.2	464.4	27.4	42.4
11	950	475	7	14,250	277.5	51.35	39.64	6.9

Supplementary Table S4. The model descriptions and Akaike information criterion corrected (AIC*c*) values (bold-italics values indicate the minimum AIC*c* value) for 10-fold dilutions of retropharyngeal lymph node tissue from white-tailed deer (WTD) #1, 4, and 11.

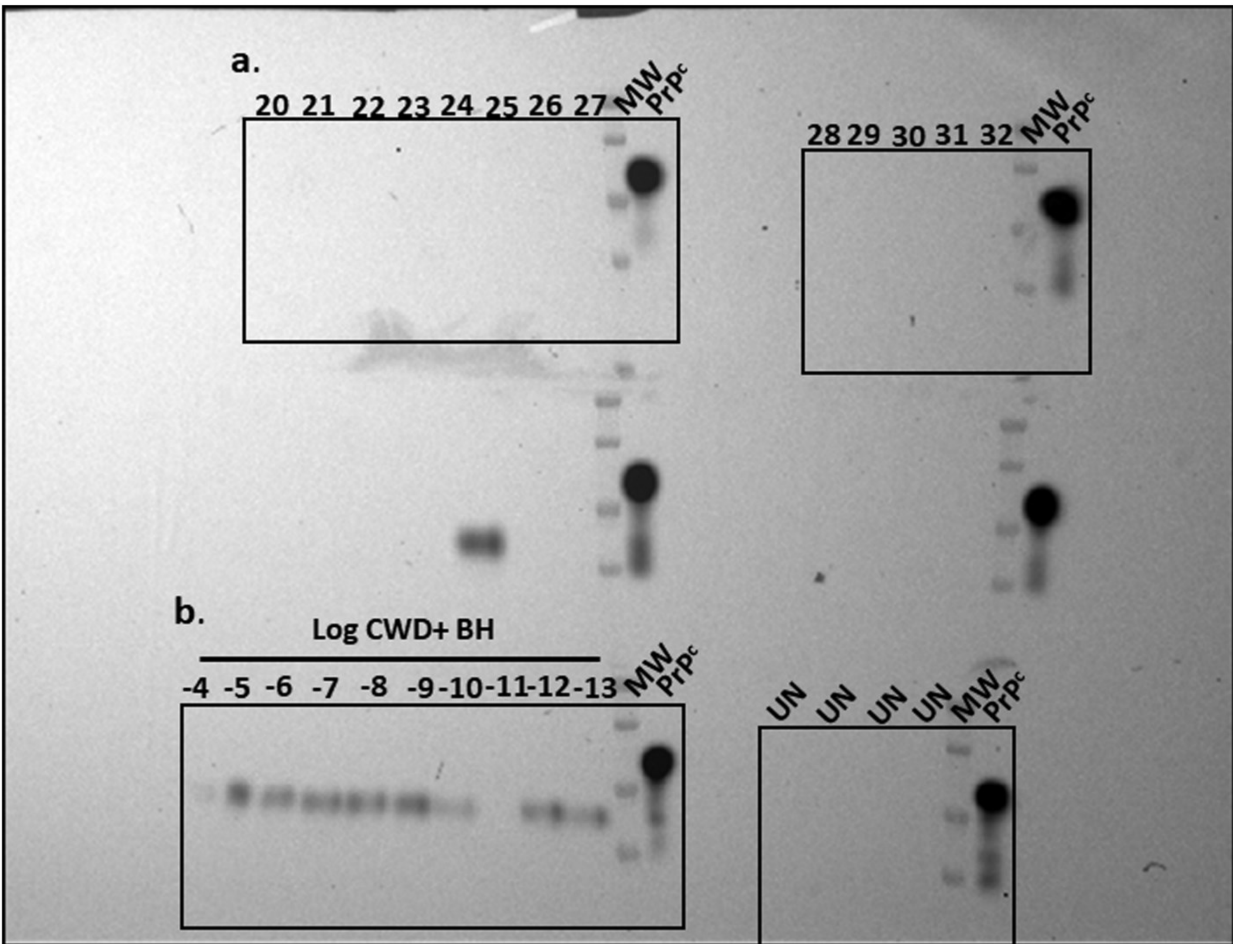
Model Selection and AICc Results				
Prediction Model	Number of Parameters	Sample #1 AICc	Sample #4 AICc	Sample #11 AICc
$\boldsymbol{a} \times \boldsymbol{e}^{(-\boldsymbol{d} \times \boldsymbol{ng})}$	2	-171.24	-162.65	-151.17
$\boldsymbol{a} + \boldsymbol{b} \times \boldsymbol{e}^{(-\boldsymbol{d} \times \boldsymbol{ng})}$	3	-230.79	-238.14	-224.38
$\boldsymbol{b} \times \boldsymbol{e}^{(-\boldsymbol{d} \times \boldsymbol{ng})} + \boldsymbol{c} \times \boldsymbol{e}^{(-\boldsymbol{f} \times \boldsymbol{ng})}$	4	-239.16	***-238.52***	***-228.59***
$\boldsymbol{a} + \boldsymbol{b} \times \boldsymbol{e}^{(-\boldsymbol{d} \times \boldsymbol{ng})} + \boldsymbol{c} \times \boldsymbol{e}^{(-\boldsymbol{f} \times \boldsymbol{ng})}$	5	***-247.37***	-235.83	-225.82

Supplementary Table S5. Estimated parameters for the prediction models with the lowest Akaike information criterion corrected (AIC*c*) value for 10-fold dilutions of retropharyngeal lymph node tissue from WTD #1, 4, and 11. Parameter names in bold indicate names of variables in the prediction models.

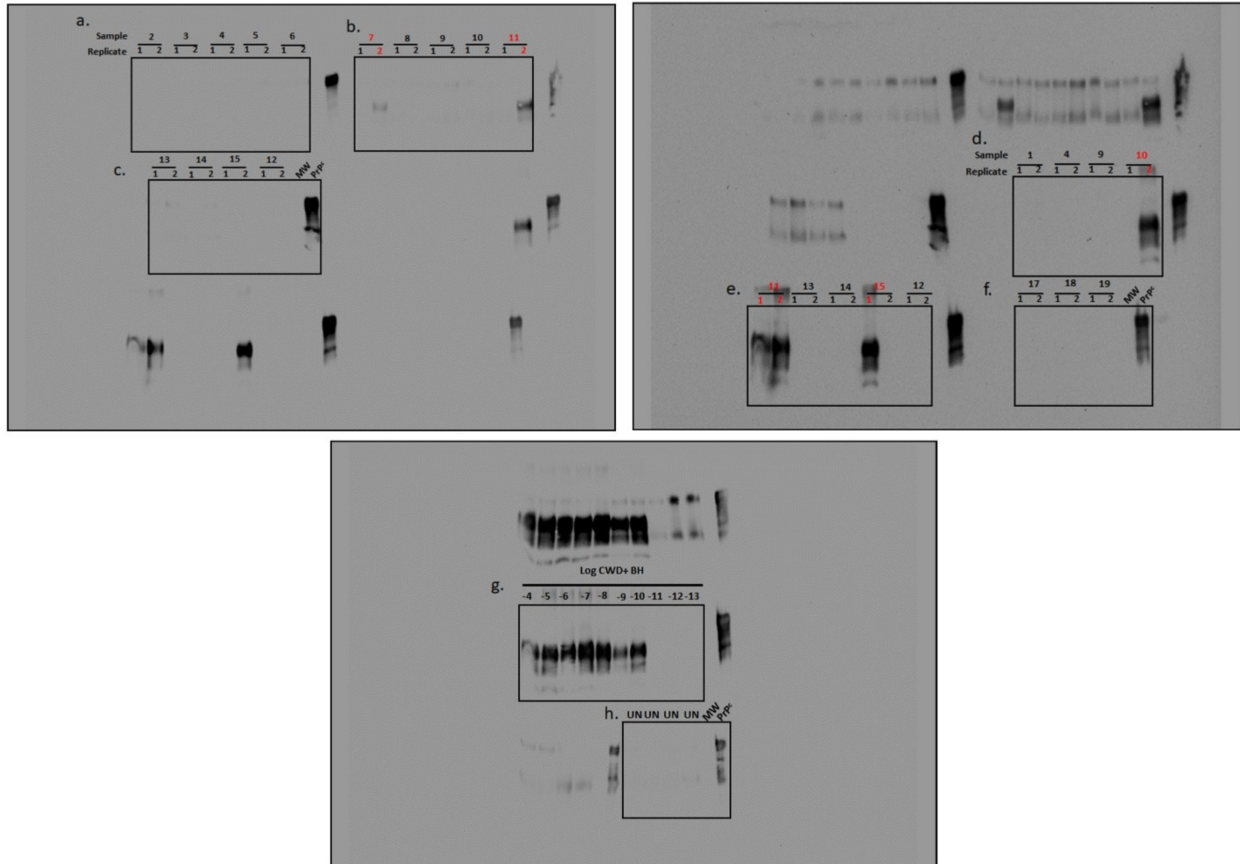
Estimated Parameters					
Sample #	Asymptote - **a**	Scale 1 - **b**	Scale 2 - **c**	Decay Rate 1 - **d**	Decay Rate 2 – **f**
1	0.15050	-0.04842	9.8922 e-6	-0.09284	0.01059
4	NA	0.10758	-0.10321	1.485 e-7	0.00012
11	NA	0.12772	-0.11567	-2.093 e-7	0.00013



Supplementary Figure S1. Additional testing of pooled tick samples collected from chronic wasting disease negative white-tailed deer (WTD) by the protein misfolding cyclic amplification (PMCA) assay. (a) Tick homogenates prepared from 13 separate pooled tick samples collected from 13 CWD-negative WTD (ID 20-32); **(b)** serial dilutions of a CWD-positive (CWD+) brain homogenate (BH) (PMCA positive control), and unseeded (UN) or cellular prion (PrP^C) (PMCA NCs). Samples analyzed in this figure were tested singularly and represent a third PMCA round. Numbers at the right of each panel represent molecular marker in kilodaltons (kDa).



Supplementary Figure S2. Uncropped Western blot images of pooled tick samples collected from chronic wasting disease (CWD)-negative white-tailed deer (WTD) by the protein misfolding cyclic amplification (PMCA) assay as depicted in Supplementary figure S1. **(a)** Tick homogenates prepared from 13 separate pooled tick samples collected from 13 CWD-negative WTD (ID 20-32); **(b)** serial dilutions of a CWD-positive (CWD+) brain homogenate (BH) (PMCA positive control), and unseeded (UN) or cellular prion (PrP^C) (PMCA NCs). Samples analyzed in this figure were tested singularly and represent a third PMCA round. Uncropped blots pertinent to this study are boxed in with labels.



Supplementary Figure S3. Uncropped Western blot images of pooled tick samples collected from white-tailed deer (WTD) by the protein misfolding cyclic amplification (PMCA) assay as depicted in **(a-c)** “figure 2g”, **(d-f)** “figure 2h”, and **(g-h)** “figure 2i”. Uncropped blots pertinent to this study are boxed in with labels.

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CHAPTER 3: Detection tools and carnivore-based surveillance for chronic wasting disease

ABSTRACT

Chronic wasting disease (CWD) is a highly contagious, fatal neurodegenerative disease caused by infectious prions (PrP^{CWD}) affecting wild and captive cervids. Although recent experimental feeding studies have demonstrated infectious prions in feces of crows (*Corvus brachyrhynchos*), coyotes (*Canis latrans)*, and cougars (*Puma concolor)*, the role of scavengers and predators in CWD epidemiology remains poorly understood. Here we adapted detection tools for feces from cervid consumers to advance surveillance approaches, thereby improving disease modeling, and adaptive management of CWD. Using the real-time quaking-induced conversion (RT-QuIC) assay, we demonstrate recovery and assess detection sensitivity of PrP^{CWD} from experimental spiking of PrP^{CWD} into predator and scavenger feces sourced from CWD-free populations and captive facilities. We then applied this technique to detect PrP^{CWD} from feces of predators and scavengers in free-ranging populations. Our results demonstrate that i) spiked PrP^{CWD} is detectable from scats of free-ranging carnivores using RT-QuIC ii) PrP^{CWD} is detectable in feces from free-ranging carnivore scats, and iii) that PrP^{CWD} rates of detection in carnivore scat reflects relative prevalence estimates observed in the corresponding cervid populations. This study adapts an important surveillance tool for CWD, allowing investigation of the epidemiology of CWD at the community-level.

INTRODUCTION

The breakdown of detritus is important for regulating the movement of energy through food webs[1], structuring ecological communities and ecosystem function[2], and can also influence the dynamics of disease systems[3]. Obligate and/or facultative scavenger species participate in scavenging, or the consumption of carrion, as a resource acquisition strategy, where the latter are often also predators, consuming live prey. Scavenging and predation processes may have important influences on disease epidemiology through removal of infectious individuals or materials that could transmit pathogens to new hosts[4, 5]. Yet simultaneously, scavengers and predators may also spread infectious diseases by transporting the pathogen to other areas (through consumption, subsequent fecal deposition, or translocation or contaminated material)[6]. Determining whether predators and scavengers reduce or enhance disease transmission risk in the host species they consume is important for understanding the ecological and ecosystem context of infectious diseases in wildlife. The role of these consumers in disease dynamics will be affected by factors such as fecal deposition patterns of host consumers, pathogen characteristics and diversity (i.e., environmental stability, infectious dose), and how and where hosts encounter and are exposed to the pathogen.

Chronic wasting disease (CWD) is a highly contagious fatal neurological disease caused by a misfolded prion protein (PrP^{CWD}) that affects several domestic and free-ranging cervid species. Currently, cervids are the only wildlife species monitored in routine CWD surveillance and the effects of other species and the environment on CWD dynamics is relatively unknown. For prion diseases, pathogen detection from abiotic and biotic environmental sources is an impeding challenge and for years has limited our ability to address the sort of ecological questions readily possible for many other infectious agents. Such a detection tool could be used not only to begin

understanding how other species influence CWD processes but would also help to improve current surveillance efforts since sampling bias may limit our understanding of current CWD distribution[7].

Although the first observations of CWD were shown in captive mule deer (*Odocoileus hemionus*) and black-tailed deer (*Odocoileus hemionus columbianus*) in Colorado[8], the disease has since spread broadly in cervid species across North America and Europe, including white-tailed deer (*Odocoileus virginianus*, hereafter referred to as WTD), elk (*Cervus canadensis*), reindeer (*Rangifer tarandus*), and moose (*Alces alces*[9-12]). Through the disease course, PrP^{CWD} accumulates in body fluids and tissues[13, 14] of presymptomatic and symptomatic CWD-infected individuals, is shed in excreta and secreta[15-18], and ultimately results in neuroinvasion causing clinical disease and death[19, 20]. Transmission of CWD can occur either directly (i.e., contact between infectious and naive individuals) or indirectly through contact with contaminated environments[21]. Given that PrP^{CWD} can remain stable and infectious for years in the environment[22], deposition and accumulation of PrP^{CWD} into the environment from infected carcasses or via PrP^{CWD} shedding from infected individuals may lead to increased environmental disease exposure risk[23]. Consumption of CWD-infected host, dead or alive, suggests a role for predators and scavenging wildlife in CWD epidemiology.

Recent studies demonstrate that coyotes (*Canis latrans*)[24], American crows (*Corvus brachyrhynchos*)[25], and cougars (*Puma concolor*)[26] shed misfolded prion following ingestion of misfolded prion-infected material. Gut residency times appear to be fairly short, lasting ≤ 3 days in coyotes and cougars, to just several hours in crows. Results reported by both VanCauteren *et al.*[25] and Nichols *et al.*[24] suggest that consumers of contaminated cervid carrion could contribute

to translocation and contamination of PrP^{CWD} in the environment, however Nichols *et al.*[24] also showed a reduction of infectious PrP^{CWD} shed in coyote scat, implying scat-associated PrP^{CWD} deposits may be reduced compared to deposits associated with unconsumed contaminated carrion. Baune *et al.*[26] suggest that most of the prions ingested by cougars are eliminated or sequestered, supporting the notion that predators may have a dilution effect on the CWD system. Further, a modeling study from Brandell *et al*.[4] suggests that cougar and gray wolf (*Canis lupus*) predation pressure may independently decrease CWD outbreak size and delay prevalence increases of deer and elk, and this cleansing effect is amplified when predator selection for infected adults is greater than uninfected juveniles. Fisher *et al*.[27] recently reported predation by cougars may have slowed the increase in prevalence in an area of high CWD prevalence.

Previous studies investigating prions in scavengers relied on detection methods that range from mouse bioassays and antibody-based tools[25], to an ultra-sensitive *in vitro* protein amplification assay, protein misfolding cyclic amplification (PMCA)[24]. Although mouse bioassay remains the gold standard for definitively assessing the presence and titer of PrP^{CWD} in a sample, these assays can take hundreds of days to years to complete. Bioassays are also often used in conjunction with PMCA, which offers the same level of sensitivity as mouse bioassay and is far more sensitive than antibody-based detection tools[28, 29]. For example, in the study by Nichols *et al*.[24], mouse bioassay and PMCA were used to characterize the fecal samples prior to use as inoculum in the mouse bioassay. However, PMCA is both costly and not adaptable for high-throughput diagnostics as it still requires the need for mouse brain tissue as a conversion substrate and can take up to two-weeks to produce a result. Recently, the development and application of an alternative ultrasensitive *in vitro* protein amplification assay, the real-time quaking-induced conversion (RT-

QuIC), has advanced high throughput detection of PrP^{CWD} from host tissues[30, 31] and various host secreta and excreta[32, 33], saliva[34, 35], urine[34] and feces[16, 31, 32] without the use of mouse tissues. Currently, there has been only one report of the application of RT-QuIC for PrP^{CWD} detection from feces of cervid consumers, however this study was limited to captive animals in a controlled laboratory environment and did not assess field collected excrement[26].

Here we evaluated the utility of RT-QuIC for detection of PrP^{CWD} from a suite of relevant predator and scavenger feces using spiking assays. We assessed limits of PrP^{CWD} recovery and detection sensitivity from nine species relative to RT-QuIC characterized CWD-positive brain tissue. We then use this assay to detect PrP^{CWD} in scat samples from two free-ranging carnivore species – coyotes and cougars –to demonstrate the utility of including scavenger and predator scats in CWD surveillance. Our findings from field collected scats suggests that this approach could be used not only to institute early surveillance of CWD, especially in locations or jurisdictions neighboring CWD endemic zones that are considered areas of concern for geographic spread or areas where hunter-harvest rates are low, but also to begin to unravel how other species influence CWD processes and geographic spread.

METHODS

Fecal sample spiking assays. Using negative control fecal samples (sources in Table 3), spiking experiments were used to demonstrate recovery of PrP^{CWD} from scavenger and predator feces. Spiking assays were carried out by using 50 mg of each fecal sample combined with 50 µL of a 10-fold dilution of a 10% (w/v) CWD-positive WTD brain homogenate (BH) (from the obex

region of WIDNR tagged WTD #5219) prepared in 1X PBS to achieve a range of final concentrations of 10^{-3} to 10^{-8} mg/mL of CWD-positive brain. For the negative control spike, 50 µL of a 10^{-3} mg/mL dilution of a 10% (w/v) CWD-negative WTD BH was used. Each spike dilution was added to feces and allowed to soak in, then placed in a 50 °C incubator without agitation for 16 h. Spiked fecal samples were then prepared as described below.

Fecal sample extract preparation for RT-QuIC assay. Feces samples (Table 3) were stored in -80°C prior to preparation for use in the RT-QuIC assay. To prepare field collected fecal samples for detection of PrP^{CWD}, each individual fecal sample had one subsample collected from three different areas of the sample (50 mg each), totaling three biological replicates. These were then extracted using 1 mL of sterile 1X phosphate-buffered saline (PBS), then processed in a thermomixer (1,400 rpm, 30 min, 25°C; Eppendorf ThermoMixer F1.5) followed by centrifugation at 16,000 x *g* for 15 min. Supernatants (~750 µL) were collected, followed by addition of 750 µL 1X PBS to scat pellet and remaining buffer. Samples were vortexed, thermomixed (1,400 rpm, 30 min, 25°C), and centrifuged again at 16,000 x *g* for 15 min, after which an additional ~750 µL supernatant was removed and added to the first supernatants. Resultant supernatants were then centrifuged again for further clarification at 16,000 x *g* for 15 min, followed by collection of 1 mL of clarified supernatant into a new sterile tube and 500 µL of 23.1 mM sodium phosphotungstate hydrate (Na-PTA) (Sigma-Aldrich, Cat. # 496626) was added. Samples were incubated without agitation for 16 hours at 4°C, then centrifuged (4 °C, 30 min, 5000 x *g*). Pellets were retained and washed with a 1:1 solution of 18 MΩ H_2O and 23.1 mM sodium phosphotungstate (Na-PTA) followed by centrifugation (4 °C, 30 min, 5000 x *g*) and aspiration of the wash solution. Pellets were resuspended in 30 µL of RT-QuIC sample buffer (0.1 g·mL-1 sodium dodecyl sulfate in

phosphate-buffered saline with N-2 cell culture supplement; filtered 0.22 µm) (ThermoFisher, Waltham, MA) and reconstituted using a Qsonica Q700 cup horn ultrasonicator (Amplitude 36 for 1 min.). A volume of 2 µL of each reconstituted sample was used to seed each reaction well for 8 technical replicates.

Real-time Quaking-induced Conversion Assay. The RT-QuIC *in vitro* prion amplification assay was performed as described by Metrick *et al*.[36] with minor modifications. Briefly, 2 µL of sample extracts were added to a given well of a 96-well format optical-bottom black microplate (Thermo Scientific, Fair Lawn, NJ, USA), each already containing 98 µL of RT-QuIC reaction mixture (0.1 $mg \cdot mL^{-1}$ 90-231 recombinant hamster prion protein, produced as previously described:[37], 300 mM sodium iodide, 20 mM sodium phosphate, 1.0 mM ethylenediaminetetraacetic acid, and 10 µM thioflavin T). Microplate-compatible spectrophotometers capable of heating, shaking, and fluorescence monitoring (BMG FLUOstar, Cary, North Carolina) were used with the following instrument settings: 50 °C for spiked samples double orbital pattern shaking at 700 rpm with 60-s shake / 60-s rest cycles, fluorescent scans ($\lambda_{excitation}$ = 448 nm, $\lambda_{emission}$ = 482 nm) every 15 minutes, at a gain of 1,600, and a total run time of 48 h.

Data Analysis. Amyloid formation rate data generated from the RT-QuIC assays were analyzed and visualized using Jmp Pro 15 (SAS Institute, Cary, NC) and Prism 8 (GraphPad, San Diego, CA). To apply a rigorous standard for distinguishing true positive samples from true negatives, the AFR threshold times (i.e., the time which amplification is determined to have occurred in the RT-QuIC assay) were calculated by adding twenty times the standard deviation of the relative fluorescence unit (RFU) values from cycles 3-14 to the mean of RFU values from cycles 3-14. We

previously applied this method to account for baseline fluorescence variation amongst samples in determining if the sample was positive for PrP^{CWD}[38]. Due to false seeding among crow, black bear, eagle, and raccoon negative control fecal samples, additional analysis was required to distinguish true seeding from false seeding events from feces for these species. To accomplish this, empirical distributions of threshold times in hours (Supplementary Figure S1) of 24 technical replicates of unspiked fecal samples were used to determine a threshold time that would yield a specificity ≥ 95% for each species fecal type. These data were then used to determine a cycle end-time that excluded ≥95% of the false seeding that occurred for each of these scat types. Cycle end-times for these four scat types were determined to be 20 h for crow (specificity of 95%), 24 h for black bear (specificity 100%), 25 h for eagle (specificity 96%), and 17 h for raccoon (specificity 100%). All spiked and surveillance fecal samples analyzed by RT-QuIC were considered positive if a sample had at least 3 out of 8 technical replicates with seeding activity and were significantly different from controls based on statistical analysis. Analyses for spiked samples were assessed with Dunnett's multiple comparison tests, while for the surveillance samples, Kruskal-Wallis tests was used to distinguish which samples had AFRs significantly different from the NYE negative control scat sample.

Using AFR as a relative measure for prion concentration in a sample, we first evaluated our ability to detect PrP^{CWD} from spiked fecal samples compared to the source material (CWD-positive brain tissue), and if the AFR differed among carnivore species. We used a two-way (factorial) ANOVA to compare AFR values among sample type (CWD-positive brain, spiked fecal samples from 9 different species). We included an interaction between species fecal type and spike dilution, to assess if detection/recovery in different feces types was sensitive to the concentration of the spike

across the 10-fold dilution series. Significant interactions were determined using the Tukey HSD for multiple comparisons.

Amyloid formation rates for field collected coyote and cougar scat samples were found to be non-normally distributed (Supplementary Figure S2). As such, the nonparametric Kruskal-Wallis test was applied to compare individual scat samples. If statistical differences were observed for each sample set, then the non-parametric Steel-dewass post hoc test was used to determine which individual samples differed from the negative control scat sample.

RESULTS

Using RT-QuIC, our experimental spiking studies aimed to test extraction efficiency and detection sensitivity of PrP^{CWD} in scats from different scavenger and predator species using a brain sample (from the obex region) from a late-stage CWD-positive WTD as the source material for spiking in all experiments. The spiked feces were sourced from grey wolf (*C. lupus,* hereafter wolf), coyote, American black bear *(Ursus americanus*, hereafter black bear*),* raccoon (*Procyon lotor*)*,* common raven (*Corvus corax*, hereafter raven)*,* cougar*,* red fox *(Vulpes vulpes*, hereafter fox*),* American crow (*C. brachyrhynchos*)*,* and bald eagle (*Haliaeetus leucocephalus*, hereafter eagle). Fecal samples for each species were spiked with 10-fold dilutions of brain-derived PrP^{CWD} (Fig. 1). Variation in assay sensitivity (i.e., the limit of relative PrP^{CWD} detection significantly different from the negative controls) for the different dilutions of brain homogenate (BH) spike was observed depending on species. The most sensitive detection was from wolf scat, with 8/8 technical replicates showing seeding activity (defined as having relative fluorescent units (RFU) above the RFU threshold) for scat spiked with only 10 ng of CWD-positive BH. This was followed

by eagle guano (6/8 seeding), which had a detection limit at 100 ng of brain-derived PrP^{CWD} (Fig. 1). Detection limits were higher for other species, at a 10^3 ng spike for crow (6/8), black bear (3/8), cougar (4/8), and raven (8/8), a 10^4 ng spike for coyote (8/8) and raccoon (4/8) (Fig. 1), and a 10^5 ng spike for fox (8/8) (Fig. 1).

The variable detection limits observed among species may be due to differences in inhibition of the assay by the fecal material, the extraction process, or a combination of both. Overall, we found species differences do account for some of the variation seen in the magnitude of amyloid formation rates (AFR) (i.e., the time above which amplification is determined to have occurred in the RT-QuIC assay) for spiked feces when compared to dilutions of a CWD-positive, WTD BH sample on its own (F (9, 420) = 155.46, $p < 0.0001$). Mean AFRs for all species were significantly lower compared to pure CWD-positive BH and there were species differences in recovery (Tukey HSD post-hoc test for multiple comparisons, Supplementary Table S1, Fig. 2a). Spiked crow, eagle guano and wolf scat had significantly higher AFRs than the other species (Supplementary Table S1, Fig. 2a). Cougar and fox scat had the lowest mean AFRs (Supplementary Table S1, Fig. 2a). Amyloid formation rate values from spiked raven did not differ significantly from wolf, black bear, coyote, or raccoon scats, however AFR values of black bear, coyote, and raccoon scats did differ from the other species (Supplementary Table S1, Fig. 2a). Amyloid formation rates for all species-fecal types decreased across the dilution series (F (5,420) = 355.69, $p < 0.0001$), with a significant interaction between scat type and spike dilution (F (45, 196) = 9.36, $p < 0.0001$). The magnitude of differences in observed AFRs varied among species and by spike dilution, however the pattern of recovery being higher at lower and higher dilutions rather than for dilutions in between appeared

to be present for all feces samples, with an exception for eagle and crow (Tukey HSD post-hoc tests, e Fig. 2b).

Following these proof-of-concept spiking experiments, we then determined if we could detect PrP^{CWD} in feces from free-ranging predators/scavengers from areas with CWD. We also evaluated how variable PrP^{CWD} presence is across a single sample and how repeatable our results for RT-QuIC were across plates by sampling and separately extracting and testing three different regions of a given sample. We examined coyote and cougar scat samples collected from three distinct geographic regions where CWD has been detected for the presence of PrP^{CWD}. In Iowa County, Wisconsin, six different coyote scat samples were collected near mortality sites for six different collared WTD in an area where CWD prevalence is ~40 to 30 % in adult males and females, respectively[39]. Of these WTD carcasses, 5/6 were confirmed CWD-positive by RT-QuIC on tissues (i.e., brain or skin from ear or belly; Fig. 3). RT-QuIC assays of three separate subsamples from each coyote scat (labeled as C1-C6) showed seeding activity in 8/8 of all subsamples from all scat samples, with exception for sample ID C3, where all eight technical replicates for the first and third extractions showed seeding activity but had no seeding activity for the second extraction (Table 1). Coyote scat AFRs were significantly greater than the controls (Kruskal-Wallis test: $X^2 = 106.29$, $p<0.0001$, df = 6; Steel-dwass post hoc test: $p \leq 0.00016$; Fig. 3; Table 1). Amyloid formation rates of coyote scats were reduced compared to those of WTD tissue from each mortality site but had similar value ranges across each primary scat sample and very little variation among technical replicates of each scat subsample (Fig. 3).

Cougar scats were collected from Niobrara Valley (2012: n=1; 2014: n=3) and Pine Ridge (2010: n=5; 2021: n=5) in Cherry County, Nebraska, USA. These areas were known to have CWD, although Pine Ridge in 2010 had 0% prevalence[40]. Amyloid formation rates of three Niobrara (one from 2012, and two from 2014) and three Pine Ridge 2021 samples were significantly greater than the controls (Kruskal-Wallis test: $X^2 = 255.25$, $p<0.0001$, df = 15; Steel-dwass post hoc test: $p \leq 0.0226$; Fig. 4). RT-QuIC assays of three separate subsamples taken from each primary scat sample showed variable seeding activity within the 8 technical replicates, ranging from 2/8 to 8/8 (Table 2). The total number of replicates (out of 24) with seeding for each primary scat sample was half or more (Table 2). The range of AFRs among these primary scat samples were variable, where values from the Pine Ridge 2021 samples were most elevated compared to the three samples from Niobrara (Fig. 4). Amyloid formation rates for the remaining primary samples were not significantly greater than the controls (Steel-dwass post hoc test: $p \geq 0.05$; Fig. 4). RT-QuIC assays of three separate subsamples taken from each of these primary scat samples also showed variable seeding that ranged from 0/8 to 6/8 (Table 2). The total number of replicates (out of 24) with seeding for each of these primary scat samples was less than half (Table 2). Overall, subsample replicate AFRs from cougar scats (Fig. 4, Table 2) were more variable that those seen from coyote scats (Fig. 3, Table 1).

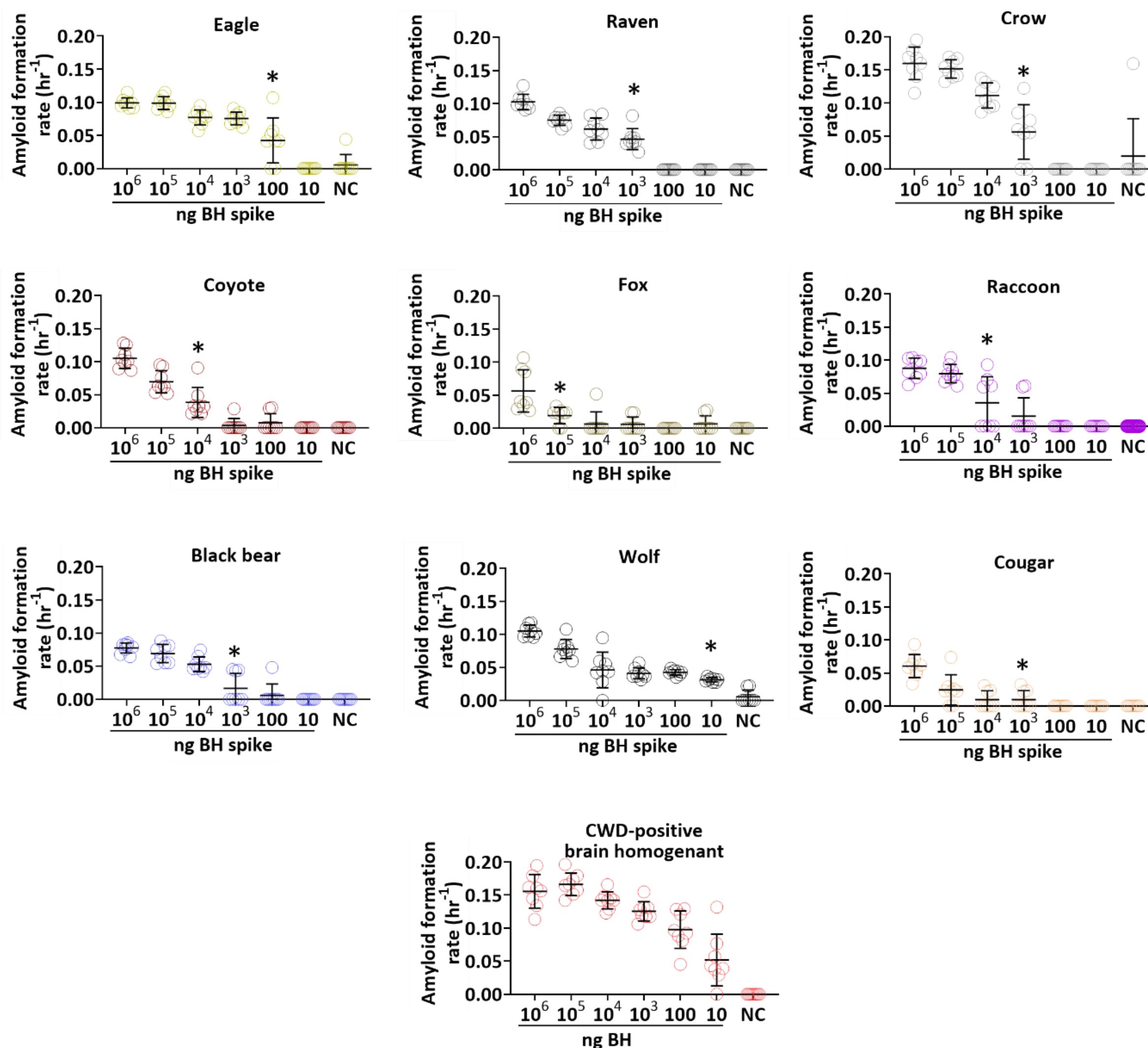


Figure 1. Recovery and detection sensitivity of chronic wasting disease (CWD) prions (PrP^{CWD}) from spiking experiments of predator and scavenger scats by the real-time quaking-induced conversion (RT-QuIC) assay. Amyloid formation rates (AFRs) by RT-QuIC for predator and scavenger scat samples spiked with 10-fold dilutions of 10% brain homogenate (from the obex region; BH) shown in nanograms (ng) from a CWD-positive white-tailed deer. Data points of AFRs ± standard deviation of eight technical replicates across each spiking dilution series in feces samples from crow (*Corvus brachyrhynchos*), raven (*Corvus corax*), eagle (*Haliaeetus leucocephalus*), raccoon (*Procyon lotor*), grey wolf (*Canis lupus*), coyote (*Canis latrans*), red fox (*Vulpes vulpes*), cougar (*Puma concolor*), and black bear (*Ursus americanus*) are

shown. The dilution curve for the BH used as spiking material is shown for comparison to PrP^{CWD} recovery and detection sensitivity in feces. * = limit of detection sensitivity of spiked material in each species fecal type.

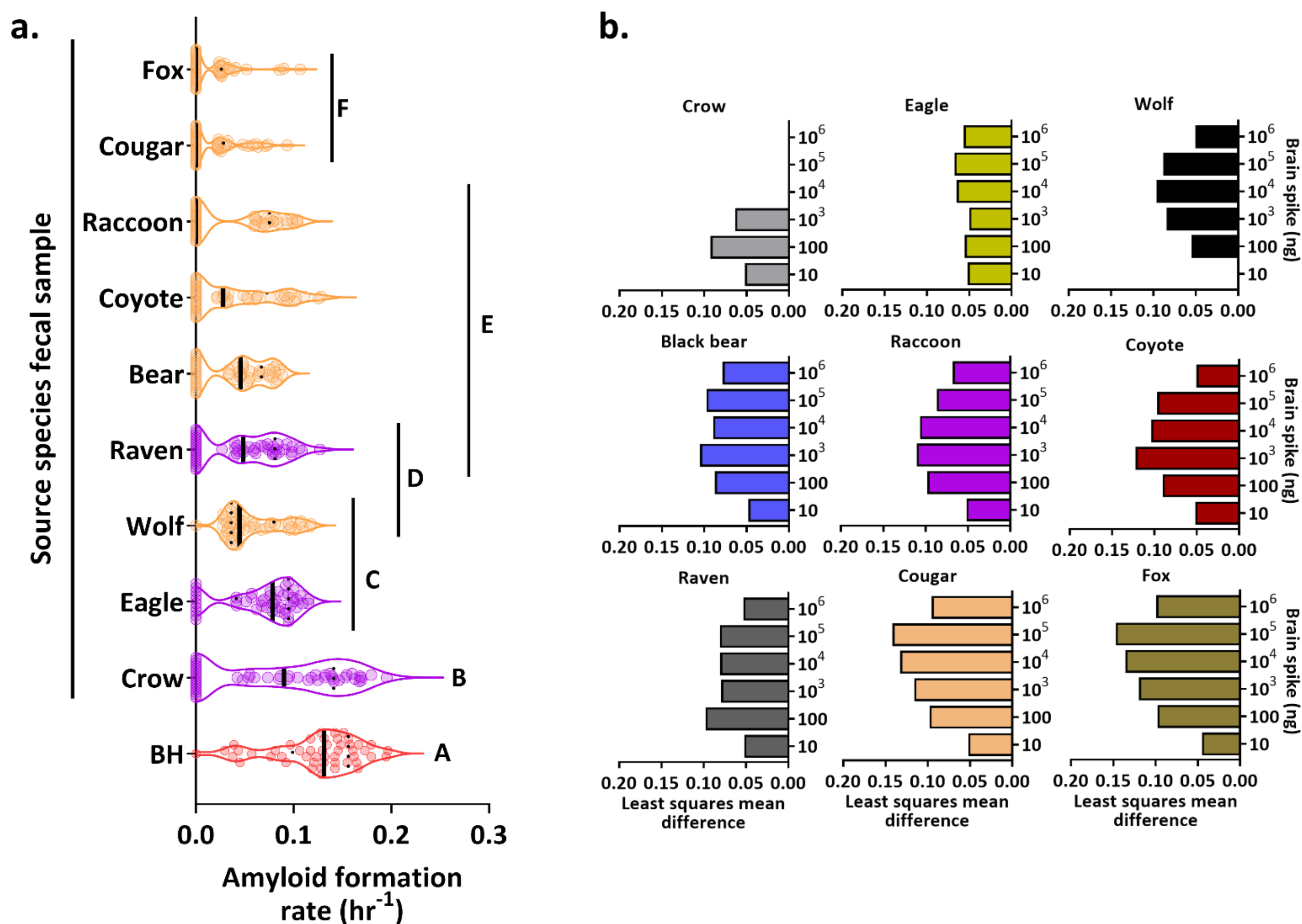


Figure 2. Differences in recovery and detection sensitivity of chronic wasting disease (CWD) prions (PrP^{CWD}) from spiked scavenger and predator scats. (a) Distribution of amyloid formations rates (AFRs) across spiked scat types showing the median (solid black line) and third and first quartiles (dotted black lines). CWD-positive brain homogenate is shown in red, mammalian species in orange and avian species in purple. Letters show which species are statistically different by Tukey HSD post-hoc test. **(b)** Illustration of the significant least squares mean AFR value differences between 10-fold dilutions of CWD-positive brain homogenate and recovered brain-derived PrP^{CWD} from spiked fecal samples by species type and brain spike dilutions using the Tukey HSD post-hoc test. Dilutions with no bars indicate no significant difference in AFR from feces compared to BH at that dilution.

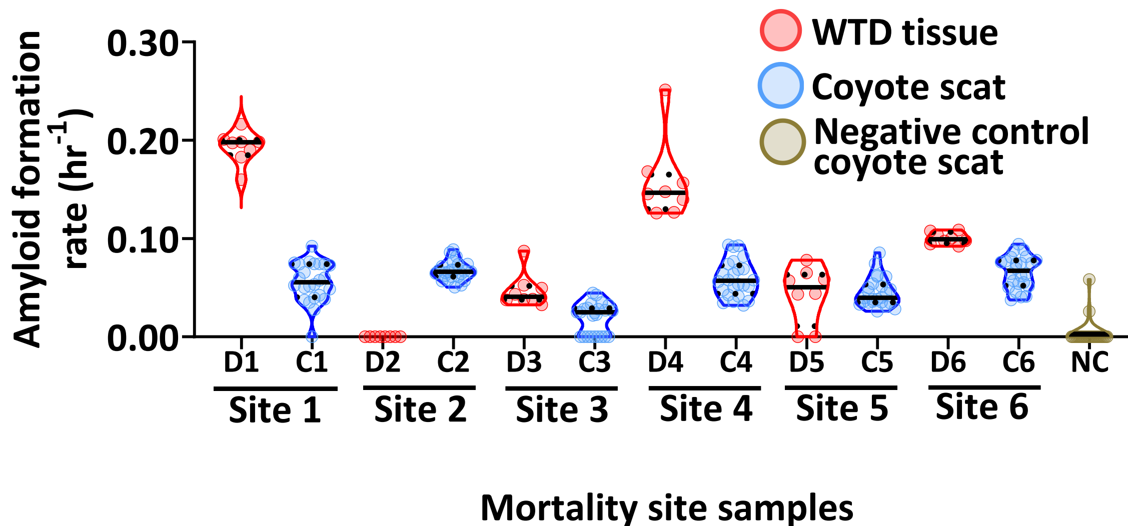


Figure 3. The presence of chronic wasting disease (CWD) prions (PrP^{CWD}) in coyote scat samples collected near collared white-tailed deer (WTD) mortality sites within Iowa County, Wisconsin, USA. Comparisons of real-time quaking-induced conversation (RT-QuIC) amyloid formation rates (AFR) of tissues (D1, obex; D2, obex; D3, belly skin; D4, ear pinna; D5, obex; D6, skin) from six different WTD carcasses (red) with six different coyote scat samples found near each respective mortality site (blue). Samples are grouped by WTD mortality site ID (by number; D=deer and C=coyote from each site) on the x-axis. Data points show 8 and 24 (3 separate extractions, 8 technical replicates each) replicates of WTD tissue or coyote scat, respectively, with the median (solid black line) and third and first quartiles (dotted black lines). Negative control (NC; tan) represents 3 separate extraction results of 8 technical replicates each for coyote scat from 1 individual collected from the Northern Yellowstone Ecosystem.

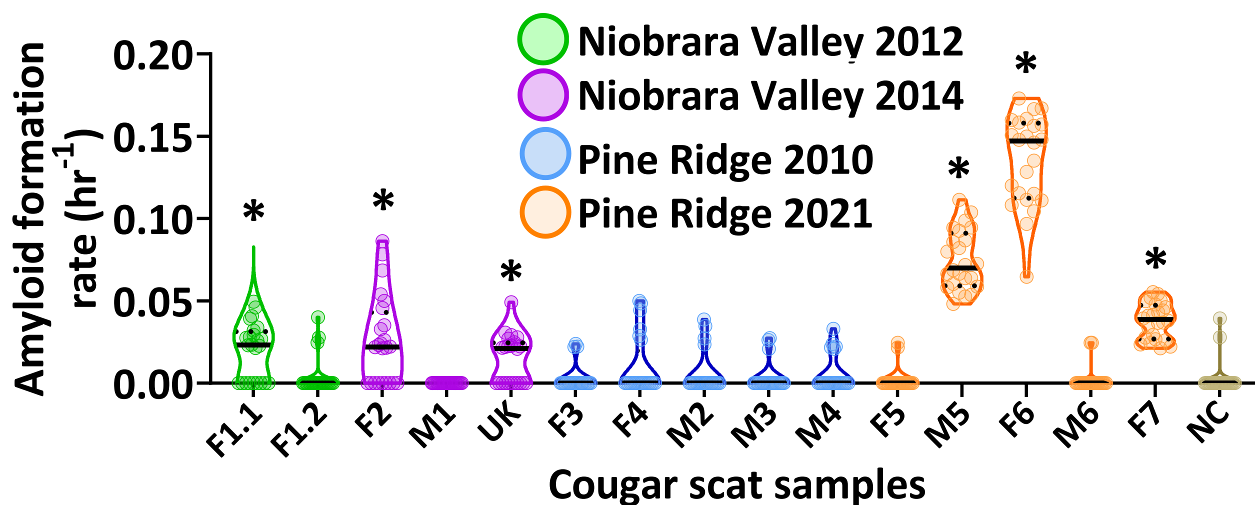


Figure 4. Presence of chronic wasting disease (CWD) prions (PrP^{CWD}) in cougar temporal and spatial scat samples collected from two locations within Cherry County, Nebraska, USA. Real-time quaking-induced conversation (RT-QuIC) amyloid formation rates (AFR) from cougar scats collected from different individuals from the Niobrara Valley in 2012 (green) and 2014 (purple) and in 2010 (blue) and 2021 (orange) from the Pine Ridge region. Samples are grouped by individual cougar ID on the x-axis (F1.1 and F1.2 are two separate samples for the same individual). Data points shown are for 24 technical replicates (3 separate extractions of 8 technical replicates each), with the median (solid black line) and third and first quartiles (dotted black lines). Negative control (NC; tan) represents triplicate assay results for CWD-negative cougar scat from 1 individual collected from the Northern Yellowstone Ecosystem. * = $p \leq 0.0226$ by Steel-dwass post-hoc test.

Table 1. Presence or absence of chronic wasting disease (CWD) prions (PrP^{CWD}) for six different coyote (*Canis latrans*) scat samples collected from within Iowa County, Wisconsin, USA and one negative control (NC) coyote scat collected from the Northern Yellowstone Ecosystem (as depicted in Figure 3) assessed by the real-time quaking-induced conversion (RT-QuIC) assay. Values indicate the proportion of seeding activity for three separate extractions, 8 technical replicates each (24 total replicates) for each primary scat sample.

Sample ID	Extraction 1	Extraction 2	Extraction 3	Total seeded reactions	PrP^{CWD} +/- for each extraction
C1	8/8	7/8	8/8	23/24	+/+/+
C2	8/8	8/8	8/8	24/24	+/+/+
C3	7/8	0/8	8/8	15/24	+/-/+
C4	8/8	8/8	8/8	24/24	+/+/+
C5	8/8	8/8	8/8	24/24	+/+/+
C6	8/8	8/8	8/8	24/24	+/+/+
NC	0/8	1/8	1/8	2/24	-/-/-

Table 2. Presence or absence of chronic wasting disease (CWD) prions (PrP^{CWD}) for scats from 15 different cougars (*Puma concolor*) from the Niobrara (N) of Pine Ridge (P) deer management units within Cherry County, Nebraska, USA and one negative control (NC) cougar scat (collected from the Northern Yellowstone Ecosystem (NYE), as depicted in Figure 4) assessed by the real-time quaking-induced conversion (RT-QuIC) assay. Values indicate the proportion of seeding activity for three separate extractions, 8 technical replicates each (24 total replicates) for each scat sample.

Sample ID	Collection Year/location	Extraction 1	Extraction 2	Extraction 3	Total seeded reactions	PrP^{CWD} +/- for each extraction
F1.1	2012/N	3/8	4/8	8/8	15/24	+/+/+
F1.2	2012/N	2/8	0/8	1/8	3/24	-/-/-
F2	2014/N	8/8	2/8	5/8	15/24	+/-/+
M1	2014/N	0/8	0/8	0/8	0/24	-/-/-
Unknown	2014/N	5/8	4/8	4/8	13/24	+/+/+
F3	2010/P	3/8	0/8	0/8	3/24	+/-/-
F4	2010/P	0/8	0/8	6/8	6/24	-/-/+
M2	2010/P	4/8	0/8	0/8	4/24	+/-/-
M3	2010/P	3/8	0/8	0/8	3/24	+/-/-
M4	2010/P	2/8	2/8	1/8	5/24	-/-/-
F5	2021/P	0/8	2/8	0/8	2/24	-/-/-
M5	2021/P	8/8	8/8	8/8	24/24	+/+/+
F6	2021/P	8/8	8/8	8/8	24/24	+/+/+
M6	2021/P	0/8	1/8	1/8	2/24	-/-/-
F7	2021/P	8/8	8/8	8/8	24/24	+/+/+
NC	2022/NYE	1/8	1/8	0/8	2/24	-/-/-

Table 3. Species fecal sample collection location information, use, and storage conditions for samples included in this study. All samples were stored at –80°C.

Species	Year collected	Location of collection
	Fecal samples used for RT-QuIC spiking assays	
Wolf	2020	Northern Yellowstone Ecosystem
Coyote	2022	Northern Yellowstone Ecosystem
Fox	2022	Northern Yellowstone Ecosystem
Raven	2022	Northern Yellowstone Ecosystem
Cougar	2022	Northern Yellowstone Ecosystem
Bear	2022	Minnesota Wild and Free Wildlife Rehabilitation Center (MWF) in Garrison, Minnesota.
Raccoon	2022	MWF
Eagle	2022	MWF
Crow	2022	Dane County Humane Society Wildlife Center, Madison, Wisconsin, U.S.A.
	Fecal samples used for RT-QuIC diagnostics assays	
Coyote	2021-2022	Iowa County, Wisconsin, U.S.A.
Cougar	2012 & 2014	Niobrara Valley, Cherry County, Nebraska, U.S.A.
Cougar	2010 & 2021	Pine Ridge region, Cherry County, Nebraska, U.S.A.

DISCUSSION

The role of sympatric wildlife in CWD epidemiology remains incompletely understood, but cervid-consuming predators and scavengers may alter rates of disease spread through removal or dispersal of PrP^{CWD} on the landscape. Determining the effects of cervid consumers on CWD ecology and epidemiology could be facilitated with development of non-invasive and sensitive methods with high throughput capacity for PrP^{CWD} detection. We presented results from laboratory spiking experiments that scat-based CWD surveillance is possible from a range of mammalian and avian species. Such scat-based surveillance could expand our toolkit of available approaches, offering a non-invasive alternative to current surveillance approaches. We then evaluate our approach using field-collected scats from CWD endemic areas, determining that this approach is able to detect PrP^{CWD} in the quantities present in coyote and cougar scats. Lack of tools that can be used to detect the presence of PrP^{CWD} in abiotic and biotic environmental has hampered our ability to address relevant ecological questions. Our study directly addresses this need through adapting an important surveillance tool for CWD, allowing investigation of the epidemiology of CWD at the community-level.

The spiking experiments provided a baseline assessment of how compatible feces from different cervid consumers is with RT-QuIC. Limits of relative PrP^{CWD} detection ranged from 10^5 to 10 ng of spiked material, depending on species and spike dilution (Fig. 1, Fig. 2a, b). These dilutions of CWD-positive material match the relative PrP^{CWD} loads seen in scats of cougars experimentally fed CWD-positive cervid tissue[26]. We posited that collecting and analyzing feces from certain cervid consumers could be a promising addition to CWD surveillance programs. Based on our findings from the spiking assays, wolf and eagle allowed for the most sensitive detection of brain

derived PrP^{CWD} (Fig. 1), suggesting these species may be best to sample as early sentinels of CWD. However, an assay cutoff time of 25 hours had to be applied to eagle results to remove false seeding events from unspiked samples, which could potentially result in loss of sensitivity. This was also the case for bear, crow, and raccoon, where assay cutoff times necessary to reduce false positives varied for each species. While spiking experiments help provide a baseline of compatibility for different fecal types with the RT-QuIC assay, they are not a perfect representation of what one may find from excrement composed of output from a natural diet collected from the field. It is also worth noting that feces from animals in rehabilitation centers needed an assay cutoff time to reduce false seeding, whereas fecal samples from free-ranging animals did not require an assay cutoff time to avoid false positives. Thus, dietary variation among species and individuals may affect assay results. Further analyses of how differences in diet and fecal composition affect the assay sensitivity and specificity would improve our understanding of assay strength and limitations.

Recovery differences of spiked brain derived PrPCWD in feces compared to pure brain based on AFR values followed a similar trend for most species. Smaller differences were observed between positive controls and spiked samples at the most and least concentrated spike dilutions compared to larger differences in detection at the mid-concentration dilutions (Fig. 2b). This hump-shaped variation in prion recovery across dilutions may have been an effect of the extraction process used for the feces samples following spiking, where some loss of seeding material likely occurred and was most sensitively observed at these spiking dilutions for each feces sample. It is also possible that the variability in AFRs that occurred for the 106 ng and 10 ng concentrations of pure CWD-positive BH was similar enough to that observed in many of the spiked fecal samples, that the differences were lower by comparison to the less variable AFRs from all other dilutions of pure

CWD-positive BH (Fig. 1, Fig. 2b). Additionally, the compounds that a given sample is composed of may influence the RT-QuIC reaction itself, as has been demonstrated with the inhibitory effect of certain sample types41, 42. Thus, it remains possible that this trend may be due to similar fecal biochemical compositions from each species that are resulting in similar matrix-specific recovery effects.

Detection of brain-derived PrP^{CWD} from wolf scat was the most sensitive across all spiked feces, did not require an assay cutoff time to distinguish true seeding from false seeding events, and would be relatively easy to collect on a seasonal basis in more temperate CWD endemic areas such as the upper Midwest. The sensitivity and specificity results from the spiking assay and ease of sample collection suggest that wolf would be an optimal candidate for incorporating into CWD surveillance strategies. However, there are other considerations about species most readily sampled in different environments, such as access/ease of collection and whether the species is rare or more common. From the collection of species evaluated here, most of the mammal, with the exception of possibly fox, and avian species may be candidates for further evaluation by RT-QuIC. Although spiking assays for coyote and cougar scat revealed mid-level sensitivity compared to wolf (Fig 1), these species also did not require an assay cutoff time and exhibited 100% specificity, compared to ~90% specificity for wolf (1/8 false seeding rate) (Fig. 1), suggesting they may also be good candidates for CWD surveillance. Raven may also be a good candidate due to lower differences in detection of PrP^{CWD} compared to pure CWD-positive BH, however ease of collection of this feces type may present restrictions on utility of this feces type for determining CWD occurrence in a given area compared with mammalian feces. In this study, we readily had access to coyote and cougar scats from areas where CWD is either endemic or has been detected

but have low prevalence. While spiking assay results for these two species was not as sensitive compared to wolf, we were still able to demonstrate detection of PrP^{CWD} from samples. Because these larger scavenger and predator species tend to consume larger amounts in one feeding—and by proxy, presumably more PrP^{CWD}— detection of PrP^{CWD} from their scats may be more likely than in avian. This suggests that carnivore scats in general may offer a way to advance CWD surveillance strategies.

Detection of PrP^{CWD} within some of the individual field collected NE cougar scat subsamples was more variable than WI coyote subsamples (Fig. 3, Fig. 4, Table 1, Table 2). This may have been the result of differences in sample age, as increased sample age may reduce detection by RT-QuIC for some sample types[43]. WI coyote scats were less than a week old and had seeding ratios similar to the Pine Ridge 2021 cougar scats. Given that the more recently collected NE cougar scats (Pine Ridge 2021) had higher over AFRs in samples with PrP^{CWD} present and the most consistent detection among all other samples in that group compared to the other groups of NE cougar scats (Niobrara 2012, 2014; Pine Ridge 2010) suggests that this variation could have been due to differences in sample age. In addition, lower amounts of seeding material have been shown to result in more variable AFRs using RT-QuIC[28], thus it is also possible that this variability in earlier NE cougar scat subsamples could be the result of overall lower levels of PrP^{CWD} present in the sample compared to WI coyote samples. Homogenizing the whole sample prior to RT-QuIC might yield more consistent seeding from a given sample. However, because microparticles can lead to complications in the RT-QuIC[37], we chose to initially evaluate subsampling of primary samples rather than whole sample-homogenization in this study. Future studies comparing homogenized vs unhomogenized samples for a given species would help determine how homogenization of a

whole sample influences RT-QuIC reactions, recovery of PrP^{CWD}, and detection sensitivity and specificity. Additionally, factors such as amounts of biomass being consumed, gut residence time of PrP^{CWD} in these species, if they are scavenging how much time they might spending at a carcass site, or whether they are visiting a site repeatedly and feeding over the course of several days are all factors that warrant further investigation.

The results of the WI field collected coyote scats found on the periphery of deer mortality sites demonstrate that triplicate subsampling of each scat sample was sufficient to overcome the variability of sensitivity for a given sample and demonstrate if PrP^{CWD} was present or not using the RT-QuIC assay (Fig. 3, Table 1). Sample ID C3 had the most variable seeding activity and also had the lowest average AFRs compared to all other WI coyote samples, suggesting that the variability was likely due to lower overall levels of PrP^{CWD} present in this sample, a common response of the RT-QuIC assay to samples with lower prion loads[28] (Fig. 3, Table 1). In addition, relative loads of PrP^{CWD} of each WI coyote scat sample were reduced compared to tissues from the CWD-infected deer carcass they may have been scavenging. Because coyotes reportedly leave numerous scent marking scats within 1 to 80 m of the carrion they are consuming, we find it reasonable to consider that some of these scat samples came from coyote that had scavenged on the respective carcass near the location their scats were collected[44]. It is also worth considering that since one of the coyote scat samples was collected on the periphery of a CWD-negative carcass site—yet still had PrP^{CWD} present, some of these individuals may have been feeding on more than one carcass, making it difficult to interpret the reason for reduced loads of PrP^{CWD} found in each scat sample and ascertain if reduced PrP^{CWD} loads are an effect of digestion by coyote or an effect of mixed consumption of different tissues from healthy and CWD-infected carcasses. Reduced

amounts of PrP^{CWD} detected in these coyote scats compared to PrP^{CWD} present in carcasses could also be an effect of the scat type reducing assay sensitivity or extract, considering the results from the spiking experiments and that feces from cervids has been shown to have reduced sensitivity by the RT-QuIC assay[45]. It is also possible that the reduced amounts present may be an effect of loss of seeding material as a result of the extraction process. Because preparation of the deer tissues is a much more simplified process compared to what is needed for feces, there is greater potential for reducing amounts of PrP^{CWD} present in fecal samples., making it difficult to infer the meaning of these differences. Lastly, PrP^{CWD} loads may also be reduced through digestion (Fig. 3), especially considering that PrP^{CWD} loads and infectivity present in coyote scat following digestion of PrP^{CWD} contaminated material was shown to be substantially reduced by mouse bioassay[24]. Additional studies assessing coyote or other scavenger scat field samples and biological mass consumption at CWD-infected deer carcass sites would help further decipher the effects of scavenging CWD-infected carcasses in structuring PrP^{CWD} environmental removal or deposition. Overall, these results support our hypothesis that CWD surveillance using carnivore feces may provide a useful estimate of CWD occurrence.

Subsampling of the field collected NE cougar scats confirmed the presence of PrP^{CWD} in 6 samples (Fig. 4, Table 2). Results of PrP^{CWD} occurrence in cougar scats from the 2010 and 2021 Pine Ridge samples reflect the increase in WTD CWD prevalence seen over 2010 to 2021 within this DMU[40]. Prevalence of CWD in WTD and cougar scats for the Pine Ridge DMU were both 0% in 2010. Although host CWD surveillance was not conducted in 2021 for the Pine Ridge DMU, surveillance in 2019 and 2022 estimated WTD CWD prevalence of 26% (69/264) and 27% (62/244), respectively, which is underestimated compared to the 60% (3/5) CWD prevalence in cougar scats

from that area (Fig. 4)[40]. Interestingly, the DMU where the Niobrara Valley cougar scats were collected has had host CWD prevalence estimates of under 1% for the past 18 years. Surveillance in 2012 estimated prevalence of 0% (0/512), and although no surveillance was conducted from 2013-2016 for this DMU, 2017-2019 prevalence was reported to be 0% (0/1), 0.41% (1/243), and 0% (0/1), respectively[40]. The consistently low prevalence rate estimates garnered from deer culling efforts contrast with the 50% (1/2; 2012) and 67% (2/3; 2014) CWD prevalence estimates based on the results from cougar scats collected for the Niobrara DMU (Fig. 4, Table 2). This suggests that incorporation of carnivore scats in CWD surveillance may help more accurately capture jurisdictional CWD prevalence. Our findings from both the Pine Ridge and Niobrara Valley DMUs highlight the usefulness of incorporating carnivore scats in CWD surveillance strategies. If predators select infected prey at a greater rate than uninfected prey, as has been observed with cougars[46], predator scats may serve as early sentinels of CWD detection in areas where CWD is not yet endemic or where areas where surveillance efforts are limited, as we have demonstrated here with cougar scats. (Fig. 4, Table 2).

Areas with more biodiverse predator and scavenger guilds may have different effects on the CWD system compared to areas where species richness and abundance of cervid consumers is less robust. Nonetheless, the presence of cervid consumers would alter CWD epidemiology compared to hypothetical systems lacking their presence. For example, predators could be killing infected prey before clinical CWD kills them, as is the case with cougar predation on infected mule deer[46, 47], thus reducing environmental prion deposition and accumulation from host shedding or carcass decay. Portions of predator kills that are not immediately consumed may be consumed by competitors and scavengers[48, 49]. This may allow increased opportunity for avian and mammalian

scavengers to consume contaminated carrion, some of which (i.e., coyote) utilized cervid carrion is one of the primary food resources[50, 51] consuming up to two-thirds cervid carrion in one feeding[51]. Further, clinical stages of CWD inevitably render the host physically and neurologically compromised and can result in hosts dying directly from CWD or increased vulnerability to other causes of death vehicle collisions[52] or being targeted during hunting season[7]. Regardless of the ultimate cause of death for CWD-infected hosts, mortality is inevitably followed by ingestion of contaminated carcass remains by both avian and mammalian predators and scavengers[53]. In addition, there is also evidence that certain avian species consume blood-feeding ectoparasites (i.e., deer keds, ticks) attached and feeding on cervids[54-56] and that both avian and small mammals (i.e., voles, shrews) may predate blood-feeding ectoparasites found in cervid bedding sites[54, 57, 58], some of which have been shown to harbor PrP^{CWD} [38, 59], suggesting that avian and small mammal species not typically associated with scavenging could also play a role in CWD epidemiology.

Cervids have been shown to visit conspecific carrion sites[53, 60] and the presence of carnivore scat deposits elicits anti-predator response behaviors (i.e., avoidance, vigilance) from cervids[61]. Where contaminated carrion is present, it remains plausible that carnivore scats could serve as a deterrent for any naïve cervids that may potentially visit these sites, which would reduce risk of direct interactions between infected carcasses and naïve individuals and indirect CWD exposure. Further, this apparent response that scavenger and predator scats elicits from prey suggests that scat deposits and other predatory cues may be a structuring force underlying general cervid movement and spatial distribution, as prey distributions are the result of a cost-benefit trade-off across space and time that is largely influenced by food resources and predation risk[62, 63].

Additionally, wide-range dispersal of PrP^{CWD}-contaminated scat by terrestrial predators and scavengers likely isn't any greater than what migratory cervid hosts are depositing on the landscape. Occasional dispersal events by some cervid consumers may result in PrP^{CWD} deposition to new areas via feces. However, cervids and the translocation of cervids or carcasses through anthropogenic recreational activities has the potential to introduced relatively larger quantities of PrP^{CWD} into new areas. Similarly, although avian scavenging strategies vary seasonally from species to species and potential exists for dispersal of PrP^{CWD} in guano following consumption of CWD-infected carcasses or other PrP^{CWD} contaminated material, long distance flight (>75 meters) following intake of large amounts of carrion may be unlikely for some obligate scavenger species[64]. Further detailed ecological studies are warranted to help disentangle the complexities of predator and scavenger involvement in mitigating direct CWD exposure and transmission as well as reducing deposition of PrP^{CWD} on the landscape through the removal of individuals actively shedding prions.

The developments we have reported here are critical first steps in elucidating the roles of scavengers and predators in CWD epidemiology and for advancing and complimenting existing CWD surveillance strategies. Adding or altering ongoing surveillance efforts to include collection and analysis of predator and scavenger scats may call for additional funding, personnel, and logistical considerations by wildlife management agencies. However, CWD surveillance often relies upon hunter-harvest submission of tissues, followed by costly testing programs, typically funded by the state or province wildlife management agency. The ability to detect PrP^{CWD} in feces from cervid-consuming predators and scavengers may offer a cost-effective surveillance alternative for potentially early CWD monitoring and management action and could provide an

efficient means to surveille at-risk areas neighboring CWD outbreak zones and/or areas where hunter-harvesting or hunter-submitted sampling is low, or where noninvasive/nonlethal approaches are desirable.

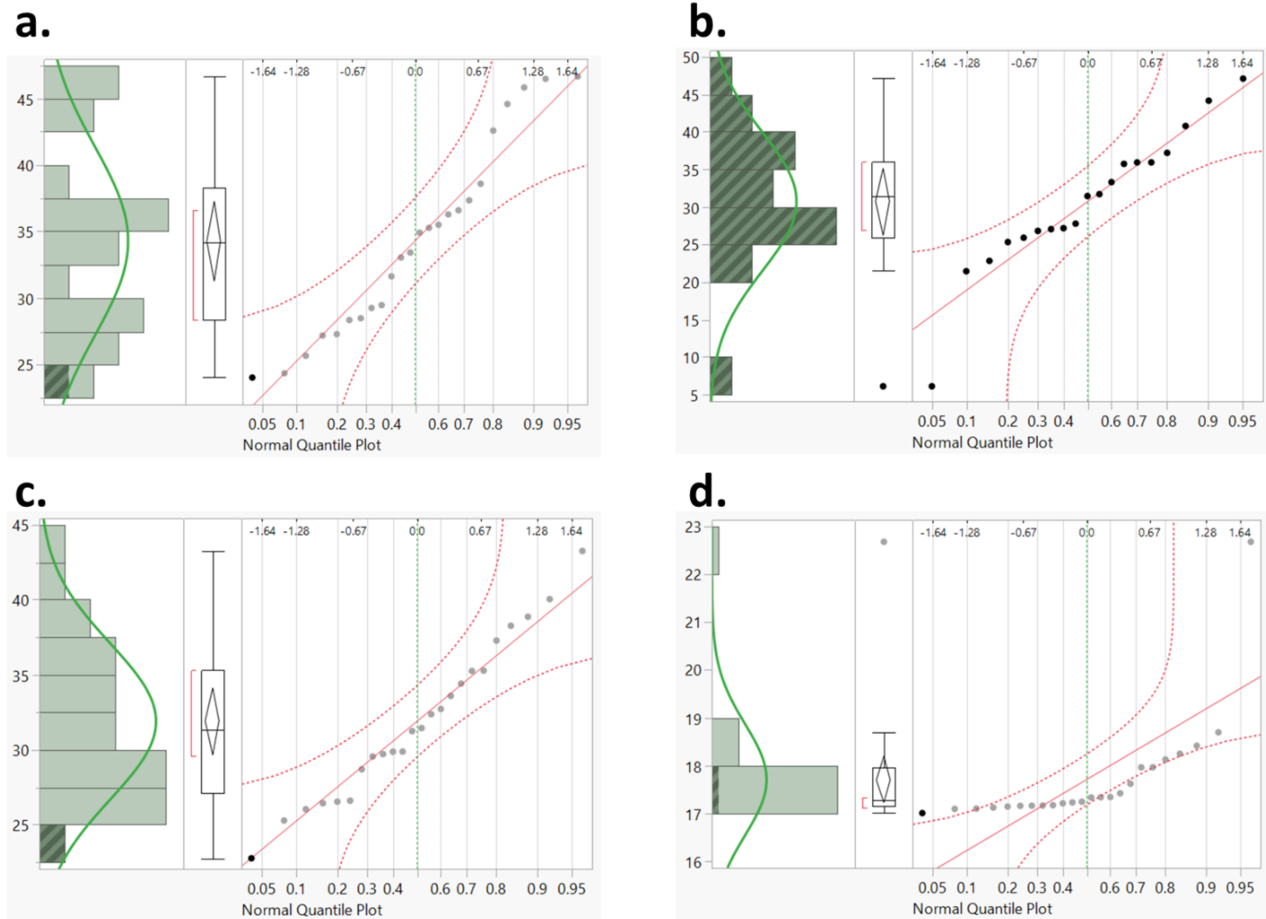
SUPPLEMENTARY MATERIAL

TABLES AND FIGURES

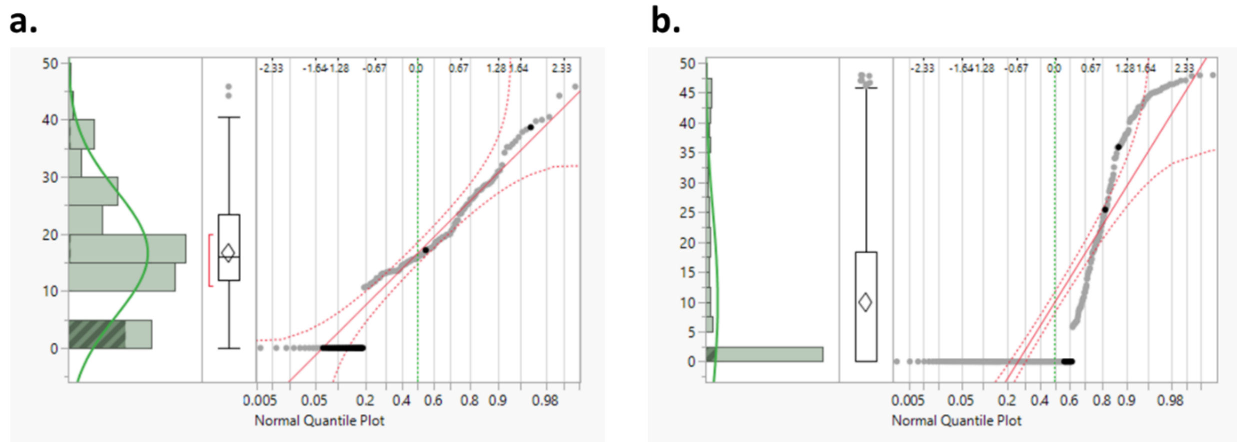
Supplementary Table S1. Ordered differences of the Least Squares mean amyloid formation rates between all spiked feces and chronic wasting disease (CWD)-positive brain homogenate used for spiking scats. Statistical significance was determined by Tukey HSD multiple comparisons test.

Scat type	Scat type comparison	Difference	Standard error difference	Lower CL	Upper CL	p-value
Raven	Fox	0.031677	0.003655	0.020052	0.043301	0.00E+00
Raven	Cougar	0.030095	0.003655	0.018471	0.04172	0.00E+00
Raven	Raccoon	0.011151	0.003655	-0.00047	0.022775	7.25E-02
Raven	Coyote	0.010118	0.003655	-0.00151	0.021743	1.51E-01
Raven	Bear	0.008386	0.003655	-0.00324	0.020011	3.95E-01
Raven	Wolf	0.003546	0.003655	-0.00808	0.015171	9.94E-01
Cougar	Fox	0.001582	0.003655	-0.01004	0.013206	1.00E+00
Wolf	Fox	0.028131	0.003655	0.016506	0.039755	0.00E+00
Wolf	Cougar	0.026549	0.003655	0.014925	0.038174	2.22E-11
Wolf	Raccoon	0.007605	0.003655	-0.00402	0.019229	5.42E-01
Wolf	Coyote	0.006572	0.003655	-0.00505	0.018197	7.36E-01
Wolf	Bear	0.00484	0.003655	-0.00678	0.016465	9.48E-01
Wolf	Eagle	0.021598	0.003655	0.009973	0.033222	3.17E-07
Eagle	Fox	0.049729	0.003655	0.038104	0.061353	0.00E+00
Eagle	Cougar	0.048147	0.003655	0.036523	0.059772	0.00E+00
Eagle	Raven	0.018052	0.003655	0.006427	0.029676	4.94E-05
Eagle	Raccoon	0.029202	0.003655	0.017578	0.040827	0.00E+00
Eagle	Coyote	0.02817	0.003655	0.016545	0.039794	0.00E+00
Eagle	Bear	0.026438	0.003655	0.014813	0.038062	4.05E-11
Bear	Fox	0.023291	0.003655	0.011666	0.034915	2.18E-08
Bear	Cougar	0.021709	0.003655	0.010085	0.033334	2.67E-07
Bear	Raccoon	0.002765	0.003655	-0.00886	0.014389	9.99E-01
Bear	Coyote	0.001732	0.003655	-0.00989	0.013357	1.00E+00
Raccoon	Fox	0.020526	0.003655	0.008902	0.032151	1.58E-06
Raccoon	Cougar	0.018945	0.003655	0.00732	0.030569	1.49E-05
Crow	Fox	0.065923	0.003655	0.054299	0.077548	0.00E+00
Crow	Cougar	0.064342	0.003655	0.052717	0.075966	0.00E+00
Crow	Raven	0.034246	0.003655	0.022622	0.045871	0.00E+00
Crow	Raccoon	0.045397	0.003655	0.033773	0.057022	0.00E+00
Crow	Coyote	0.044365	0.003655	0.03274	0.055989	0.00E+00
Crow	Bear	0.042633	0.003655	0.031008	0.054257	0.00E+00

Crow	Wolf	0.037792	0.003655	0.026168	0.049417	0.00E+00
Crow	Eagle	0.016195	0.003655	0.00457	0.027819	5.03E-04
Coyote	Fox	0.021559	0.003655	0.009934	0.033183	3.36E-07
Coyote	Cougar	0.019977	0.003655	0.008353	0.031602	3.50E-06
Coyote	Raccoon	0.001033	0.003655	-0.01059	0.012657	1.00E+00
Brain	Fox	0.107407	0.003655	0.095782	0.119031	0.00E+00
Brain	Cougar	0.105825	0.003655	0.094201	0.11745	0.00E+00
Brain	Raven	0.07573	0.003655	0.064105	0.087354	0.00E+00
Brain	Raccoon	0.086881	0.003655	0.075256	0.098505	0.00E+00
Brain	Coyote	0.085848	0.003655	0.074223	0.097472	0.00E+00
Brain	Bear	0.084116	0.003655	0.072491	0.09574	0.00E+00
Brain	Wolf	0.079276	0.003655	0.067651	0.0909	0.00E+00
Brain	Eagle	0.057678	0.003655	0.046054	0.069303	0.00E+00
Brain	Crow	0.041483	0.003655	0.029859	0.053108	0.00E+00



Supplementary Figure S1. Histogram plots depicting empirical distributions of threshold times for 24 technical replicates of unspiked feces from **(a)** bear (34.3, ±7.1; goodness of fit P, 0.25), **(b)** crow (M,30.7; SD, 9.2 Goodness of Fit P, 0.46), **(c)** eagle (M, 31.9; SD, 5.2; Goodness of Fit P, 0.80), and **(d)** raccoon (M, 17.7; SD, 1.2; Goodness of Fit P, < .0001).



Supplementary Figure S2. Non-normal distributions of the time (hours)-to-threshold data for predator and scavenger scat samples. Histogram and normal quantile plot depicting distribution of hours to threshold for (a) coyote scat sample data depicted in “Figure 3” (N, 168; M, 16.6; SD, 10.9; Goodness of Fit P, < .0001) and (b) cougar scat sample data depicted in “Figure 4” (N, 384; M, 9.9; SD, 15.27, Goodness of Fit P, < .0001)

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CHAPTER 4: Artificial mineral sites that pre-date endemic chronic wasting disease become prion hotspots

ABSTRACT

Chronic wasting disease (CWD) is a highly contagious, fatal neurodegenerative disease caused by infectious prions (PrP^{CWD}) affecting wild and captive cervids. As CWD continues to spread broadly over North America, environmental factors influencing geographic expansion remain poorly understood. CWD-infected cervids shed infectious prions in urine, feces, and saliva. Locations or behaviors where cervids congregate and shedding PrP^{CWD} may result in 'hot spots' of environmental PrP^{CWD} aggregation, however empirical data regarding the impact of deer attractants on environmental PrP^{CWD} deposition is lacking. Filling this knowledge gap could inform deer and disease management. In 2018, CWD was first detected in the southwest region of Tennessee, U.S.A. The Ames Research and Educational Center property, centrally located within the CWD zone of southwest Tennessee, contains 49 historical mineral supplementation sites that were established prior to the regional CWD outbreak and decommissioned in 2011. Here, we demonstrate that despite a decade of no additional supplementation, 32 of the 49 (65%) historical mineral sites within Ames have environmental PrP^{CWD} contamination. Detection of PrP^{CWD} in soils from these artificial mineral sites was not dependent on site-specific management efforts or intensity of deer use. Soil textures were very similar across sites and no correlation between PrP^{CWD} detection and soil properties was found. The detection of PrP^{CWD} in soils at historic attractant sites within a newly established CWD zone advances our understanding of environmental PrP^{CWD} accumulation dynamics, providing valuable information for advancing adaptive CWD management approaches.

INTRODUCTION

Chronic wasting disease (CWD) is a highly contagious and fatal neurodegenerative disease affecting free-ranging and domestic members of the Cervidae family (e.g., deer, elk, moose, caribou) that was first identified in captive mule deer (*Odocoileus hemionus*) and black-tailed deer (*Odocoileus hemionus columbianus*) in 1967 in Colorado, U.S.A.[1]. The geographic range of CWD continues to advance in cervid populations across North America[2] and has been detected in Norway, Finland, Sweden[3, 4] and South Korea[5]. Prevalence of CWD can reach 30% in free-ranging populations in endemic areas[6] and some instances in captive herds have reported rates up to 90%[7]. Areas with consistently high or increasing CWD prevalence see reductions in cervid populations[6] as increasing prevalence in wild cervid herds is expected to affect recruitment[8] and herd viability[9]. Additionally, regional CWD zones place neighboring jurisdictions at a high risk for CWD invasion.

The causative agent of CWD is a misfolded protein, referred to as a prion (hereafter PrP^{CWD})[1]. Cervid hosts with a CWD infection can shed PrP^{CWD} into the environment via urine[10], saliva[11], and feces, or from decomposition of infected carcasses[12-14], which may be relevant sources of infectivity for indirect exposure and transmission in the wild. Studies done to demonstrate risk of exposure to secreta and excreta of infected cervids report that white-tailed deer (WTD) orally exposed to 50 mL of urine 50 g of feces from CWD-infected deer resulted in disease[15] and that oral exposure to three 10-mL doses sufficient to cause disease[16]. The minimum oral infective dose (ID_{50}) of urine from CWD-infected deer is 1-5 (ID_{50}; the minimum inoculum required to induce a prion infection in 50% of the challenged population) per 10 mL[17] and 30 µL of 10% fecal

homogenates contain 0.03 and 1 ID_{50} PrP^{CWD} [14]. It is estimated that cumulative PrP^{CWD} shed in feces over the disease course is approximately equivalent to the relative quantity of PrP^{CWD} found neural tissue of a CWD-infected individual in the late stages of disease[12, 14].

Environmental accumulation and bioavailability of PrP^{CWD} appears to play a role in facilitating transmission events and geographic spread of CWD[13, 18]. Prions bound to soil remain infectious[19], and possess increased infectivity compared to free prions[20]. Point resources and features on the landscape that facilitate cervids to congregate may generate foci of accumulated, bioavailable PrP^{CWD} deposits, creating areas that possess high CWD exposure and transmission risk for naïve individuals. Soil found at natural and artificial mineral licks can serve as a reservoir of PrP^{CWD} in CWD zones[21]. Cervid attractants are not lawful in all jurisdictions, and when allowed there are often restrictions on when attractant use can occur, and under what circumstances[22]. Deer can consume 0.05 kg of soil monthly[23] and often do so at natural and artificial mineral licks to supplement mineral uptake during physiologically demanding periods (e.g., fawning of young, lactation, new antler development)[23-26] and will revisit historical attractant sites, even following discontinuation of supplementation[27].

Soil characteristics of artificial mineral licks following the monthly addition of attractant tend to accumulate high mineral salt concentrations[28]. This may influence bioavailability of prions in soils as ionic strength can impact protein adsorption and electrostatic interactions[29]. Conditions of high ionic strength result in tighter adsorption and increased attachment of prions to quartz soil particles[30], suggesting that higher levels of salt minerals present in soils may limit soil-bound prion mobility and result in potential aggregation of prions in the organic and surface soil horizons.

Interactions of prions with soil and the subsequent ability to extract and detect PrP^{CWD} from soil may be influenced by a variety of soil factors and conditions. Soil texture and mineral composition influence prion binding capacity, where prions have a strong affinity for silicate clay minerals in particular (i.e., montmorillonite), compared with quartz sand (silicate). This association enhances prion infectivity[19, 20], but at the same time can inhibit detection and accurate assessment of relative amounts of prion present in clay rich soils. Adding to the complexity of soil-prion interactions and hampering detection efforts is the presence of soil organic matter (OM)[31]. The precise composition of OM compounds (i.e., humic substances and non-humic substances) are highly variable from one soil source to another. Some humic substances have been shown not only to have a strong affinity for prion proteins[32-35], but can also enhance sorption capacity of montmorillonite and kaolinite clays[34]. In addition, soil pH can directly influence prion structure and interactions with soil organic and mineral compounds[36], suggesting that pH may also mediate detectability of prions in soils.

In 2018, CWD was detected for the first time in Fayette and Hardeman counties in western Tennessee, U.S.A. State-wide CWD surveillance in Tennessee had been ongoing since 2002, since the first detections on CWD in Wisconsin, U.S.A. and intensified in 2016, after CWD was first detected in Arkansas, U.S.A. [2, 37]. Similar to other areas, CWD prevalence in this region has rapidly increased over the intervening years and has since been detected in 14 other counties that surround Fayette and Hardeman County[37]. Within CWD endemic zone in Fayette and Hardeman Counties is our study area, the Ames Research and Education Center (Fig. 1a) (hereafter referred to as Ames). CWD prevalence among the white-tailed deer population within Ames is about ~30-60%[38]. The Ames property encompasses 49 artificial mineral attractant sites that were established in 2004.

Mineral supplement was added annually to each of the 49 sites from 2004-2011, after which supplementation was discontinued.

Recent reports demonstrate that the presence of PrP^{CWD} in mineral lick site soils[21] using the Protein Misfolding Cyclic Amplification (PMCA) protein amplification assay, suggesting that either artificial or natural mineral licks can serve as possible environmental reservoirs of PrP^{CWD}. Previously, we demonstrated the use of an ultra-sensitive protein amplification assay — the real-time quaking-induced conversion (RT-QuIC) assay, for detection of PrP^{CWD} in soils[39]. As RT-QuIC offers advantages over PMCA in terms of assay run times and expense and efficient production of required components for assay function, we sought to employ our soil-adapted RT-QuIC as a high throughput method for assessing PrP^{CWD} presence in soils from historical artificial mineral lick locations within our study area. We then assessed the influence of soil texture, conditions, and compounds on probability of PrP^{CWD} detection and whether intensity of deer use, or site-specific management approaches were also predictors of PrP^{CWD} detection and conduced a spatial analyses to determine if sites that were close in space were also more likely to have the same detection results. The detection of PrP^{CWD} in soils at attractant sites within an endemic CWD zone significantly advances our understanding of environmental PrP^{CWD} accumulation dynamics within this southwest region of Tennessee. The results of this study provide state and regulatory agencies with valuable information for advancing adaptive CWD management approaches to limit and reduce further increase in regional CWD prevalence and reduce the risk of geographic spread to neighboring jurisdictions.

METHODS

Study Area. The Ames Research and Education Center (hereafter Ames), a University of Tennessee agricultural research facility, is a 74.5 km^2 (~18,400 acre) area in southwestern Tennessee (35.12 N, -89.21 W) in Fayette and Hardeman counties (Fig 1a). Habitat types at Ames include commodity row crops (i.e., cotton, soybean, wheat, and corn), forests (i.e., loblolly pine, upland hardwoods, and bottomland hardwoods), and pastures (i.e., horse and beef cattle pastures) and is managed to maintain a variety of native habitat types for a wide range of wildlife species, forbs, grasses, and mixed-hardwoods[40-42].

Forty-nine historic mineral lick sites located within the property boundary were included in this study (Fig. 1a). The mineral lick sites were established by Ames in 2004 for deer herd management and were replenished annually with loose mineral supplement for WTD up until 2008, followed by intermittent replenishment until 2012 due to associated cost, at which point baiting of WTD with minerals ceased due to state regulations and CWD concerns[37]. From 2012-2020 all 48 sites were left undisturbed by Ames management. CWD was identified in free-ranging WTD populations southwestern TN in 2018; consequently, in the summer of 2020, Ames management initiated remediation efforts to deter or prevent WTD visitation, interest, and consumption of mineral-laden soils at these sites. These efforts began by randomly covering nine of the 49 mineral lick sites with enough soil to create an ~ 3-foot mound on top of the mineral lick area. Soils used to fill/cover depressions were collected within ~25 yards of the mineral lick site. The original intention by Ames management was to cover all of the historic mineral licks within Ames, however these efforts were not systematic and ceased shortly after they began to preserve the remaining

uncovered mineral licks for additional research efforts. These covered sites were included in this study to explore if any differences in PrP^{CWD} existed compared with uncovered sites. Approximately one year following the discontinuation of these remediation efforts, intensity of deer use was subjectively evaluated at each site and categorized as either high, low, or no deer sign as previously described[25]. High deer sign characteristics included obvious removal of soil (incisor marks in soil), lack of or limited vegetation in and around the perimeter of the mineral lick depression, and fresh deer tracks in dirt or mud within 10 feet of the mineral lick, whereas sites with low deer sign had few fresh deer tracks and vegetation withing 10 feet of the site was present and not tamped down, with little or no soil removal (Fig.1c).

Soil sample collection. We collected soil samples from the 48 historic mineral lick sites within the study area property boundaries (Fig. 1a) in April 2021. At each site eight soil samples were collected 0-3in deep, of ~40g each, five near the center of the mineral site and three on the site periphery. Center point iron stakes were located for each site and a plastic coated, wire 2 x 2 ft. grid containing 25, 6 x 6-inch grid cells was positioned over the center stake with the top leading edge of the grid facing north. The 5 samples on the mineral site were collected using a sampling grid (hereafter, grid samples; see Fig. 1b), and the 3 peripheral samples were ~20 m from the site in a N, S, E, or W coordinate direction, vegetation allowing. To prevent cross contamination, each sample was collected using a new disposable plastic spoon, which was discarded after being used, gloves were changed in between each sample, and walking on any sample point of each grid cell was avoided. All samples were stored at –20°C until extracted for use in the RT-QuIC assay.

Soil sample preparation for RT-QuIC. Sub-samples (500 mg each) of each soil sample were subjected to extraction with 1000 µL 0.6 mM myristyl sulfobetaine (MSB) zwitterionic surfactant in phosphate buffer (75.4 mM dibasic sodium phosphate and 24.6 mM monobasic sodium phosphate; Millipore Sigma, Burlington, MA) with rotation for 1 h, followed by centrifugation at 8000 xg for 10 min. Supernatants (~750 µL) were collected, followed by addition of 250 µL MSB buffer to the soil pellet and remaining buffer. Samples were vortexed and centrifuged at 8000 xg for 10 min. ~250 µL supernatant was removed and added to the first supernatants. To concentrate any precipitate any PrP^{CWD} present in a sample, 34 mg·mL-1 (final concentration) sodium phosphotungstate at pH 7.1 (Sigma-Aldrich, St. Louis, MO) was added to supernatants and allowed to incubate overnight at 4 °C, followed by centrifugation at 16000 g for 30 min at 4 °C. The resulting pellet was rinsed by addition of ultrapure water (Milli-Q system, Millipore Sigma, Burlington, MA) with 34 mg·mL-1 (final concentration) sodium phosphotungstate at pH 7.1, and centrifuged again at 16000 xg for 30 min. The final pellet was reconstituted in 50 µL of RT-QuIC sample buffer (0.1 g·mL-1 sodium dodecyl sulfate in phosphate-buffered saline with N-2 cell culture supplement; filtered 0.22 µm; ThermoFisher, 17502048, Waltham, MA) and reconstituted using a Qsonica Q700 cup horn ultrasonicator (Amplitude 36 for 1 min.). A volume of 2 µL of each reconstituted sample was used to seed each reaction well of a clear bottom 96-well plate with black sides (ThermoFisher, 265301, Waltham, MA) for 8 technical replicates.

Real-time Quaking-induced Conversion Assay and data prep. The RT-QuIC *in vitro* prion amplification assay was performed as described by Orru *et al.*[43] using sodium iodide as described by Metrick *et al.*[44] with minor modifications. Briefly, 2 µL of sample extracts were added to a given well of a 96-well format optical-bottom black microplate (Fisher), each already containing

98 μL of RT-QuIC reaction mixture (0.1 mg·mL-1 90-231 recombinant hamster prion protein (produced as previously described[43]: 300 mM sodium iodide, 20 mM sodium phosphate, 1.0 mM ethylenediaminetetraacetic acid, and 10 μM thioflavin T). Microplate-compatible spectrophotometers capable of heating, shaking, and fluorescence monitoring (BMG FLUOstar, Cary, NC) were used with the following instrument settings: 50 °C for spiked samples (unless described otherwise) double orbital pattern shaking at 700 rpm with 60-s shake / 60-s rest cycles, fluorescent scans ($\lambda_{excitation}$ = 448 nm, $\lambda_{emission}$ = 482 nm) every 15 minutes, at a gain of 1600, and a total run time of 48 h.

RT-QuIC-generated relative fluorescence unit (RFUs) values and amyloid formation rate data were analyzed using a combination of MARS Data Analysis Software (BMG Labtech) and JMP Pro 15 (SAS Institute, Cary, NC) and visualized using Prism 8 (GraphPad, San Diego, CA). To distinguish samples with true seeding activity from environmental background-induced seeding, the AFR threshold times (i.e., the time above which fluorescence amplification is determined to have occurred in the RT-QuIC assay) were calculated by adding ten times the standard deviation of the relative fluorescence unit (RFU) values from cycles 3-14 to the mean of RFU values from cycles 3-14[39]. This method accounts for baseline variation amongst samples in determining if a sample was positive for PrP^{CWD}. Due to environmental background induced seeding present in soil samples from the study area, additional analysis was required to distinguish true seeding from false seeding events. To accomplish this, the mean and standard deviation of the time to threshold was determined from a presumed-negative regional soil sample, with the assumption that the time to threshold for false seeding events would be normally distributed. This assumption of normality was assessed using the goodness of fit function in Jmp Pro 15. These data were then used to

determine a cycle end-time that would exclude 50% of the false seeding that occurred for surface soil samples collected from within the study area at sites that had no indications of deer sign (Fig. 1c). To determine this, we subtracted 0.5 standard deviations from the mean time-to-threshold for 3 separate plates, each consisting of 80 technical replicates of the representative soil sample. The cycle end-time was determined to be 30 h for soils from the study area. All soil samples analyzed by RT-QuIC were considered positive if a sample had at least 4 out of 8 technical replicates with seeding activity at or before 30 hours.

Soil characterization. The five grid surface soil samples from each mineral site were pooled for soil characterization. Mineral lick site GPS coordinates were used to determine soil textural class (e.g., silt loam, silty clay loam, silt, etc.), soil series (e.g., Lexington, Smithdale, etc.), and soil composition (percentage of sand, silt, clay) using the UC Davis California Soil Resource Lab Soil Data Explorer. Soil chemistry analysis included pH, parts per million (ppm) of phosphorus and potassium, as well as percent organic matter, total nitrogen, and total organic carbon. Samples for soil chemistry were analyzed at the University of Wisconsin Soil and Forage Analysis Lab, Madison, Wisconsin, U.S.A. For a complete list of study site soil series, textural class, and soil chemistry analysis results see Table 1 and Supplementary Table S1.

Statistical model selection and fitting. We used to generalize linear regression models to determine if site characteristics and soil properties were predictors of PrP^{CWD} detection. Our response variable was PrP^{CWD} detection (a binary outcome). For the statistical model for PrP^{CWD} detection at mineral licks, we only evaluated soil samples collected within the sampling grid and excluded the periphery samples. We predicted that the probability of PrP^{CWD} detection would be a

function of site characteristics (i.e., site specific management efforts and intensity of deer use) and soil properties (i.e., soil pH, Potassium and Phosphorus content, percent clay, percent organic matter, etc.)[45]. For the response variable of PrP^{CWD} detection, we fit logistic regressions based on specific biological hypotheses[46]. Explanatory variables (Table 2) included the site characteristics of site treatment (Covered or uncovered) and site use (high or low deer sign). Explanatory soil characteristics included percent clay, percent sand, Phosphorous (ppm), Potassium (ppm), pH, percent organic matter (OM), total organic carbon (TOC), and total nitrogen (TN) content. All continuous predictors were scaled and centered, and models were checked graphically for linearity assumptions (binned residuals for logistic regressions)[47]. Site treatment, OM, TOC, and TN were removed from the model as predictor variables as they violated model linearity assumptions (i.e., binned residuals for logistic regressions) and were not statistically significant (Table 2). All analyses were performed in R v4.2.2 [45]. To view all relevant data sources used in our analyses see supplementary materials (Supplementary Tables S1 and S2).

Spatial analysis. Study area maps were created using ESRI ArcGIS 2.5 software. To determine if our statistical models could be affected by spatial structuring of prion detections, we performed several tests for spatial clustering and autocorrelation. A local cluster analysis for the 49 mineral sites within the study area was completed using SATscan v10.1 [48] We used the multinomial scan statistic[49], a circular window, and a maximum spatial cluster size of 50% of the population at risk. The categories for the multinomial test were defined based on prion detection to determine if sites with grid samples positive for PrP^{CWD} were spatially clustered. Additionally, we performed a global cluster analysis using the Cuzick and Edward's test (global cluster detection with case-

control data) in the R package *smacpod* (1, 3, 5, 7, 9, and 11 nearest neighbors; 999 iterations)[50-52] using R v4.2.2 [45].

To test for spatial autocorrelation (i.e., spatial dependence) among site-level prion detections, we used Moran's I and Mantel tests (999 permutations). The Mantel test can inflate type I statistical error[53], but we deemed this an acceptable limitation due to our *a priori* expectation for limited spatial dependence among site-level prion detections. Both tests were conducted by constructing a geodetic distance matrix between all mineral lick site coordinate locations, and a binary pairwise distance matrix based on PrP^{CWD} presence data for each mineral lick site. Pairs of mineral lick sites that had the same PrP^{CWD} detection results (e.g., both sites had PrP^{CWD} detected) had a distance of 0, while pairs of sites with different detection results had a distance of 1. Distance matrices were calculated, and tests conducted using the *geosphere*[54], *spatstat*[55], and *ade4*[56] packages in R v4.2.2 [45].

RESULTS

Analysis of grid and peripheral soil samples by RT-QuIC showed variable deposition of PrP^{CWD} across the 49 sites. Each mineral lick site was categorized as having PrP^{CWD} in grid soil samples or not (Fig. 2a). Of the 49 sites, 25% (12/49) had positive samples from grid samples, 75% (37/49) of sites had soil samples that did not have grid detection, of which more than half had peripheral soil samples that were positive (Fig. 2, Supplementary Table 1). Spatial analysis of mineral lick detection using SatScan and the Cusik Edward's test revealed no significant clustering of PrP^{CWD} positive or negative sites (P = 0.89, 0.63). In addition, binary PrP^{CWD} detection statuses were not

spatially autocorrelated (Moran's I, $P = 0.559$; $\rho = -0.0413 \pm 0.0351$; Mantel test, $P = 0.3409$; $\rho = 0.0053, \pm .0173$).

A total of nine different soil series were present across the 49 mineral lick sites (Table 2), and soil characteristics were similar across the study area. The most abundant soil textural class within the study area was silt loam, followed by silt, sand, sandy clay loam, and silty clay loam (Table 2). Soil pH was the least variable soil property (6.14 ± 0.37), followed by TN (0.20 ± 0.055), TOC (3.23 ± 1.34), and OM (5.03 ± 1.98). Phosphorous and potassium ppm were the most variable, with values ranging from 2 to 863 ppm phosphorous (155.25 ± 189.56) and 76.58 to 272.3 ppm potassium (143.39 ± 41.06) (Supplementary Table S1).

Site and soil characteristics at mineral licks did not significantly affect the probability of detecting PrP^{CWD}. The final model, after removal of predictors that lent to violation of model assumptions (i.e., binned residuals for logistic regressions), included site treatment, OM, TN, and TOC, though none of these predictors were statistically significant (Table 2). Because only a portion of the mineral sites were covered (9/49), skewing samples sizes in analysis and because site treatment had to be excluded from our final model, we performed a *post hoc* Fisher's exact test to specifically test if site treatment was associated with PrP^{CWD} detection. Of the covered sites, 44.4% (4/9), had grid soil samples that were positive for PrP^{CWD} while 20% (8/40) uncovered sites were positive for PrP^{CWD}. No evidence was observed associating site treatment with detection status (Fisher's exact test; $P = 0.1948$; odds ratio = 0.3215), further verifying that site treatment did not influence probability of PrP^{CWD} detection. In addition, we found no association between deer sign (high vs low) and site treatment (Fisher's exact test; $P = 1.00$, odds ratio = 0.9637). Similarly, univariate

analyses of our other excluded variables (OM, TOC, and TN) showed no significant relationship with PrP^{CWD} detection.

FIGURES AND TABLES

Table 1. Summary of study area soil series, textural class and fraction of clay, silt, and sand.

Soil series	Soil textural class	Number of sites	% clay	% silt	% sand
Memphis	Silt loam	8	12	84	4
Calloway	Silt loam	2	12	80	8
Loring	Silt loam	8	12	85	3
Henry	Silt loam	1	12	75	13
Kurk	Silt loam	1	19	68	13
Falaya	Silt loam	1	17	72	11
Grenada	Silt loam	2	13	80	7
Lexington	Silty clay loam	5	15	70	15
Smithdale	Sandy loam	9	5	20	75
Gullied land	Silt/sand mix	12	0	50	50

Table 2. Results from logistic regression for the probability of detection of chronic wasting disease prions (PrP^{CWD}) based on site and soil characteristics.

Variable	coefficient	Standard error	Lower confidence interval	Upper confidence interval	p-value
Intercept	0.27	0.43	0.11	0.60	0.002
Deer sign	1.54	0.74	0.35	6.66	0.56
% sand	1.02	0.42	0.45	2.30	0.97
% clay	1.06	0.39	0.51	2.49	0.88
pH	0.81	0.36	0.38	1.64	0.56
Phosphorus	1.11	0.38	0.49	2.34	0.79
Potassium	0.89	0.40	0.38	1.88	0.77

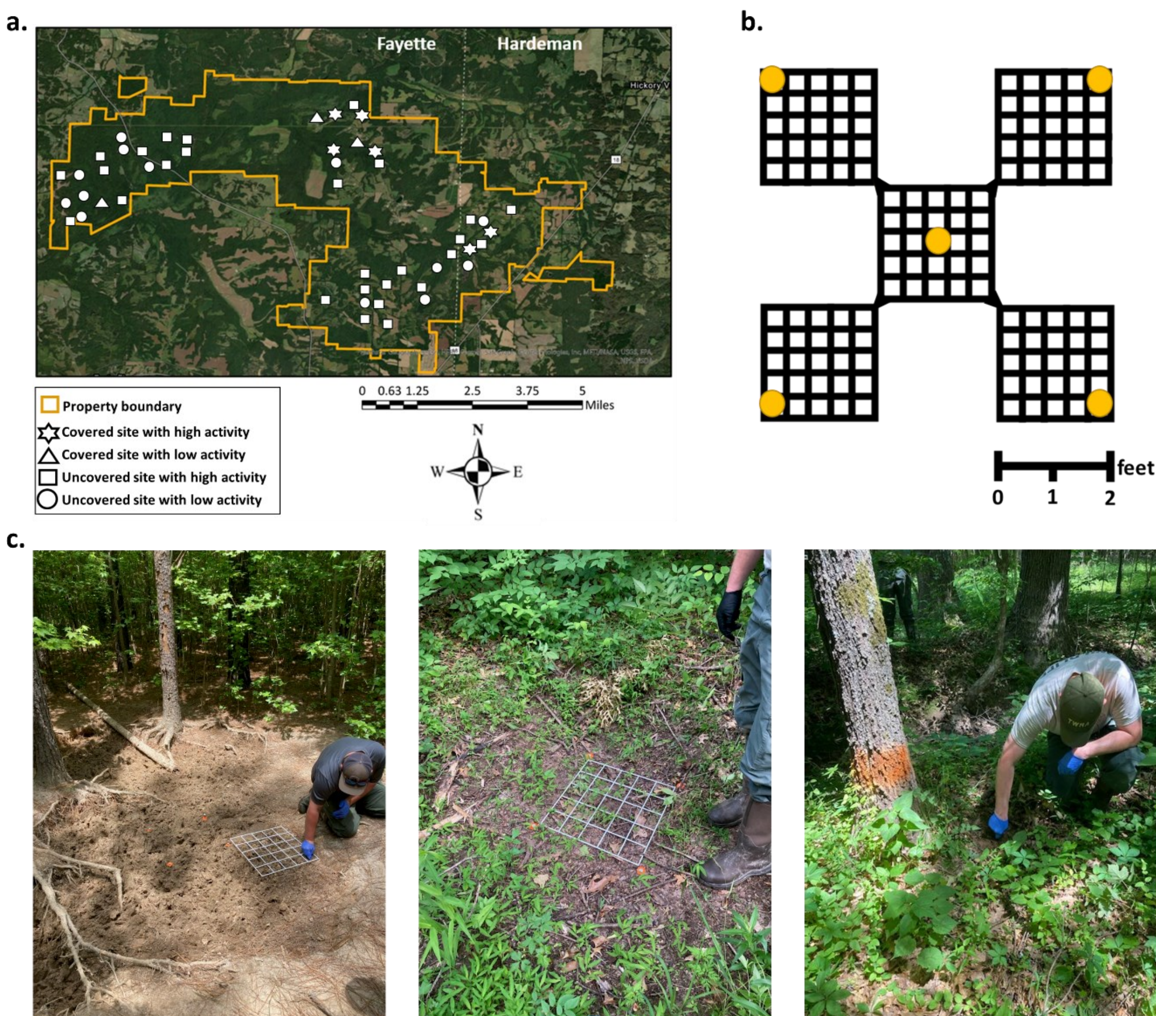

Figure 1. Study area map, mineral site locations, and soil sample grid. (a) Map of the Ames Research and Education Center depicting the study area property boundary and historic mineral site locations where soil samples were collected. **(b)** Soil sampling grid layout used at each historic mineral site. Three additional soil samples collected on the periphery 20 meters from the grid center are not depicted here. **(c)** Representative depictions of observed high (left) and low (center) deer sign, compared to a control site (right).

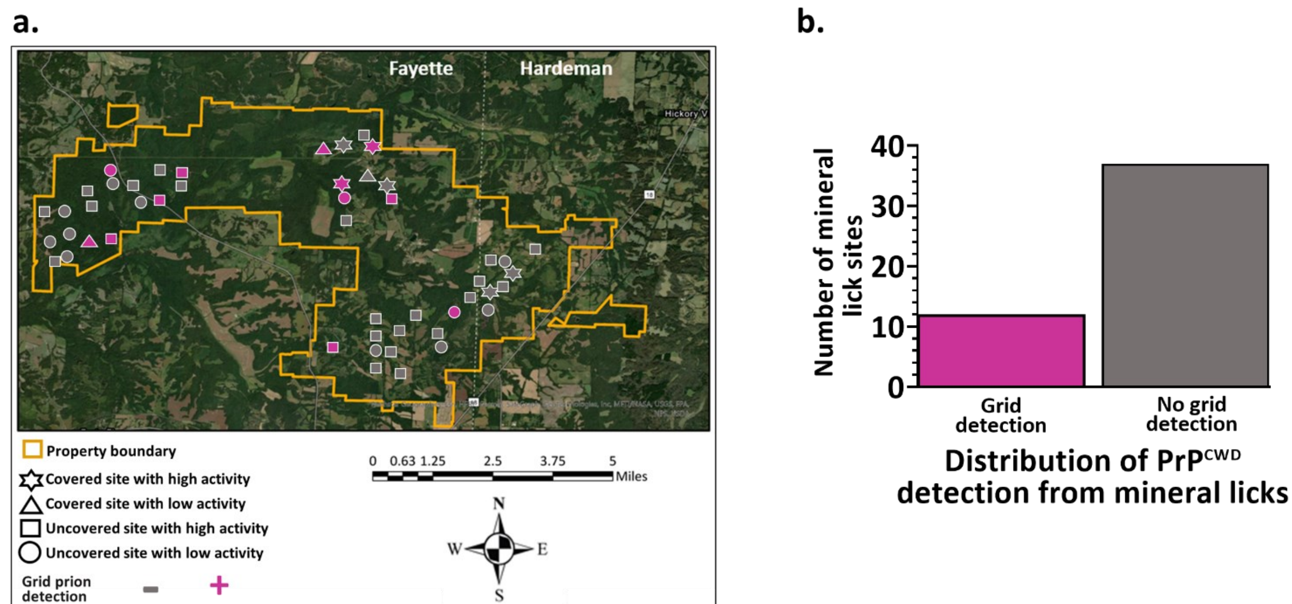

Figure 2. Distribution of grid sample detection for chronic wasting disease (CWD) prions (PrP^{CWD}) at historic mineral lick sites. (a) Map depicting spatial distribution of PrP^{CWD} occurrence in grid soil samples across the 49 mineral sites within the study area. **(b)** Comparison of the number of sites where PrP^{CWD} detection occurred or did not occur in mineral lick site grid soil samples.

DISCUSSION

Soils can serve as an environmental reservoir for PrP^{CWD} and may drive geographic spread of CWD[21]. Soils are complex multicomponent systems that consist of mineral particles (clay, silt, sand) and organic and inorganic compounds. Interactions, bioavailability, and subsequent detection of soil-bound prions are thought to be heavily influenced by different soil properties and compounds[19, 30, 57-65]. Our results demonstrate that decommissioned mineral lick sites established at least a decade prior to CWD being first detected in southwest Tennessee have PrP^{CWD} present in site soil. PrP^{CWD} detection status of each mineral lick site within the study area was not found to be clustered or spatially autocorrelated. No differences were found in probability of PrP^{CWD} detection as a result of site-specific management approaches (site treatment) or deer use intensity. Soil textures were very similar across mineral lick sites and were not a predictor for PrP^{CWD} detection. Although variability among soil characteristics was observed, there was no evidence to support that soil pH, ppm phosphorus and potassium, soil OM, TOC, or TN influenced the probability of PrP^{CWD} detection in soils from within the study area.

Soil samples collected from the grid area of the mineral licks showed 25% (12/49) as positive for PrP^{CWD} across the study area. Looking outside of the grid area to include peripheral samples, positive detection was 65% (32/49). This high percentage of PrP^{CWD} occurrence in soils was not surprising since deer densities in Ames are relatively high throughout[38] and CWD prevalence is 49% (Allan Houston, personal communication). This may explain why we didn't detect spatial differences in prion detection among sites or differences based on deer sign. Among the sets of grid and peripheral samples for each site (8 (5 grid, 3 peripheral) samples total) we saw that some sites had more samples with PrP^{CWD} detection than others (Supplementary Table S1). These

differences may be due to variation in deer behaviors at licks since duration and frequency of lick visitation can be highly inconsistent among individual WTD and defecation within the central lick area may be less likely than defecation on peripheral edges not directly associated with the central lick area[66, 67]. Whether PrP^{CWD} detection variation was due to differences in soil characteristics was difficult to discern, since within this relatively small study area soils were comparatively homogenous.

Overall, soil properties did not significantly influence the probability of PrP^{CWD} detection. This could be due to the scale of our study in the larger context of CWD, as certain soil properties that are thought to be important for PrP^{CWD} detection were at a finer scale than factors considered here. Although we report that soil properties did not significantly influence probability of PrP^{CWD} detection, it is possible that finer-scale properties may still have altered detection to some degree as 35 % of the sites had no PrP^{CWD} detection and some sites had several or all soil samples with PrP^{CWD} detection. Potential sources of variation in PrP^{CWD} detection from Ames soils may be a result of PrP^{CWD} interactions with soil OM. It's important to consider that soil OM is a mixture of humic substance (i.e., humic acids, fulvic acids, and humin) and non-humic substances (plant debris, lignins, proteins, polysaccharides) and ratios of humic and non-humic compounds can vary from one soil source to another, influencing soil-prion interactions, detection, and accurate measurements of prion loads in soils[31, 39]. Because of this potential variability in humic substance ratios, the simple measurement of total amount of soil OM may not be an adequate indicator for determining how OM constituents influence soil-prion interaction outcomes. There is much we still do not understand about the interactions of soil OM components with prions and future

research in relatively small study areas may find more variation if they describe some of these finer details of soil types than we did.

Another possible explanation for the differences in occurrence of PrP^{CWD} across the mineral lick sites may be an effect of geophagy by deer or other behaviors that limit PrP^{CWD} deposits in soil within the central lick area. For example, behavioral studies indicate that deer often prefer to feed at the edge of the lick rather than in the central lick area and are never observed licking the soil, only picking up the majority of consumed soil with their lips or gnawing it loose using their incisors, and only occasionally pawing soil loose to eat it[25, 68]. This suggests that it's possible that shed PrP^{CWD} from saliva and excretions within the central lick area may be limited or that any PrP^{CWD} deposited in soils is either being consumed or is not detectable due to assay sensitivity limitations, as has been observed with saliva from CWD-infected deer using RT-QuIC[69]. It is also possible that differences in PrP^{CWD} occurrence PrP^{CWD} may have been an effect of peripheral soil being used to cover nine of the mineral licks included in this study. This may have resulted in topping these uncovered sites with soil that possessed comparatively higher prion loads than soil at sites left uncovered, especially given that occurrence of PrP^{CWD} detection in both grid and peripheral soil occurred in four of the nine covered sites, regardless of the level of deer sign.

Detection of PrP^{CWD} at 65% of decommissioned licks demonstrates the potential of mineral licks to contribute to current and future CWD exposure and transmission events in cervids, advancing our current knowledge of the role of deer attractant sites in establishing and perpetuating CWD outbreaks. In addition to the unknowns of fine-scale soil compounds and deer behavioral effects of PrP^{CWD} soil deposits, the high level of CWD prevalence and deer density within Ames, and size

of the study area limited our ability to tease out the influence of fine scale soil factors and site characteristics as predictors for the probability of PrP^{CWD} detection or determine the reason for PrP^{CWD} detection limitations. Future research efforts could address some of the limitations of this study by comparing soil factors from deer congregation areas across different CWD endemic regions with different CWD prevalence. Also important would be to further understand and characterize humic substance ratios in soils from areas where deer congregate, what behaviors deer participate in during visits at licks and how these factors influence PrP^{CWD} deposition and soil-prion interactions and the transmission risk. In addition, because site remediation was initiated and then ceased prior to the onset of our study a more systematic assessment of the effects of site remediation efforts would help to better understand how covering sites influences PrP^{CWD} deposition and deer use.

SUPPLEMENTARY MATERIAL

Supplementary Table S1. Mineral lick site location, characteristics, and soil chemistry analysis. results.

Site location		Site type	Deer sign	Soil series	pH	P	K	Percent OM	TN	TOC	Number PrPCWD positive grid/ periphery
latitude	longitude										
35.12154	-89.23394	U	H	Memphis	6.6	83	227.6	7.5	0.31	4.76	0/0
35.10317	-89.19342	U	H	Lexington	6.5	108	130.8	3.5	0.17	2.12	0/0
35.08179	-89.22078	U	H	Calloway	5.8	85	123.3	4.7	0.21	3	0/0
35.11034	-89.31824	U	L	Smithdale	6	483	76.58	5	0.18	3.15	0/0
35.07692	-89.22512	U	H	Gullied land	5.7	60	157.7	3.7	0.17	2.54	0/0
35.12581	-89.31072	U	H	Gullied land	6.1	156	111.7	5.7	0.22	4.05	0/0
35.09823	-89.19676	U	H	Memphis	6.3	306	144.2	6.6	0.25	4.51	0/0
35.13072	-89.3121	U	H	Smithdale	6.2	16	158.5	3.3	0.15	1.88	0/0
35.13205	-89.29851	U	H	Loring	6.7	140	150.7	4.9	0.16	2.95	0/0
35.09163	-89.22482	U	H	Gullied land	6	38	213.2	5.6	0.19	3.09	0/0
35.144	-89.23502	C	H	Memphis	5.9	12	162.9	2.8	0.13	1.39	0/0
35.10158	-89.18693	U	H	Smithdale	6.3	80	194.4	3.4	0.18	2.03	0/0
35.08819	-89.21798	U	H	Henry	6.3	377	107.2	4.5	0.2	3.56	0/0
35.10889	-89.18579	U	L	Kurk	5.6	254	84.82	5.8	0.2	3.76	0/0
35.12439	-89.3191	U	L	Memphis	6.5	646	129.4	8.4	0.3	5.01	0/0
35.13696	-89.29039	U	H	Gullied land	5.9	15	114.3	2.2	0.11	1.56	0/0
35.11288	-89.17705	U	H	Lexington	5.7	335	143.8	5.5	0.22	3.56	0/0
35.12739	-89.29592	U	L	Gullied land	6.6	166	122.5	4	0.18	2.48	0/1
35.14726	-89.22854	U	H	Falaya	6.2	17	104.5	4.5	0.23	3.37	0/1
35.08734	-89.20659	U	H	Loring	6	15	134.7	2.7	0.17	1.45	0/1
35.10968	-89.19019	U	H	Lexington	5.5	6	234.7	3.2	0.14	1.63	0/1
35.09434	-89.19117	U	L	Lexington	5.7	3	158.8	3.3	0.17	1.85	0/1
35.09272	-89.213	U	H	Gullied land	6.4	394	103.1	3.1	0.12	1.82	0/2
35.11478	-89.32343	U	L	Smithdale	6.9	38	96.69	3	0.15	1.62	0/1
35.0752	-89.21738	U	H	Loring	6	46	155.1	3.2	0.17	1.86	0/1
35.12407	-89.32547	U	H	Loring	6.7	322	113	5.4	0.23	3.17	0/2
35.08203	-89.22507	U	L	Gullied land	6.4	49	138.8	4.3	0.23	2.56	0/1
35.13158	-89.22161	C	H	Memphis	6	14	179.6	2.5	0.14	1.52	0/3
35.08304	-89.20521	U	L	Gullied land	5.8	62	180.3	9.2	0.24	6.15	0/1
35.08632	-89.22506	U	H	Loring	6.3	232	154.5	7.4	0.26	4.74	0/1
35.10558	-89.18389	C	H	Smithdale	6.2	73	272.3	4.2	0.21	2.68	0/1
35.13486	-89.22755	C	L	Smithdale	6.3	25	156.7	7.8	0.28	5.17	0/1
35.09994	-89.18996	C	H	Smithdale	5.7	2	155.1	2	0.1	0.92	0/1
35.13293	-89.30451	U	L	Gullied land	6.9	612	139.1	5.8	0.22	3.98	0/1
35.1282	-89.2205	U	H	Lexington	6.3	65	152.6	4.1	0.18	2.26	0/1
35.13212	-89.2841	U	H	Grenada	6.3	227	98.01	4.3	0.17	3.67	1/1
35.11757	-89.31765	U	L	Loring	5.8	127	85.31	4.8	0.18	3.16	0/2
35.1093	-89.32217	U	H	Smithdale	6.4	103	116.7	5.9	0.21	4.28	0/2
35.08329	-89.23797	U	H	Loring	6.2	36	201.3	5.1	0.2	4.12	1/0
35.14423	-89.2259	C	H	Memphis	6.5	14	184.1	5.2	0.23	4.08	1/0
35.13622	-89.28342	U	H	Gullied land	6.1	130	117.6	4.9	0.21	2.9	1/0
35.13217	-89.23512	C	H	Smithdale	6	29	125.9	3.2	0.18	2.21	5/3
35.12772	-89.29048	U	H	Memphis	6.2	863	104.2	4.5	0.18	2.87	3/2
35.13662	-89.30499	U	L	Loring	6.7	290	157.7	7.9	0.29	4.62	1/2
35.12854	-89.23443	U	L	Memphis	6.1	297	163.7	9.9	0.32	5.99	1/1
35.0939	-89.20156	U	L	Gullied land	6.5	103	144.8	5.1	0.23	3.48	2/2
35.11593	-89.30495	U	H	Grenada	5.9	19	145.9	9	0.34	5.89	5/1
35.14275	-89.2408	C	L	Gullied land	5.3	22	106.6	4.6	0.2	2.91	4/3
35.11486	-89.31157	C	L	Calloway	5.3	13	91.38	9.2	0.3	5.72	2/2

Supplementary Table S2. Data sources used in the analysis of chronic wasting disease prion (PrP^{CWD}) detection in soils from mineral lick sites with the Ames Research and Education Center study area.

Data description	**Source**
Soil series and textural class	Soil Data Explorer California Soil Resource Lab (ucdavis.edu)
Percent sand, silt, and clay	Soil Data Explorer California Soil Resource Lab (ucdavis.edu)
Soil chemistry	University of Wisconsin Soil and Forage Analysis Lab

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CHAPTER 5: CONCLUSION

Landscape features where deer preferentially congregate are potential areas of high PrP^{CWD} deposition into soils of varying compositions, where clay-rich soils bind prions, enhancing their infectiousness and transmission. Baiting sites result in congregation and revisitation of deer, establishing foci for environmental contamination where deer deliberately ingest soil, potentially leading to indirect exposure and transmission of CWD. Consumption of CWD-infected hosts, dead or alive, suggests a role for predators and scavenging wildlife in CWD epidemiology. Further, the potential for mechanical vectors to be involved in CWD epidemiology also exists, since several ectoparasites of cervids consume PrP^{CWD}-containing blood or tissue (e.g., ticks, deer keds, biting flies) and allogrooming among cervids may result in ingestion of these ectoparasites and CWD exposure risk.

Research findings presented in *chapter 2* established that ticks can assimilate PrP^{CWD} from infected blood meals, contained transmission-relevant amounts of PrP^{CWD} after taking a blood meal from CWD-infected deer, and may therefore pose a CWD risk to cervids by acting as potential mechanical vectors mediated by cervid social grooming behaviors. Future research efforts investigating the relevance of these findings could include field surveys and analysis for detection and relative quantification of tick DNA in cervid feces, in addition to cervid behavioral studies to determine frequency of allogrooming events and observation of tick or other ectoparasite consumption. Additionally, further research is needed to determine the prevalence of CWD prions in ticks, investigate tick attachment sites on the host's body, optimize detection methods, and explore the impact of tick infestations and grooming behaviors on CWD transmission among cervids. Understanding the dynamics of CWD transmission and identifying potential vectors are crucial for effective management strategies. With changing environmental conditions and tick

distributions, the risk of CWD transmission among cervids may increase. Therefore, continued research efforts in this field are essential for disease control and management.

In *chapter 3*, I demonstrated the utility of RT-QuIC for detection of PrP^{CWD} in excrement of cervid consumers and showed that the application of carnivore-based surveillance in comparison to CWD prevalence of deer may be an effective tool for improving CWD surveillance approaches. Additionally, findings from this work provide a way to advance our understanding of the potential ecological implications of carnivore and scavenger involvement in the CWD system. Incorporating carnivore feces in CWD surveillance strategies can provide a cost-effective and non-invasive alternative to traditional testing methods that rely on hunter-harvested tissues. By analyzing predator and scavenger scats, wildlife management agencies can potentially monitor CWD in at-risk areas neighboring outbreak zones, areas with low hunter-based sampling rates, or where non-lethal approaches are desired. However, implementing such surveillance programs may require additional funding, personnel, and logistical considerations.

Predators and scavengers may consume infected prey, reducing environmental prion deposition and accumulation. The presence of carnivore scats may deter naïve cervids from visiting infected carcass sites, reducing direct interactions and indirect exposure to CWD. Additionally, the scat deposits and other predator cues may influence cervid movement and spatial distribution by eliciting anti-predator responses. Further ecological studies are needed to better understand the role of predators and scavengers in mitigating CWD exposure and transmission. Future research could use this approach coupled with camera-capture to better understand and quantify how cervid-

consuming wildlife influences PrP^{CWD} soil deposition in and around carcass sites. In addition, future efforts could also use this approach to better understand how predator-prey relationships influence CWD dynamics, which will help inform state and federal wildlife agency policy and management of large carnivores.

In *chapter 4* I presented that mineral lick sites established prior to the first detection of CWD in southwestern Tennessee, U.S.A. are foci of PrP^{CWD} contamination. Using RT-QuIC, I revealed that detection of PrP^{CWD} in soils from decommissioned artificial mineral sites was not influenced by site characteristics or soil properties, but that other factors not evaluated in this study may have influenced the distribution and accumulation dynamics of PrP^{CWD} across the different sites. The presence of PrP^{CWD} in soils indicates that soils can act as environmental reservoirs and potentially contribute to the spread of chronic wasting disease (CWD) in different geographical regions. Soils are complex and heterogenous systems and the interactions, bioavailability, and detection of soil-bound prions are believed to be strongly influenced by various soil properties and compounds.

My findings demonstrate the presence of PrP^{CWD} in soil samples from mineral lick sites that were decommissioned at least ten years before the detection of CWD in southwest Tennessee. However, there was no spatial clustering or autocorrelation observed in the PrP^{CWD} detection status among the mineral lick sites within the study area. Furthermore, the probability of PrP^{CWD} detection did not differ based on site-specific management approaches or deer use intensity. Soil textures were similar across the mineral lick sites and did not serve as a predictor for PrP^{CWD} detection. While I did observe variability in soil characteristics, there was no evidence to support the influence of soil

pH, phosphorus and potassium levels, organic matter content, total organic carbon, or total nitrogen on the probability of PrP^{CWD} detection within the study area.

The detection of PrP^{CWD} in 65% of the decommissioned mineral licks highlights the potential of these sites to contribute to current and future CWD exposure and transmission events among cervids. This finding advances our understanding of the role of deer attractant sites in the establishment and perpetuation of CWD outbreaks. In addition to the uncertainties regarding fine-scale soil factors and the effects of deer behavior on PrP^{CWD} soil deposits, the high prevalence of CWD and deer density in the Ames region, as well as the size of the study area, limited my ability to disentangle the influence of soil heterogeneities and site characteristics as predictors of PrP^{CWD} detection probability or determine the reasons for limitations in PrP^{CWD} detection.

Future research can overcome the limitations of this study by conducting comparative analyses of soil factors in deer congregation areas across different geographic regions with varying CWD prevalence. By examining heterogeneities in soil properties in diverse endemic regions, researchers can gain a more comprehensive understanding of the role of soil in PrP^{CWD} detection and transmission. In addition, exploration of deer behavior during visits to mineral licks and their impact on PrP^{CWD} deposition and soil-prion interactions is another important avenue for future research. By observing and studying the specific behaviors exhibited by deer at mineral licks, an assessment can be made for how these behaviors influence the spread and persistence of PrP^{CWD} in soil. Incorporation of camera-capture at sites to better understand how PrP^{CWD} deposition and accumulation in soil over time is influenced by duration and frequency of cervid visitation, prion-

shedding behaviors (e.g., urinating, salivating, or defecating), and exposure-risk behaviors (e.g., scraping, snorting, eating, or licking soil) or if point resources result in more frequent and riskier direct interactions. Additionally, a systematic assessment of the effects of site remediation efforts on PrP^{CWD} deposition and deer use would be valuable. Examining the influence of covering sites and other remediation techniques on PrP^{CWD} presence in soil and deer behavior can provide insights into the effectiveness of these strategies in reducing CWD transmission. By addressing these research gaps and expanding our understanding of soil properties, deer behavior, and site remediation efforts, we can enhance our knowledge of PrPCWD detection, transmission dynamics, and develop more effective strategies for managing and preventing the spread of CWD in cervid populations.

The purpose of this dissertation was to expand upon existing tools that can help advance our current understanding of what environmental factors influence CWD ecology. The research I present here demonstrates that RT-QuIC can help us understand some important environmental factors in the CWD system. These findings and future research that expands upon this work will incorporate more of a dynamic approach for evaluating potential risk, help build more complete prediction models for disease spread, and advance effective CWD management approaches.