THESIS

ELUCIDATING MOTHER TO OFFSPRING TRANSMISSION OF CHRONIC WASTING DISEASE USING A TRANSGENIC MOUSE MODEL

Submitted by

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ABSTRACT

ELUCIDATING MOTHER TO OFFSPRING TRANSMISSION OF CHRONIC WASTING DISEASE USING A TRANSGENIC MOUSE MODEL

Chronic wasting disease (CWD) is a transmissible spongiform encephalopathy (TSE), or prion disease, of free-ranging and farmed cervids (deer, elk and moose). CWD is the only TSE in a wildlife population, which was initially discovered in a captive mule deer herd in a study shared between Colorado State University and University of Wyoming in 1967. CWD is the most readily transmitted of all the prion diseases and since its discovery has been identified in cervid populations in 24 states, 2 Canadian provinces, and the Republic of Korea. Horizontal transmission of prion diseases is thought to account for its exceptional transmission efficiency [2-10]. Recent studies published by our group provide evidence that transmission from mother to offspring may also be a contributing factor.

In the work of this thesis, we employed a transgenic mouse system that expresses the cervid prion protein Tg(CerPrP-E226) to help elucidate the role of mother to offspring CWD transmission via hemochorial placentation. Females were inoculated with known CWD-positive material and subsequently bred with CWD-naïve males at various timepoints post inoculation to investigate if maternal/vertical transmission occurs in this host, as well as to further understand how this might occur. We examined the likelihood of prion trafficking *in utero* by analysis of mother: offspring pairs at different timepoints in CWD-infection and gestation, in addition to looking for infectious prions in milk collected from CWD-positive dams.

We have demonstrated that CWD-infected Tg(CerPrP-E226) females successfully breed and bear offspring irrespective to TSE disease stage. Offspring born to CWD- infected females did not exhibit signs of TSE disease and lacked detectible PrP^{res} via conventional methodologies.

Interestingly, conversion competent prions were identified in the brains and spleens of offspring by highly sensitive amyloid seeding assays. The lack of symptoms in these offspring indicates covert prion transmission from mother to offspring, resulting in a potential silent-carrier status.

As for our studies to further the understanding of the mechanisms behind this transmission, we identified CWD-prions in reproductive and mammary tissue, and spleen of Tg(CerPrP-E226) mouse mothers as early as 72 days post inoculation. In addition, we found minute quantities of amyloid conversion material in placenta and fetal tissues from mother:offspring pairs at varying timepoints in CWD-infection. We were unable to detect prions in milk collected from CWD-positive transgenic dams, leading us to hypothesize that the route of TSE transmission to offspring is likely a combination of environmental exposure, and/or very low concentrations of prions breaching the feto-maternal interface.

ACKNOWLEDGMENTS

Throughout this journey, I have encountered a unique plethora of bright minds. Though each distinctive in their approaches to research, areas of expertise, and personalities, there is not one individual I've met along the way, who has not impacted my experience as a graduate student in one way or another. Collectively and separately, these people have helped to sculpt my own research approach, increase my areas of expertise, and influence my personality. There is no doubt that I will walk away from this work a different bird, as they say… and all for the better.

I thank Dr. Candace Mathiason for her listening ear, honesty, encouragement, and praise. Her determination in her research endeavors is nothing short of inspiring. Our mutual history as women raised in Wyoming who eventually followed their hearts to science (and Colorado) has been a great foundation for the connection we have built over the years, and I am truly lucky to have her as my mentor.

I thank the members of my committee for their unique contributions to this project. Dr. Quint Winger for his knowledge of reproductive science—there has been quite the learning curve for me on this topic and he makes it seem more accessible. Dr. Erica Suchman for her advocacy for my future as an educator. Dr. Mark Zabel for his friendship, approachability, and for making science look cool— "don't make it weird!"

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DEDICATION

I dedicate this work to—

The lovely Katherine Sloan. Your endless support in every sense of the word will never be forgotten. Our shared laughs, smiles, adventures, and, interestingly enough, tears, are the reason I finished this thing. I've never met a person who can weave words of encouragement together so beautifully that I am able to forget my self-doubt almost instantaneously. You are unlike any other, and I strive to be more like you every day.

My students—past, present and future. After all, you're the reason I decided to pursue this endeavor. Even on my worst days, I walk away from the classroom with a smile on my face and confidence in my pocket…full of hope to change the way the world views science, one student at a time.

Mom, dad, and Stacie. From day one you've assured me that I'm capable of achieving anything I want in life if I just reach for the stars-- this is a much easier feat knowing I have you three as my cheerleaders.

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CHAPTER ONE:
INTRODUCTION

Protein misfolding diseases

Proteins are invaluable biological molecules that are responsible for the structure, function, and regulation of tissues and organs. Critical to proper protein function is the three-dimensional structure, or folding, enciphered by the protein's unique amino acid sequence. Protein structure is central to protein function. Several mechanisms are employed by the cell to aid in proper folding and recycling of misfolded and/or dysfunctional proteins. Unfortunately, these mechanisms are not without failure, and thus protein misfolding and aggregation is the root of several neurological and systemic disorders.

Protein misfolding diseases (PMDs) are the consequence of the conformational change in a protein's structure, resulting in its aggregation and insoluble fibril deposition within tissues. The etiology of PMDs includes both sporadic and infectious forms. In a recent review, comparison is drawn between PMDs— including Alzheimer's, Amyotrophic Lateral Sclerosis, Parkinson's, and Huntington's disease, and the transmissible spongiform encephalopathy (TSE) or prion diseases [1]. Disease presentation depends upon the protein involved, as well as the location of its deposition (Table 1.1). PMDs are a diverse group of diseases that can result in a range of disease outcomes including islet amyloid polypeptide buildup in the pancreas associated with diabetes mellitus type 2 , and amyloid-beta and tau aggregation in the brain of Alzheimer's patients [2].

Table 1.1. Clinically relevant protein misfolding disorders, including transmissible spongiform encephalopathies.

Adapted from Treherne and Scopes, *EBR* 2012

Amyloid protein	Clinical syndrome
Amyloid-ß peptides (1-40, 1-42)	Alzheimer's disease Inclusion body myositis (orphan)
Amylin or Islet amyloid polypeptide (IAPP)	Type 2 diabetes
β2-Microglobulin	Dialysis-related amyloidosis
α-Synuclein	Parkinson's disease
Prion protein	Transmissible spongiform encephalopathies (for example Creutzfeldt-Jakob disease (CJD))
Tau	Fronto-temporal dementias
Huntingtin (polyQ expansion)	Huntington disease
Superoxide dismutase	Amyptrophic lateral sclerosis
Transthyretin	Senile systemic amyloidosis
	Familial amyloidotic polyneuropathy
γ-Crystallin	Cataract
Lysozyme	Lysozyme systemic amyloidosis
Ig light chains	Primary systemic amyloidosis
Serum amyloid A	Secondary systemic amyloidosis

History of prion diseases

Human TSEs

Kuru

Kuru (derived from the Fore word for "shake") is a TSE that is believed to have affected the Fore tribes of Papua New Guinea since the early twentieth century. Kuru is characterized by an extremely long asymptomatic incubation period, which can last decades. Carleton Gajdusek and colleagues began studying the disease in Papua New Guinea in 1957. Soon after, it was realized that the neuropathology of the disease closely resembled scrapie, the prion disease that affects sheep [3]. Soon after, the brains of infected individuals were experimentally inoculated into chimpanzees, which succumbed to the disease no more than two years later [4]. Eventually, epidemiological data revealed that ritualistic cannibalism associated with Fore funeral practices was responsible for the transmission of Kuru [5] .

Creutzfeldt-Jakob disease (CJD)

There are 4 types of Creutzfeldt-Jakob disease, characterized by the cause of prion protein dysfunction in the host. Sporadic CJD (sCJD) is thought to account for the largest number of cases of CJD (85%) of which no cause has been found. sCJD is commonly referred to as classical CJD [6]. Familial CJD (fCJD) is genetically acquired CJD, which is a result of a mutation or polymorphism in the genetic code for the normal cellular prion, PrP^c [7] and accounts for 5–15% of CJD cases. Iatrogenic (iCJD) accounts for 5% of cases, and occurs when a human has acquired CJD via medical procedure. For example, iCJD was found in patients who received pooled human growth hormone from the pituitary glands of human cadavers [8]. Perhaps the most readily recognized human prion disease, Variant (vCJD) is thought to be acquired by the ingestion of BSE-infected meat products, resulting in approximately 1% of all CJD cases [9].

Gerstmann –Sträussler–Scheinker Syndrome

Gerstmann–Sträussler–Scheinker syndrome (GSS) was first reported in the 1920's in an Austrian family, and is an inherited prion disease. GSS is a result of mutations in the *prnp* gene and can be recognized by its extended clinical phase (up to ten years) which includes dementia and chronic progressive ataxia [10].

Fatal familial insomnia

Fatal familial insomnia (FFI) was previously known as thalamic dementia, due to its characteristic pathology, which includes neuronal loss and astrogliosis in the thalamus. Damage to this area often results in sleep disturbances, muscle spasms, seizures, disrupted speech, and difficulty swallowing. Another inherited prion disease, clinical onset often begins in an individual's late forties or fifties, and can last for over a year until the patient succumbs to terminal disease [11].

Animal TSEs

Scrapie

Scrapie is the oldest known prion disease, dating back to at least 1732 [12]. Named for one of its hallmark symptoms, scrapie causes neurological deficits in sheep that causes itchiness in the affected animals, resulting in a patchy appearance of the skin. Additional signs include altered gait due to ataxia, lip smacking, and eventual weight loss and death [12]. Although scrapie does not appear infectious to humans, it is readily transmissible from sheep to sheep and can result in widespread dissemination within flocks. Studies have shown that scrapie prions can persist in contaminated environments for at least 16 years [13]. Genetic selection is now the primary means of scrapie control [14].

Bovine spongiform encephalopathy

Bovine spongiform encephalopathy (BSE) is the TSE that most are familiar with. This disease affects cattle between the ages of 3 and 5, with an incubation period of up to 8 years. The disease is characterized by lameness, incoordination, and in some cases violent or nervous behavior [15]. In the 1990s a BSE epidemic occurred among cows in the United Kingdom, due in part to feeding practices that included supplementing cattle's diets with ovine bone meal. This resulted in an extremely large slaughtering of cows across the country (an estimated 4.4 million), and a necessary change in slaughterhouse and meat processing practices [16].

Chronic wasting disease

Chronic wasting disease (CWD) was first reported in a captive mule deer herd in a conjoined study shared between Colorado State University and University of Wyoming in 1967 [17]. It was officially reported as a TSE in 1980, with clinical signs that include weight loss, polydipsia, polyphagia, as well as gait impairment. Rocky Mountain elk [18], white-tail deer [19] and moose [20] have also been identified as susceptible hosts. CWD is recognized as the most

readily transmitted TSE and since its discovery, has been detected in free-range and farmed cervid populations in 24 states, 2 Canadian provinces, and the Republic of Korea [21].

Cellular prion protein

The normal cellular form of the prion protein (PrP^c) is an extracellular, glycosyl-phosphatidylinositol-(GPI)-anchored protein. It is predominantly expressed on mammalian cells, is in close association with lipid rafts on cellular membranes, and can be found in the highest concentrations in neuronal tissue [22]. The final, processed version of PrP^C consists of amino acids 23-231 (peptide 1–22 is cleaved as signal peptide during trafficking, and peptide 232–253 is replaced by the GPI anchor) which contains two asparagine-linked glycosylation sites, giving rise to a profile containing three glycoforms (non, mono, and diglycosylated) (Figure 1.1, 1.2). As shown in Figure 1.1, the N-terminus of PrP^c is relatively unstructured, and contains several copper binding octapeptide repeats [10].

PrP^c is synthesized in the rough endoplasmic reticulum, and is subsequently transported to the plasma membrane via the Golgi apparatus. During synthesis in the endoplasmic reticulum, several posttranslational modifications occur to achieve the mature form of the protein. These include the cleavage of an N-terminal signal peptide, addition of N-linked oligosaccharides in two positions, formation of a single disulfide bond, and attachment of the GPI-anchor [23].

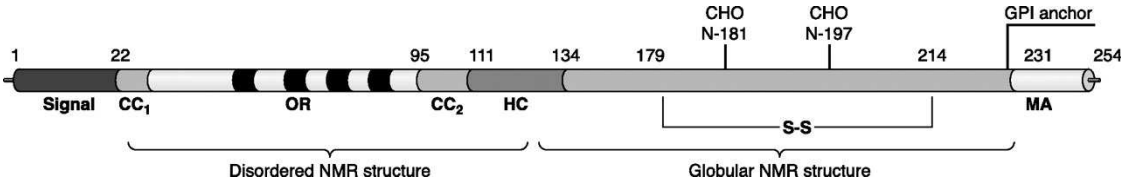

Figure 1.1. Primary structure of the normal cellular prion protein.

Aguzzi and Calella, *Phys. Reviews* 2009

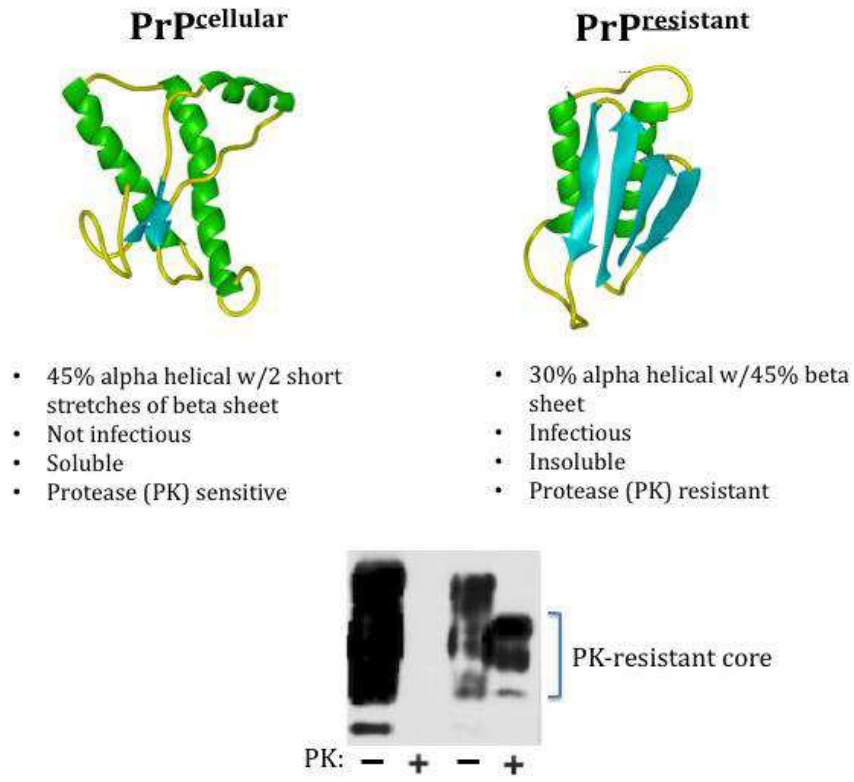

Figure 1.2. Cellular PrP vs. infectious prion.

To elucidate the function of mammalian PrP^c, cohorts of PrP^c knockout mice have been monitored for phenotypic changes. Although changes in circadian rhythm have been noted, there have been no reports indicating that the prion protein is essential for life [24-26]. This suggests that the role of PrP^c in cells is inducible, or is minor. Proposed functions of PrP^c include neuroprotection against oxidative stress, prevention and/or promotion of apoptosis, cellular adhesion and cell signaling [23, 27, 28].

Establishment of prion disease

Prion conversion and aggregation

The mechanism of prion conversion is not well understood. It is known that the normal prion protein is essential to act as a substrate for conversion to PrP^{res}[29], and that the native

conformation of the prion protein (PrP^{C}) becomes misfolded, taking on the beta-pleated sheet rich conformation associated with prion disease (PrP^{res}). This abnormal form, PrP^{res}, is partially resistant to protease (PK)-digestion [30] and can be readily differentiated from its normal cellular counterpart by assays employing PK (i.e. western blot) (Figure 1.2).

Conversion from the normal to the aberrant form of the prion protein is energetically unfavorable, and thus conversion from the alpha helical structure to that of a beta sheet is rare. This initial misfolding is either spontaneous or is aided by the addition of a "seed" of PrP^{res} that acts as a template for conversion of PrP^{c} (Figure 1.3). In vitro evidence suggests that a higher concentration of misfolded seed increases the likelihood of a conversion event [31]. PrP^{res} molecules aggregate with one another to form a more stable structure, called an amyloid. Amyloids are defined as protein aggregates that accumulate as extracellular fibrils of 7-10 nm and have the ability to bind Congo red dyes and thioflavins S and T [1].

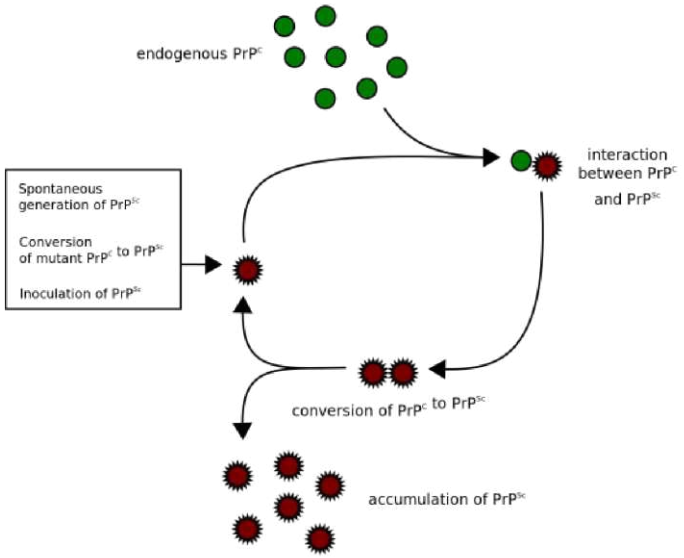

Figure 1.3. Proposed mechanism for prion conversion [32].

Routes of prion transmission

Infectious prions are present in the tissues, bodily fluids (saliva, blood) and excreta (urine, feces) of animals, irrespective of overt clinical disease [13, 33-40]. Additionally, it has been shown that infectious prions can be transmitted horizontally from infected to naïve host by oral ingestion, environmental and iatrogenic exposure, and blood transfusion [41-45]. Evidence is also available that suggests mother to offspring transmission of prions may play a role in the spread of prions from one individual to another, which is the primary focus of this thesis. The first recognition of maternal transmission was in sheep scrapie, and PrP^{res} has been identified in maternal and fetal tissues of infected ewes and their lambs [46-51]. Evidence for maternal transmission of FSE was reported in 2009 when a cheetah cub born to a FSE-infected cheetah presented and was diagnosed with prion disease, even though she was housed in a TSE-free environment and fed TSE-free meat after separation from her mother post weaning [52]. In addition, a higher incidence of BSE has been reported in calves born to BSE-infected cows, though the methodology used at the time of those studies was unable to detect prions in pregnancy related tissues [53-55]. Finally, we have recently reported maternal transmission of CWD in experimental studies utilizing Muntjac deer [56] and in free-ranging elk naturally exposed to CWD [57].

Susceptibility to prion diseases

It is suspected that the acquisition of a TSE depends on PrP^{res} acting as a template for PrP^{c} conversion, and research has shown that amino acid sequence is a key factor in both susceptibility and resistance to certain prion diseases. This has been well described in sheep, where polymorphisms at amino acid 136, 154 and 171 of the *prnp* gene seem to confer resistance or susceptibility to scrapie [58, 59]. Similarly, humans possessing a polymorphism at amino acid 129 are more susceptible to variant Creutzfeldt-Jakob disease [60, 61]. These parameters, while neither conveying total susceptibility nor complete resistance, have also been defined in cattle for susceptibility to BSE, which include polymorphisms within noncoding regions of promoter

sequences for the *prnp* gene,[62, 63], and for deer (96 GS deer show less PrP^{res} accumulation than 96GG deer) and elk (132 MM display shorter incubation times than those with 132LM or 132LL) in cases of chronic wasting disease (CWD) [64].

It has also been established that some infectious prions are capable of crossing species barriers. For example, exposure to BSE-contaminated meat products (presumably by oral ingestion) has been attributed to the development of variant form of Creutzfeldt-Jakob disease (vCJD) in humans [9], feline spongiform encephalopathy (FSE) in cats [65] and neurologic disease in zoo ungulates [66]. Several factors are likely responsible for the propensity of a TSE to cross the species barrier into a new host species. According to recent research in the Kurt laboratory, "cross-species prion transmission is influenced by (a) the sequence similarity between the cellular prion protein (PrP^{C}) and the misfolded, aggregated conformer (PrP^{Sc}) and (b) the PrP^{Sc} conformation" [67]. These findings corroborate with the thought that PrP^{res} acts as a template for prion conversion, but more research is needed to completely solve the question of species barriers.

Clinical signs

Clinical signs of TSEs include memory loss, lack of coordination, ataxia of the limbs, and invariably, death [1]. These signs are consistent with the distribution of the normal cellular form of the prion protein (PrP^{C}), with the highest concentrations found in the central nervous system (CNS), particularly in neurons [68]. Although the mechanism is not completely understood, the accumulation of PrP^{res} is correlated with neurotoxicity, causing characteristic clinical neurological features regardless of the species inflicted [69].

PrP^{res} invasion and dissemination

Two types of neuroinvasion have been hypothesized for the routes of prions invasion; neural and hematogenous. Neural neuroinvasion occurs when the agent travels along peripheral nerves until it reaches its destination, whereas hematogenous neuroinvasion involves the agent traveling in the blood [70]. Although TSEs can be transmitted via various routes, it has been

hypothesized that a majority of natural infections are acquired by ingestion of prion-laden materials [71, 72]. It is generally accepted that neuroinvasion involves amplification of infectious prions in lymphoid tissues (lymphoreticular system or LRS), followed by retrograde trafficking to the brain using autonomic nerves (i.e., neural neuroinvasion) [70]. Interestingly, the ability to locate pathogenic prions within the periphery of animals is variable. For example, CJD, scrapie and CWD prions can be found within the periphery of infected animals, including spleen and muscle, whereas BSE prions are not. BSE is limited to the central nervous system with very little lymphoid tissue involvement [73]. As previously mentioned, it has been established that prions are present in the blood of infected individuals, regardless of inoculation route [43-45, 74]. Transfusion of blood from prion-infected donors results in recipient infection and establishment of both neural and hematogenous prion transport [41-45, 74].

Detection of prions

In vivo bioassay

The gold standard for detection of infectious prions is the bioassay, which involves inoculation of suspect tissues/bodily fluids into native hosts or transgenic mouse models over-expressing host specific prion protein. For example, to assess CWD infectivity transgenic mice over-expressing the elk prion protein are employed [75]. Although bioassay is a sensitive and powerful tool, studies are costly and time consuming.

Conventional in vitro methodologies

Conventional *in vitro* methodologies for the detection of prions are dependent upon the use of antibodies derived against the normal prion protein in combination with protease digestion permitting PrP^{res} detection (Figure 2).

Western blot

Western blot analysis permits distinction between the cellular and aberrant forms of the prion protein; the normal cellular prion protein is ablated by PK digestion, whereas the aberrant

form is only partially digested, revealing a PK-resistant core of approximately 27-30 kilodaltons in size (Figures 1.2, 1.4) [76].

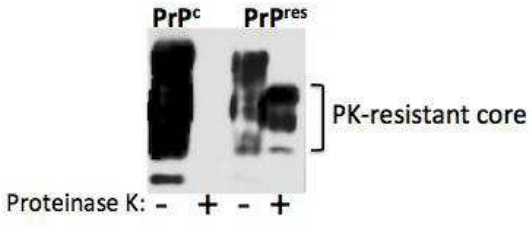

Figure 1.4. Western blot analysis of PK-resistant material.

Histopathology and immunohistochemistry (IHC)

Histopathology and IHC analysis are common methods used to characterize the pathology *caused* by TSE disease and detect the biological marker associated with prions, PrP^{res}. IHC is dependent upon formic acid/PK digestion of the normal cellular prion protein and subsequent antibody specific detection to reveal PrP^{res} deposition within tissues [77]. Common tissue pathology in the brain of TSE-infected hosts includes astrocytosis, neuronal degeneration, and widespread distribution of PrP^{res} within the grey matter [78]. Example: PrP^{res} deposition within the hippocampus of a Tg5037 mouse IC-inoculated with 30ul 1% CWD-positive brain homogenate (Figure 1.5). The current means for diagnosis of CWD in North American cervid populations is IHC of lymphoid (tonsil and/or rectal) biopsies [79].

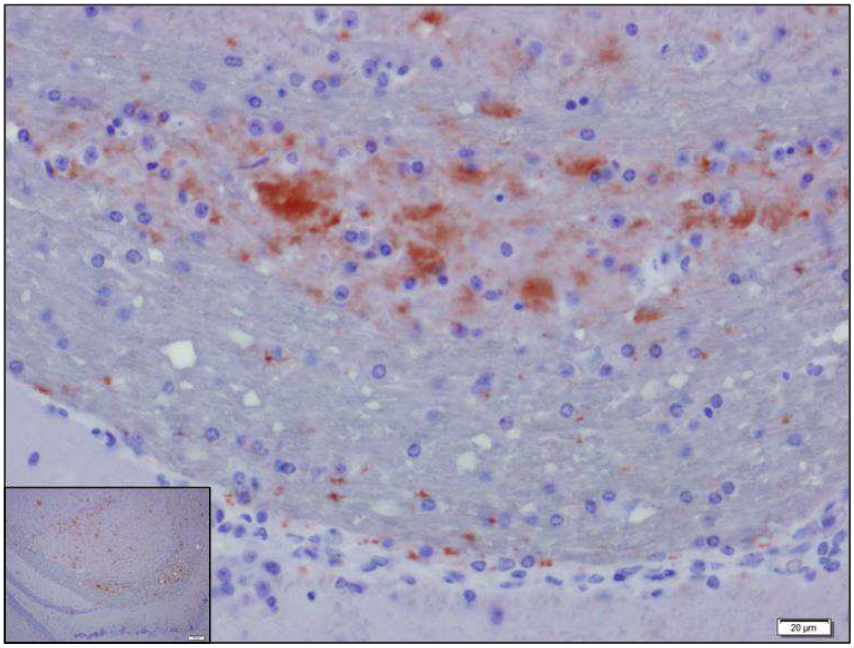

Figure 1.5. Immunohistochemical detection of PrP^{res}.

Inset is 4X, larger image at 40X (scale bar = 20 um). PrP^{res} deposits are indicated by rust color at locations of anti-prion antibody BAR-224 binding.

Enzyme-linked immunosorbent assay (ELISA)

Similar to western blot and IHC, ELISA relies upon PK-digestion, as well as the use of an antibody specific to cellular prion protein for detection. This assay can be used for antemortem diagnosis of prion diseases such as scrapie and CWD, but often requires a 2nd method (IHC) for confirmation [80].

Highly sensitive in vitro methodologies

Serial protein misfolding cyclic amplification (sPMCA)

sPMCA, developed by the Soto laboratory [81], is an established method for the detection of prions. sPMCA is used when the amount of prions are below the limit of detection by conventional assays, and is similar to polymerase chain reaction (PCR) in that a minute quantity of material is amplified over several rounds, facilitating its detection. Minute quantities of prions can be detected by demonstrating their ability to amplify normal cellular prion protein within a prion-

naïve normal brain homogenate substrate (NBH). Fluid or tissue homogenates containing minute quantities of prions (referred to as the seed) are added to NBH substrate and placed into a sonicator. Sonication serves to break up growing amyloid fibrils, aiding in the exposure of seed to substrate throughout the conversion process. Several 24-hour rounds of this method can be performed, in each instance serially diluting the previous sample into a new source of NBH substrate [82]. The resultant material is analyzed by western blot for the presence of PK resistant prions (Figure 1.6).

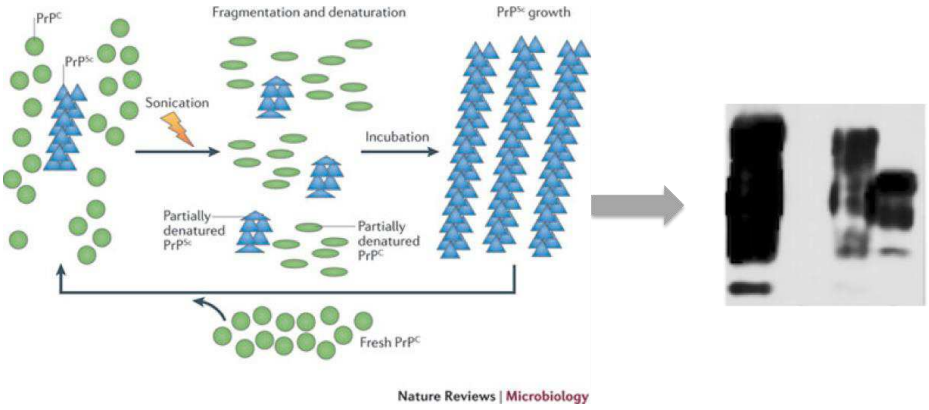

Figure 1.6. Schematic representation of protein misfolding cyclic amplification.
Adapted from Colby & Prusiner, *Nature Reviews Microbiology* 2011.

Real-time quaking induced conversion (RT-QuIC)

A second-generation amplification methodology, RT-QuIC, was recently developed by the Caughey laboratory [31]. The basis of RT-QuIC is similar to PMCA in that a prion seed is added to a naïve substrate, and is tested for its ability to generate conversion competent amyloid. RT-QuIC employs the use of a *recombinant* prion protein as the conversion substrate vs. animal derived PrP^c in PMCA. Recombinant prion protein substrate (rPrP) is readily produced in the laboratory and is based on host PrP^c sequence, permitting questions to be asked about native host species conversion properties as well as those that occur across species barriers. RT-QuIC uses a 96 well plate format

with shaking and a fluorescent dye Thioflavin T (ThT), which fluoresces upon its intercalation into growing amyloid fibrils [83]. This intercalation is detected in real-time using a BMG FLUOstar Fluorometer (Figure 1.7) [31].

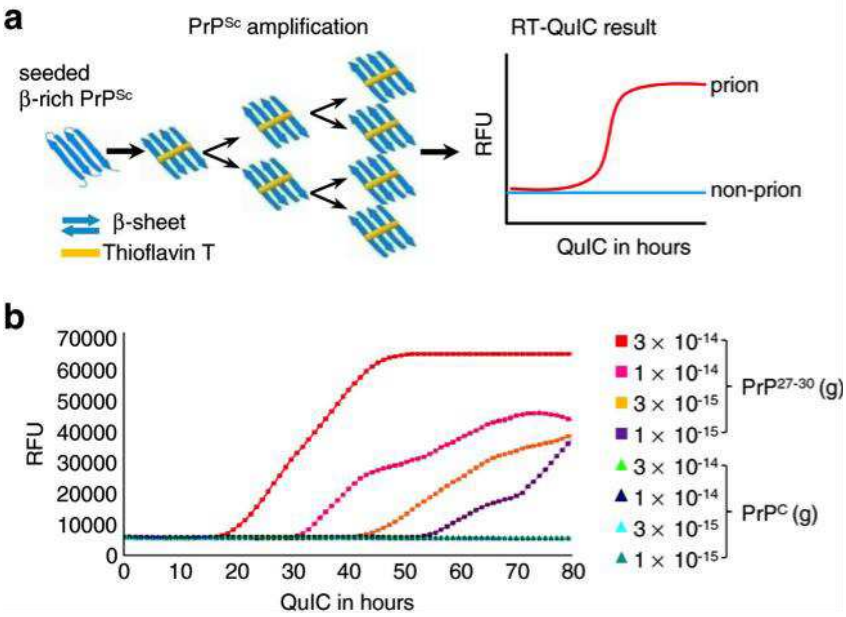

Figure 1.7. Schematic of real-time quaking induced conversion assay.

Shi et al. *Acta Neuropathol Commun* 2013.

Introduction to this work

Recent work in our laboratory has characterized maternal transmission of CWD in Muntjac deer [56], resulting in low offspring viability and clinical signs leading up to terminal clinical disease in offspring that survived post birth. Interestingly, there are no confirmed cases of maternal transmission of the human prion disease, CJD, even though 125 children have been born to parents with the disease [84]. Scrapie has been highly characterized with regard to maternal transmission, and information gathered from those studies has led us to hypothesize that the infectious prion's route of travel is likely through the placenta *in utero* and/or post birth via milk.

In this specific study, we seek to identify whether CWD is maternally transmissible using a transgenic mouse model (whose placental structure is more similar to human than cervid [85]) and

to ascertain whether the transmission efficiency and features of the disease in offspring are similar to what we have previously reported. Additionally, we aim to identify potential mechanisms by which this transmission may occur by collecting maternal/fetal tissues and milk from lactating, gravid, CWD-positive mouse mothers at different timepoints in gestation and clinical disease.

Aim 1

Identify whether CWD is maternally transmissible using this model and compare the transmission efficiency and features of the disease in offspring to what has previously been reported.

Aim 2

Characterize potential mechanisms by which this transmission may occur: either *in utero,* or through milk .

Hypothesis

Maternal transmission of CWD-prions occurs in the Tg mouse model, causing disease in the offspring. Transmission of these prions either occurs *in utero*, via milk, or by both of these routes.

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CHAPTER TWO:
MATERNAL TRANSMISSION OF CHRONIC WASTING DISEASE RESULTS IN ASYMPTOMATIC CARRIERS

Introduction

Chronic wasting disease (CWD), the transmissible spongiform encephalopathy (TSE), or prion disease, of free-ranging and farmed cervids (deer, elk and moose) was first reported in a captive mule deer herd in a conjoined study between Colorado State University and University of Wyoming in 1967 [1]. CWD, the only TSE in a wildlife population, is the most readily transmitted of all the prion diseases and is now detected in free-range and farmed cervid populations in 24 states, 2 Canadian provinces, and the Republic of Korea [2].

The presence of infectious prions in the tissues, bodily secretions/excreta (urine, saliva, feces and blood) in sufficient amounts to cause disease, in addition to shedding of these prions into the environment is thought to account for CWD's high transmission efficiency [3-11]. Recently it has been recognized that transmission from mother to offspring may contribute to this facile spreading behavior [12, 13]. Although the mechanism of how this happens is yet completely understood, the extended asymptomatic TSE carrier phase, lasting years to decades, suggests that maternal transmission may have implications in the spread of prions. Our work aims to identify whether prions are transmitted from a CWD-positive mother to her offspring via hemochorial placentation, and identify whether there are sufficient prions to establish disease in these offspring.

We employed a transgenic mouse system that expresses the cervid prion protein Tg(CerPrP-E226) [14] to further understand the role of placentation in mother to offspring CWD transmission. Females were inoculated with known CWD-positive material and subsequently bred with CWD-naïve males at various timepoints post inoculation to investigate maternal/vertical transmission in an animal model with hemochorial placentation. The resultant offspring were

monitored over a period of 500 days for clinical signs, after which time they were sacrificed and multiple tissues were analyzed for CWD-prion deposition.

We have demonstrated that CWD-infected Tg(CerPrP-E226) females successfully breed and bear offspring irrespective to TSE disease stage. While offspring born to CWD-infected females did not exhibit signs of TSE disease and lacked detectible PrP^{res} via conventional methodologies, conversion competent prions were identified in their brains and spleens by highly sensitive amyloid seeding assays. Their lack of symptoms indicates covert prion transmission from mother to offspring, resulting in a potential silent-carrier status. While maternal transmission of CWD in the transgenic mouse model does not directly reflect what is observed in the native host, the information gained from this work may provide insight into the potential for covert maternal transmission of prions in other TSEs, such as Creutzfeldt-Jakob disease.

Background

The transmissible spongiform encephalopathies (TSEs) or prion diseases are neurodegenerative disorders that are uniformly fatal, and can affect several species including cattle (bovine spongiform encephalopathy), sheep and goats (scrapie), domestic and nondomestic cats (feline spongiform encephalopathy), mink (transmissible mink encephalopathy), cervids (chronic wasting disease) and humans (Creutzfeldt-Jacob disease and Kuru). Prion diseases are the result of the conversion of the normal cellular form of the prion protein (PrP^{c}) into a disease-associated misfolded, aggregation-prone conformer (PrP^{res}) [15]. CWD was first observed in a captive mule deer population in 1967 in Fort Collins, Colorado and was officially reported as a TSE in 1980 [1]. Clinical disease signs include weight loss, polydipsia, polyphagia and gait impairment [1, 16, 17]. The disease has since been identified in 24 states, 2 Canadian provinces, and the republic of Korea [2].

CWD's rapid diffusion throughout cervid populations (CWD is now known as the most readily-transmissible prion disease) has sparked the curiosity of several researchers to investigate

mechanisms associated with the spread of this disease from one host to another. The presence of infectious prions has been demonstrated in tissues, bodily fluids, and excreta of CWD-positive animals, irrespective of clinical disease [4-11, 18]. These studies provide vast insight into the highly infectious nature of CWD. We extend these studies with our current work, which aims to explore an additional means by which infectious prions may be acquired: maternal transmission.

Maternal transmission (i.e., transmission from mother to offspring) has most notably been demonstrated in sheep scrapie, as infectious prions have been detected in maternal and fetal tissues harvested from scrapie-positive ewes, and are in sufficient quantity to infect and cause progressive clinical TSE disease in lambs born to infected ewes [19, 20]. Additional support for maternal transmission of prions has been documented in a cub born to a feline spongiform encephalopathy-infected cheetah queen. The cub suckled on the mother (euthanized due to clinical TSE disease), and was subsequently held in a prion-free environment and fed a TSE-free diet. At the age of 7, the cub presented with clinical signs consistent with TSE infection and was found to be FSE positive by histopathology upon termination. The cub's only known exposure to the infectious agent was that associated with her mother pre- and post-parturition [21]. Studies conducted using conventional assays with less sensitivity than current *in vitro* detection methodologies did not detect PrP^{res} in maternal or fetal tissues harvested from Bovine spongiform encephalopathy (BSE) infected cattle. Yet, epidemiological findings indicate a correlation between BSE-infected cow: calf pairs [22-24]. A more recent study performed in bovidized transgenic mice (BoTg) suggests that vertical transmission of BSE does occur in the transgenic model when females are bred at clinical stages in BSE infection. However, IC-inoculations of BoTg mice failed to show infectivity in milk collected from the BoTg mice [25]. In addition, we have recently reported that CWD maternal transmission can occur in experimentally-inoculated Reeves muntjac deer [12] and in free-ranging elk naturally-exposed to CWD [13].

The studies undertaken for this thesis were conducted to establish and characterize maternal transmission of CWD in a widely used transgenic mouse strain, Tg(CerPrP-E226)[14, 26]. This work will further our understanding of transmission of CWD in a model with a hemochorial placenta, and by extension, maternal transmission for other prion diseases.

Materials and Methods

Study design

Transgenic mice (TgCer(PrP-E226)) were intracranially inoculated with known CWD positive material, and bred at various timepoints in infection. These dams were sacrificed at onset of severe terminal disease, characterized by hind limb ataxia, extreme hypersensitivity, inability to reach food or water, and a loss in body mass greater than 20 percent. Their tissues were subsequently collected for PrP^{res} analysis. Their offspring were monitored for 500 days or until onset of terminal clinical disease, followed by tissue analysis for CWD-prion deposition. (Figure 2.1.)

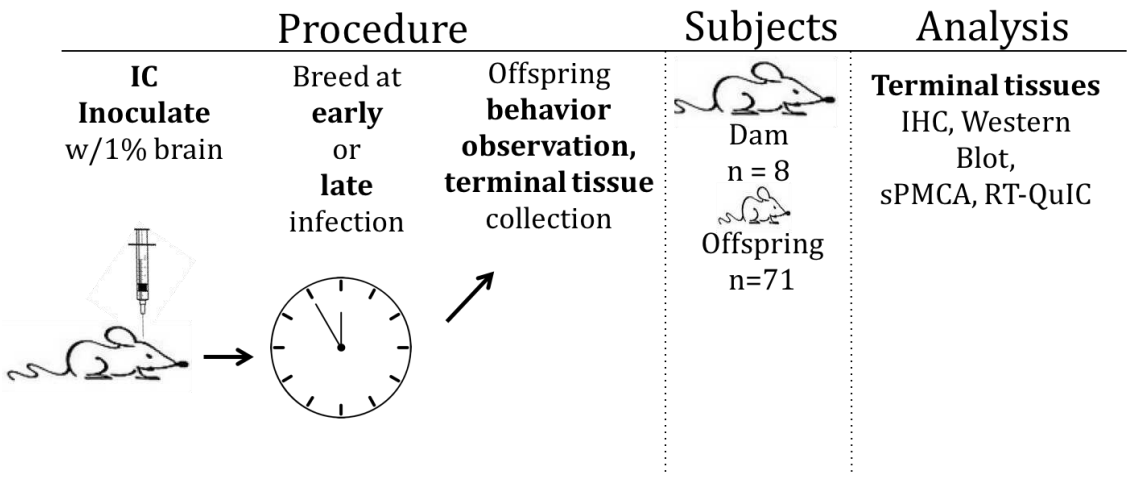

Figure 2.1. Experimental design.

Source of Tg(CerPrP-E226) 5037$^{+/-}$

Tg(CerPrP-E226) mice, developed by our long time collaborator Dr. Glenn Telling [26], were made to express the elk PrP coding sequence via site directed mutagenesis at codon 226 of the deer PrP gene (*PRNP*) via a Q to E mutation. This coding sequence was inserted into a MoPrP.Xho expression vector, followed by microinjection of the transgene into pronuclei of fertilized PrP knockout oocytes. PCR screening of genomic DNA identified three Tg(CerPrP-E226) founders: Tg5029, Tg5034, and Tg5037. Each was mated to PrP knockout mice to produce hemizygous transgenic lines. PrP expression levels were estimated using immuno-dot blotting and western blotting using the monoclonal antibody (MAb) 6H4. [14, 26]

Genotypic confirmation of elk PRNP insert

DNA was isolated from tail tips collected at weaning (Qiagen), and amplified using Polymerase Chain Reaction with the primer pair 158F TCATGGTGAAAAGCCACATAGG and 159R CATCCTCCTCCAGGTTTTGG [12].

Tg(CerPrP-E226) mouse breeding scheme:

CWD positive IC-inoculated female mice (n=3/cohort) and CWD negative IC-inoculated female mice (n=3/cohort) were bred at specific timepoints (45 and 120 days post inoculation (dpi)) by exposure to intact naïve Tg(CerPrP-E226) male mice to assess the association of clinical disease status in maternal transmission of CWD. (Figure 2.1.)

Tg(CerPrP-E226) mouse offspring cohorts:

Offspring born to the above females were split into cohorts based on their time of conception post inoculation: Cohorts 1 and 2 include offspring born to CWD-positive and/or sham-inoculated females; Cohort 1 conceived approximately 45 dpi, Cohort 2 conceived after 120 dpi. (Table 2.1.)

Table 2.1. Cohort placement and their respective breeding data.

Cohort	# of Tg(CerPrP-E226) dams	# live offspring	Average dpi at breeding	# non-viable offspring
1 (Sham-inoculated)	3	16	56.3	1
1 (CWD-inoculated)	3	16	45.6	1
2 (Sham inoculated)	3	20	126	0
2 (CWD-inoculated)	3	19	121.3	0

Inoculation

Tg(CerPrP-E226) adult female mice were inoculated via the intracranial (IC) route under isofluorane anesthesia with a 30 ul of 1% brain homogenate in 1X phosphate buffered saline (ThermoFisher Scientific) and a 2% penicillin/streptomycin (Gibco) antibiotic mixture. The source of inoculum was brain material collected from previously inoculated muntjac deer that were confirmed positive (MJ 11/15) or negative (MJ 62/64) for CWD prions by western blot.

Breeding practices

Two to three Tg(CerPrP-E226) females were housed together without the presence of males for several weeks prior to breeding in order to stimulate the influence of pheromones and social factors on the estrous cycle. By doing this, the diestrus cycle is prolonged and estrus is suppressed, a phenomenon known as the Whitten effect [27]. Two females were placed with one intact male to restart the estrus cycle at the specific breeding timepoint associated with their cohort. Intact males remained with the females during pregnancy and parturition until weaning of the mice at 21 days post birth.

Observation for clinical TSE signs in offspring

Offspring born to CWD-infected and naïve Tg(CerPrP-E226) mice were weaned at 21 days post parturition and were monitored for 500 days. Each mouse was weighed weekly until it was

determined that they were no longer actively gaining weight; at this point the mice were monitored weekly for the onset of TSE clinical disease and weighed monthly. Video recording was collected on a bi-weekly basis to compare behavior among cohorts.

Euthanasia/tissue collection

Animals were sacrificed upon terminal clinical disease, characterized by a 20% weight loss, or inability to reach water or food due to hind limb ataxia or 500 dpi. Carbon dioxide inhalation was used for euthanasia procedures at a flow rate of 1.3L/min (IACUC Protocol #14-4890A.) Immediately upon euthanasia, blood samples were collected from the dam using cardiac puncture. Brain, salivary gland, tongue, heart, spleen, gastrointestinal tract, muscle, kidneys, adrenal glands, mammary, and reproductive tissues (uterine horns, ovaries, and vagina) were collected from dams; each tissue was divided, half for frozen storage and half for fixation in a periodate-lysine-formaldehyde solution. Fixed tissues were moved to 60% ethanol after 48 hrs.

Analysis of tissues for CWD prions

A battery of tissues including brain, tongue, salivary gland, heart, lung, liver, spleen, kidney, adrenal gland, gastrointestinal tract, bladder, ovary, uterus, vagina, and muscle were collected from offspring upon their sacrifice at >500 days post birth. Tissues analyzed for PrP^{res} deposition and/or amplification competent prions include brain and spleen by amplification methods described previously (PMCA, RT-QuIC.)

Western blot

Brain homogenates were made at a 10% (w/v) concentration in 1X phosphate-buffered saline (ThermoFisher Scientific) using a bead ruptor (Omni). Homogenates were mixed with proteinase K (PK) (Invitrogen) at a final concentration of 1 mg/ml and incubated at 37°C for 30 minutes, followed by 10 minutes at 45°C in a shaking thermomixer (Eppendorf). Samples were mixed with Reducing Agent (10X)/LDS Sample Buffer (4X) (Invitrogen) at a final concentration of

1X, heated to 95°C for 3 minutes, then run through a NuPAGE 10% Bis-Tris gel at 135 volts for 1 hour. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane with a Trans-Blot Turbo™ blotting system for 7 minutes. The membrane was loaded into a pre-wetted SNAP i.d. blot holder (Millipore) then placed in the SNAP i.d. system (Millipore). Blocking buffer (Blocker Casein in TBS [Thermo Scientific] with 0.1% Tween 20) was added to the blot holder for 3 minutes followed by vacuum removal. Primary antibody BAR224 (Cayman Chemical) conjugated to horseradish peroxidase (HRP) was diluted in blocking buffer to 0.2 ug/ml and added to the blot holder for a 12-minute incubation. The antibody was pulled through the membrane via vacuum and was followed by three washes with 30 ml volumes of wash buffer (50% Blocker Casein in TBS, 50% 1X Tris-buffered saline (TBS), 0.1% Tween 20 (Sigma) each). Proteins were visualized by using ECL Plus™ (GE Healthcare) and a digital Gel-Doc™ capturing chemiluminescent signals using ImageGauge™ software.

Immunohistochemistry

Tissues were stored in a Periodate-Lysine-Paraformaldehyde (PLP) solution for a minimum of 48 hours, and switched to 60% ethanol prior to paraffin embedding. Paraffin-embedded tissues were cut into 6um sections and transferred to positively charged glass slides (Unifrost.) Slides were incubated at 37°C for one hour to remove paraffin which was followed by graded alcohol-rehydration and a 40-minute immersion in 88% formic acid to digest cellular PrP. The slides were then subjected to heat-induced epitope retrieval in a 2100-Retriever (Prestige Medical) in sodium citrate buffer (0.01 M sodium citrate, 0.05% Tween 20, pH 6.0) followed by incubation with primary antibody BAR224 (Cayman Chemical) at 1 mg/ml* and Envision+™ anti-mouse HRP labeled polymer (Dako) and rinsed twice with TNT (0.1M Tris (pH 7.5) + 0.15M NaCl + 0.05% Tween-20). Slides were incubated with AEC (3-Amino-9-Ethylcarbazole)Substrate-Chromagen (Dako), counterstained with hematoxylin and bluing reagent (0.1% sodium bicarbonate), and coverslipped with an aqueous mounting medium (Vector Laboratories) [28].

*In cases where detection of PrP^{res} was not achieved using the protocol above, a Tyramide Signal Amplification (TSA) kit (Fisher Scientific) was used to amplify any signal potentially missed by the conventional IHC methodology. This differs from the standard protocol in that a 30-minute PK-digestion at 1 mg/mL in a CaCl/Tris buffer was added following rehydration of the slides in combination with a shorter (5 min) 88% formic acid treatment. Following addition of anti-mouse-HRP (secondary antibody) as described above, all slides were treated with a Dinitrophenyl (DNP) Amplification Reagent (stock solution diluted 1:50 in amplification diluent), washed twice in TNT buffer, and incubated for 30 minutes in anti-DNP-HRP diluted 1:100 in TNB. These additional steps are then followed by incubation with AEC and counterstained with hematoxylin and bluing reagent as described above.

Preparation of normal brain homogenate (NBH)

An 8.5% normal brain homogenate (NBH) in 0.1 M PBS buffer (pH 7.5, with 1 % Triton X-100) was prepared from whole brains collected from naïve Tg(CerPrP-E226) mice no older than 4 months of age. These homogenates were then frozen at -80C, serving as a substrate for subsequent protein misfolding cyclic amplification (PMCA) experiments.

Serial protein misfolding cyclic amplification (sPMCA)

The initial round of sPMCA incorporated 30 ul of 10% tissue homogenate sample plus 50 ul of 8.5% w/v normal Tg(CerPrP-E226) brain homogenate (NBH) placed in individual 0.6ml thin-walled PCR tubes (USA Scientific) containing two 2.38 mm and three 3.15 mm Teflon beads (McMaster-Carr). The PCR tubes were sealed with parafilm, vortexed for 6 seconds and subjected to one round of PMCA. Each round of PMCA is equal to 288 cycles of sonication (Misonix) (10 seconds separated by 5 minute incubations) at 37°C over 24 hours. Seven total rounds of sPMCA were completed in duplicate per tissue by transferring 30 ul from the previous round into 50 ul fresh NBH (1:1.6 dilution). Standard CWD positive and negative assay controls, consisted of 10% homogenates made from brain harvested from Tg(CerPrP-E226) mice IC-inoculated with CWD-

positive or negative muntjac brain. These samples were incorporated into the first run at a different ratio than samples, 2 ul sample to 98 ul NBH, and subsequently treated like a sample in the following runs. The seventh round of sPMCA was analyzed by conventional PK digestion and western blot analysis as above.

Sodium phosphotungstic acid precipitation (NaPTA)

10 ul of a 10% (w/v) tissue homogenates were added to 90 ul of 0.1%SDS/1X PBS buffer, followed by a 7 ul addition of a NaPTA (Sodium phosphotungstic acid + $MgCl_2$ 6-hydrate) solution [29]. This mixture was incubated while shaking for 1 hour at 37°C in a thermomixer (Eppendorf), and spun at 14,000 x rpm for 30 minutes in a Beckman centrifuge at room temperature. The resultant pellet was diluted into 10 ul of 0.1%SDS/1X PBS buffer prior to RT-QuIC analysis.

Ethanol precipitation of brain homogenate for analysis by RT-QuIC

A 10% (w/v) brain homogenate was added to 90 ul 100% ethanol, followed by a 5-minute incubation at room temperature and centrifugation at 14,000 x rpm for 5 minutes to form a pellet. The resulting supernatant was removed, and the pellet diluted into 100ul 1X PBS (ThermoFisher Scientific). This mixture was then centrifuged at 14,000 x rpm for 5 minutes to form a pellet, and resuspended in 100 ul 0.1% SDS/1X PBS. 2 ul of this material was added to each well of a 96 well plate prior to fluorescent analysis (Hoover C et al. 2016, manuscript in preparation).

Real-time quaking induced conversion assay (RT-QuIC)

Recombinant truncated Syrian hamster prion protein (rPrP) containing amino acid sequences 90-231 was prepared as previously described [9]. RT-QuIC premixed reaction buffer containing 20 mM $NaPO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 320 mM sodium chloride (NaCl) was mixed with the 0.1 mg/ml rPRP, and 10 μM thioflavin T (Sigma). 95 ul of this mixture was added to each well of a 96-well plate. Plates were shaken in a microplate fluorometer (BMG) for 1 min (700 rpm, double orbital) followed by 1 min of rest for a total of 250 shake:rest cycles.

Fluorescence (450-nm excitation and 480-nm emission, 20 flashes/well) was recorded every 15 min using a gain of 1,700.

Rate calculations in RT-QuIC

In order to determine the rate at which a given sample is able to convert rPrP, the threshold in which a sample is deemed positive is calculated by the following equation:

Threshold = average baseline fluorescence + 5 X the standard deviation of baseline fluorescence

The rate of conversion is defined by *1/time* at which a positive sample crosses this threshold. This was calculated for each sample and compared to negative tissue-matched controls, and statistically analyzed using the Mann-Whitney test.

Results

CWD-positive Tg(CerPrP-E226) females give birth to healthy offspring that are comparable in litter size and mortality to their CWD-negative cohorts.

Although mouse breeding habits can be highly variable depending on genetic background and the age of the breeders [30], we found that the Tg(CerPrP-E226) females did not exhibit abnormal breeding behavior. That is, we did not see a decline in successful breeding, litter size, or an appreciable increase in fetal mortality at time of birth (Table 2.1.)

PrP^{res} is readily identified in hippocampus and cerebellum of CWD-positive Tg(CerPrP-E226) females.

To confirm successful inoculation and CWD-positive status in the dams, we assayed brain from each IC-inoculated female via several methods: western blot, RT-QuIC, and IHC. Using anti-PrP antibody (BAR-224) to locate PrP^{res} in this area, we determined that the majority of PrP^{res} deposition was located in the hippocampus and cerebellum (Figure 2.2A-F.) We confirmed the presence of CWD prions using western blot analysis and RT-QuIC (Figure 2.2G, 2H.)

Conversion competent prions are present in the brain of offspring; detected by highly sensitive amyloid seeding assays.

It has been shown that the concentration of PrP^{c} is greatest in neuronal tissue [31] therefore we hypothesized that the most likely site of PrP^{res} conversion and detection would be in the brain of these offspring. Initial evaluation of these tissues by IHC revealed a lack of PrP^{res} (Figure 2.3C, 3D.) We thus concluded that prions might be below the limit of detection by conventional assays and further analyzed tissues by ultrasensitive amplification methods. Ultrasensitive amplification methodologies (PMCA or RT-QuIC), where tissue harvested from prion-exposed hosts is used as a seed to amplify conversion competent substrate (mammalian or recombinant PrP^{C}, respectively) results in an increase in amyloid formation, permitting the detection of very low levels of amyloid or prions in the test sample. In our study we detected PMCA amyloid conversion in 1/16 brains collected from offspring born to dams during early CWD infection, and in 1/16 brains harvested from offspring born to dams during late CWD infection (Figure 2.3E.) RT-QuIC revealed amyloid formation in the brains of 2/16 offspring born to dams during early CWD-infection, and in 8/16 offspring born to mothers exhibiting late-stage clinical CWD. (Figure 2.3F, 2.3G.)

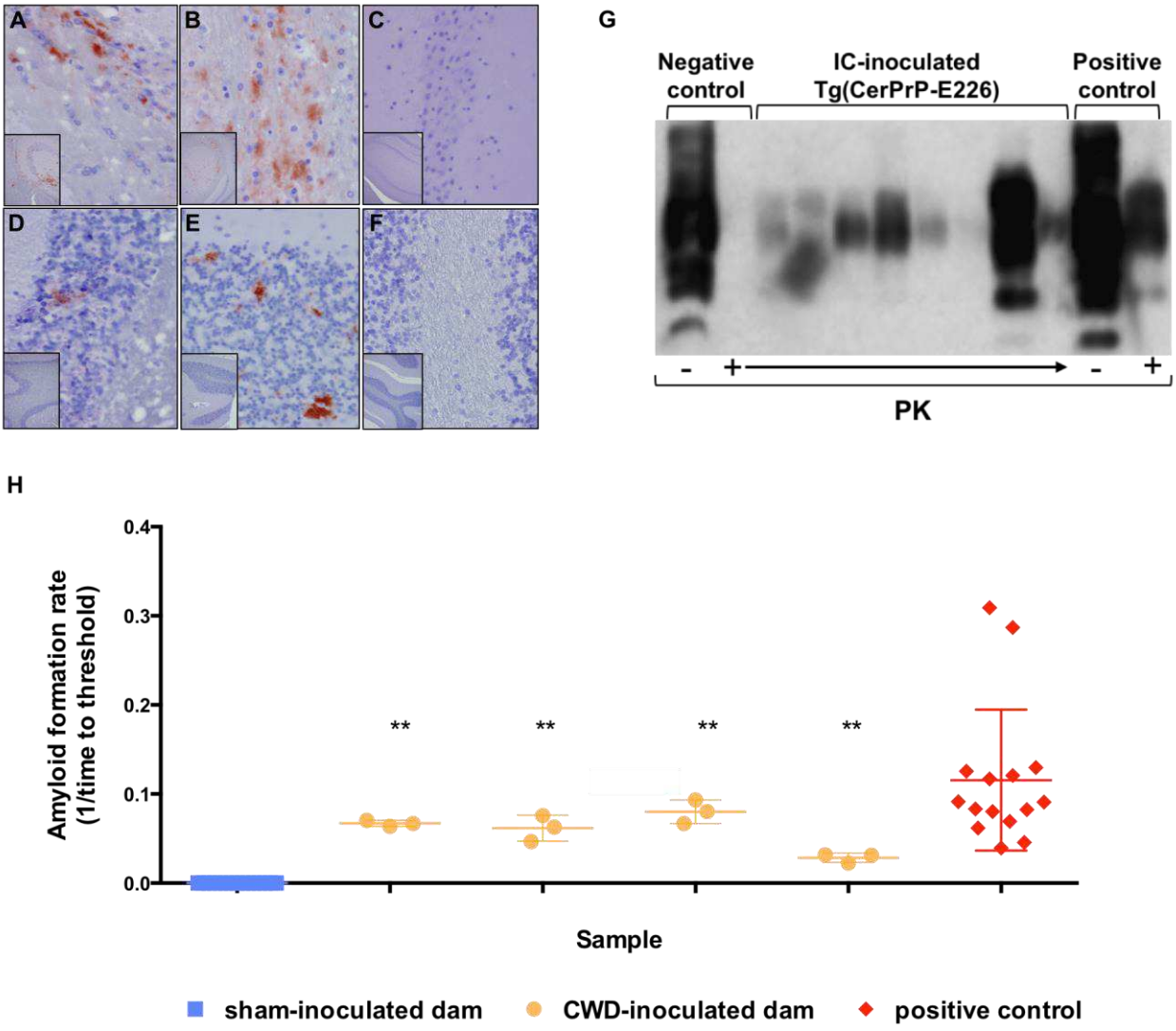

Figure 2.2. Confirmation of amyloid conversion activity (CWD prions) in brains of IC-inoculated Tg(CerPrP-E226) dams.

A-F) IHC staining with anti-PrP antibody BAR-224. Picture objectives are 40X with insets at 10X. A-C) Hippocampus. A) Positive control. B) CWD-inoculated dam at >120dpi. C) Negative control. D-F) Cerebellum. D) Positive control. E) CWD-inoculated dam at >120dpi. F) Negative control. G) Western blot analysis of 10% brain homogenates from CWD-positive dams following PK digestion at 1 mg/ml. H) RT-QuIC rate analysis of 0.01% brain homogenate. Each group represents tissue collected from one individual. P values: **** $\geq$ 0.0001, *** $\geq$ 0.001 ** $\geq$ 0.01 * $\geq$0.05.

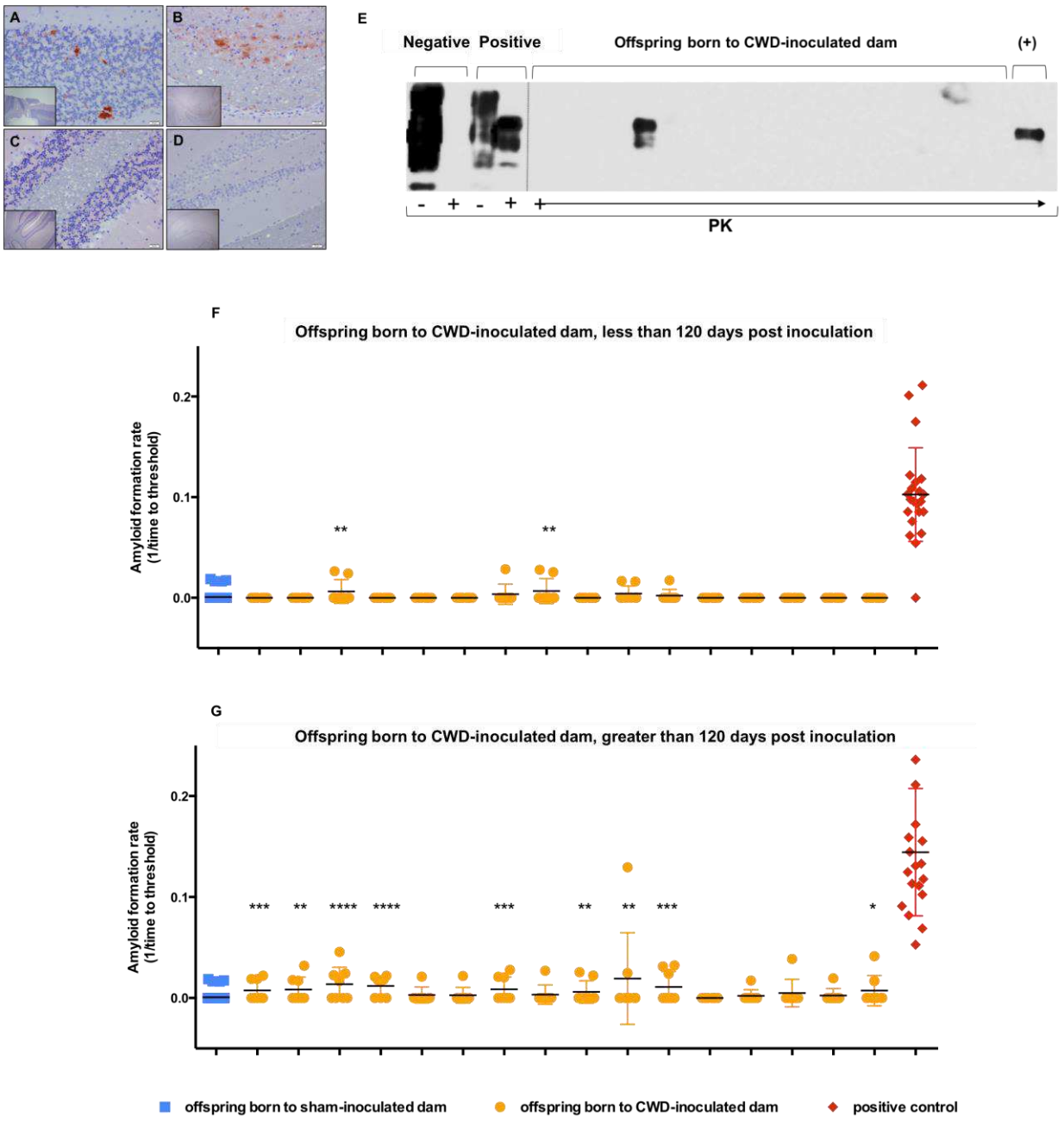

Figure 2.3. Detection of amyloid conversion activity (CWD-prions) in brains of offspring.

A-D) IHC staining does not detect CWD-prions. Picture objectives are 40X (scale bar = 20µm) with insets at 4X. A) CWD-positive control, cerebellum. B) CWD-positive control, hippocampus. C) Offspring born to CWD-positive female bred at >120 dpi, cerebellum. D) Offspring born to CWD-positive female bred at >120 dpi, hippocampus. E) Western blot of offspring brain following 7 rounds of amplification by PMCA. (+) indicates known positive, PMCA amplified brain material. F) RT-QuIC analysis of early offspring brain following etoh precipitation. G) RT-QuIC analysis of late offspring brain following etoh precipitation.
P values: **** $\underline{\geq}$ 0.0001, *** $\underline{\geq}$ 0.001 ** $\underline{\geq}$ 0.01 * $\underline{\geq}$0.05.

Offspring born to early and late stage CWD-infected mothers harbor prions in the spleen

The lack of clinical disease in offspring born to CWD-infected dams prompted further exploration for prions in peripheral and lymphoid tissues. In particular, previous studies have demonstrated the presence of PrP^{res} in spleen [32, 33] tissue of asymptomatic carriers. Here we report that RT-QuIC analysis revealed the presence of conversion competent prions in spleen tissue of 2/14 offspring born during early CWD-infection and 3/11 offspring born during late CWD infection (Figure 2.4.)

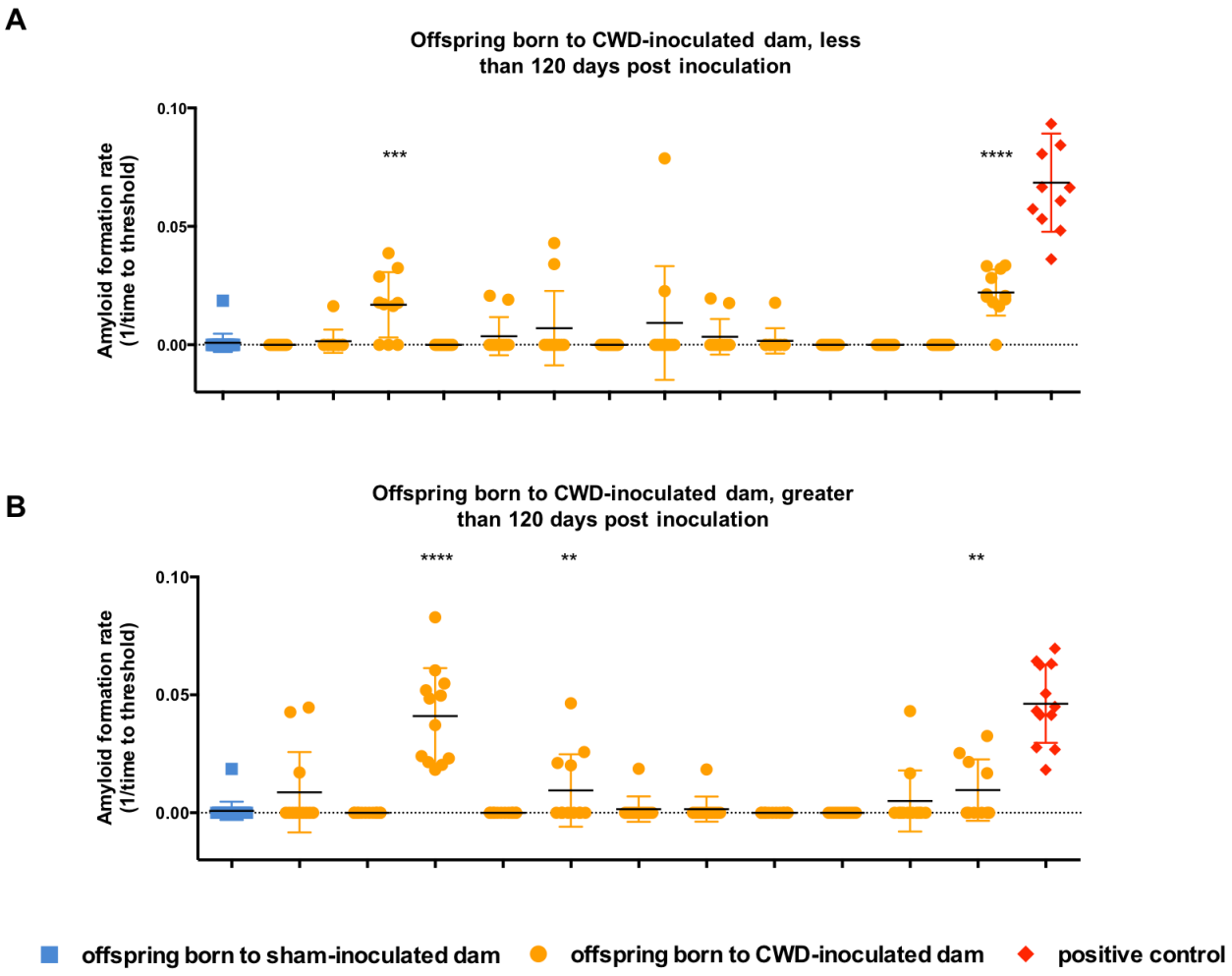

Figure 2.4. Detection of conversion competent amyloid (CWD-prions) in spleens of offspring.

A) RT-QuIC analysis of spleens collected from offspring born to dams bred >45 dpi, following NaPTA precipitation of 1% homogenate. Each group represents tissue collected from one individual. B) RT-QuIC analysis of spleens collected from offspring born to dams bred >120 dpi, following NaPTA precipitation of 1% homogenate. Each group represents tissue collected from one individual. P values: **** $\leq$ 0.0001, *** $\leq$ 0.001 ** $\leq$ 0.01 * $\leq$0.05.

Discussion

Chronic wasting disease is undoubtedly one of the most difficult prion diseases to control, with transmission dynamics including direct dissemination from animal-to-animal, as well as indirect transfer via contaminated environments. Prior to this study our laboratory demonstrated CWD maternal/vertical transmission in muntjac deer and a free-range elk population with epitheliochorial placentation. Here we sought to establish an experimental model to further studies to assess the efficiency of prion maternal transmission via hemochorial placentation.

The initial part of this study entailed observing offspring born to dams at different timepoints of CWD-infection; breeding the females at 45 dpi to serve as a pre-clinical state, and greater than 120 dpi as a timepoint in which the dams begin to display clinical signs, with potential to reach near-terminal disease around the time of weaning. Our original hypothesis was that we would see similar characteristics within the mother: offspring pairs as those that have been observed in the cervid host. For example, we previously reported that the incidence of morbidity in offspring born to CWD-positive muntjac does was increased by 60% when compared to CWD-negative animals [12]. Conversely, in the Tg(CerPrP-E226) model, nonviability was not an issue—1 nonviable pup was born to a CWD positive dam and 1 nonviable pup was born to the negative control counterpart dam.

From previous studies, we hypothesized that we would observe clinical disease associated with CWD in the offspring born to CWD-positive females, particularly those born at the later timepoints, as prion load increases throughout infection [9]. In these studies, offspring did not show consistent signs of clinical TSE infection, regardless of the prion status of their mother at the time of breeding. Offspring born to early and late stage CWD-infected dams survived well past the time-period in which a CWD-positive individual (by some form of inoculation) would succumb to disease**,** leading us to question whether we would find prions within their tissues.

The current "gold standard" for detection of prions within cervid populations is IHC of post-mortem collected brain tissue. Our inability to detect PrP^{res} in brain tissue of offspring by this method indicated that the prion load in this tissue was below the limit of detection by conventional means. Thereafter, we chose to pursue prion detection in tissues via amplification methodologies, including PMCA and RT-QuIC.

Subsequent analysis by PMCA revealed that amyloid seeding activity was present in brain tissue of 1/16 offspring from each cohort born to CWD-positive females, indicating that there was no difference in prion distribution across litters born at early *or* late timepoints in infection. This was a surprising result, given our previous hypotheses correlating prion load in the dam with the propensity for maternal transmission. However, in attempts to confirm our PMCA result in a more recently developed amyloid-seeding assay, RT-QuIC, we found that there *was* an appreciable difference between cohorts. We determined through statistical analysis of RT-QuIC reactions that 2/16 mice born to mothers during preclinical stages of CWD-infection had significant amounts of conversion-competent prions in their brains, compared to 8/16 offspring born to late stage CWD-infected mothers. From this work we are able to make two conclusions: 1) Stage of CWD infection in Tg(CerPrP-E226) dams *does* affect the propensity of prions to be maternally transmitted and 2) RT-QuIC is more sensitive than PMCA for the detection of trace amounts of prions associated with asymptomatic carriers.

The low levels of prions within the brain of these offspring led us to question if prions might be replicating within other tissues prior to neural-invasion. It is well established that subclinical carriers of prion disease often demonstrate detectable levels of PrP^{res} within lymphatic tissues, including spleen [32, 33]. Taking this into account, we assayed spleen tissue collected from the offspring by RT-QuIC. We detected amyloid seeding activity in a small number of spleens collected from each cohort; 2/14 from offspring born to preclinical mothers, and 3/11 spleens from offspring born to clinical mothers.

The noticeable trend within this data is that prions *are* transmitted from mother to offspring in this model, resulting in very low levels of detectable prions demonstrated in brain and splenic tissue of the offspring. Amplification techniques, as well as methods employed to enhance detection/remove potential inhibitors, i.e., NaPTA and ethanol precipitation were necessary for prion detection. Furthermore, offspring born to CWD-infected mothers did not display clinical signs associated with TSE disease. This leads us to address several important questions with regard to prion detection and more importantly, transmission.

Are current techniques used to assess prion-status in natural infection sufficient to identify prion-positive animals? The answer is clearly no: Our recently published work demonstrates that the incidence of CWD in elk localized to Rocky Mountain National Park is comparably higher when the animals are tested by PMCA (79%) vs. IHC (12.9%) [13]. Had we analyzed offspring tissues in the current study using only the methods that are used in the field today, we would have overlooked prion deposition entirely. This being the case, how many prion-exposed animals in native habitats that display no indication of clinical TSE disease are asymptomatic carriers that harbor amyloid-seeding material? Given a longer period of time would these animals begin to show clinical signs, or would they continue to live their lives with no observable indication of their prion status? And finally, and arguably most importantly, what are the implications behind this "silent-carrier" status? In other words, is there potential for these animals to shed prions into the environment unknowingly, and are these shed prions infectious?

These studies lead us to examine other TSEs in which there is potential for maternal transmission that has yet to be characterized, and/or for those in which clinical TSE signs are not readily apparent. For instance, a report published in 2009 by the National Creutzfeldt-Jakob disease Surveillance Unit stated "there have been 125 children born to parents diagnosed with variant Creutzfeldt-Jakob disease, and nine of these children were born to mothers who were symptomatic at conception, birth, or within a year of clinical onset" [34]. These children (now aged

10-52 years old) have not, to the knowledge of the authors, progressed to clinical vCJD. The extended incubation period of prion diseases, including vCJD, can last decades or even the entire life span of an infected host. It is therefore not possible to diagnose all prion disease based on presentation of symptoms. In fact, according the findings of this current study, the children born to CJD-infected mothers may never display clinical disease. The question remains, do they harbor infectious prions?

In conclusion, we have demonstrated that maternal transmission occurs, albeit at low levels, in the Tg(CerPrP-E266) mouse model. Identification of PrP^{res} in these tissues required a diligent search aided by amplification methods that are not currently used in the diagnoses of individuals naturally exposed to prions. The mechanism(s) by which prions are passaged from mother to offspring remains unknown, although it appears that prion load and placental structure are variables that affect maternal transmission efficiency of CWD. Finally, we have observed that offspring born to CWD-infected dams, while lacking the development of overt TSE clinical disease, harbor amyloid-conversion competent prions. This data further supports the presence of a silent carrier population for all prion diseases, potentially including those that affect humans.

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CHAPTER THREE:
PLACENTAL STRUCTURE PLAYS A KEY ROLE IN THE EFFICACY AND OUTCOME OF MATERNAL TRANSMISSION OF CHRONIC WASTING DISEASE

Introduction

Transmissible spongiform encephalopathies or prion diseases, have been characterized as uniformly fatal in nature, to which no cure exists. Thus understanding transmission of these diseases in hopes of prevention is a widespread goal of many prion researchers. Previous research has characterized numerous sources of infectious prions, as well as several potential mechanisms by which these sources may cause disease. In chapter two of this work, we identified the occurrence of mother to offspring transmission of chronic wasting disease within a transgenic mouse system that expresses the cervid prion protein Tg(CerPrP-E226). In this study, less than half of offspring born to CWD-positive mothers harbored amplification-competent prions in brain and spleen tissues, yet did not display clinical signs of disease. This differs from what is observed in maternal transmission of CWD in a cervid host, such as increased morbidity, and clinical signs eventually leading to terminal CWD disease in Muntjac deer offspring. The intent of this current study is to continue studies of CWD transmission in an animal with a hemochorial placental structure (as opposed to the epitheliochorial placentation of cervids) to further our understanding of the mechanism(s) associated with covert transmission of prions from mother to offspring. More specifically, we aim to assess potential routes of maternal transmission in the Tg(CerPrP-E226) model. We hypothesize that there are two routes by which maternal transmission of prions may occur: *in utero* before birth, or post birth during nursing, should prions be present in the milk of infected dams.

Tg(CerPrP-E226) females were inoculated with known CWD-positive material and bred with CWD-naïve males at various timepoints post inoculation. We examined: 1) CWD prion transmission via milk by collecting milk during the post-parturition suckling period (i.e., up to 20

days post birth) for PrP^{res} analysis and 2) *in utero* prion transmission by harvesting gestational reproductive and fetal tissues from mother: offspring pairs for the evaluation of CWD prions. In this study, we have identified prion deposition and amyloid seeding activity in several tissues collected from Tg(CerPrP-E226) mouse mothers, including brain, reproductive, mammary, and spleen. Although we identified amyloid conversion in tissues collected *in utero*, which include placenta, fetus, and fetal sac, seeding activity was not higher than negative control conversion in RT-QuIC in the majority of these samples. This suggests low levels of prions in these tissues. We were unable to detect prions in milk collected from CWD-positive transgenic dams, leading us to hypothesize that the route of TSE transmission to offspring is likely a combination of horizontal or environmental exposure, and/or very low concentrations of prions breaching the feto-maternal interface.

Background

Prion diseases, or transmissible spongiform encephalopathies (TSEs) are fatal neurodegenerative disorders that are caused by conversion of the normal cellular form of the prion protein (PrP^{c}) into a misfolded confirmer (PrP^{res}) that is prone to aggregation, resulting in neuronal death. TSEs can affect several species including sheep and goats (scrapie), domestic and nondomestic cats (feline spongiform encephalopathy), mink (transmissible mink encephalopathy), cattle (bovine spongiform encephalopathy), cervids (chronic wasting disease) and humans (Creutzfeldt-Jacob disease and Kuru) [1]. The prion disease of cervids, chronic wasting disease (CWD), was identified in a captive mule deer population in 1967 in Fort Collins, Colorado and was subsequently reported as a TSE in 1980 [2]. This disease is characterized by weight loss, polydipsia, polyphagia and gait impairment [2-4], and since its discovery, has been recognized in 24 states, 2 Canadian provinces, and the republic of Korea [5].

In fact, CWD is unique compared to other TSEs in its ability to rapidly transmit from host to host, which has led to increased interest in elucidating routes of transmission. Current knowledge

of CWD-prion transmission includes the prions deposition in tissues, bodily fluids, and excreta of CWD-positive animals, irrespective of clinical disease [6-14], which are sufficient to spread the infectious protein from one host to another. In this study, we aim to understand the mechanisms behind maternal transmission—assessing the likelihood of *in utero* transmission via a hemochorial placenta structure, and/or post parturition via prion-contaminated milk.

Current knowledge of maternal transmission falls within two extremes depending on the TSE: 1) This route has been established, and thought to impact the number of prion-positive individuals in a population, or 2) relatively little is known. The majority of what is known about maternal transmission of prion diseases has been characterized in sheep scrapie, the oldest known TSE. There is ample evidence of both pre- and postnatal transmission, especially given the lamb's genetic susceptibility to scrapie [15-22]. Studies dating back as far as the seventies have shown that prions are in sufficient quantity within the placenta to infect and cause progressive clinical TSE disease in lambs [23]. More recently, Timm Konold and colleagues published a study demonstrating infectivity in milk collected from scrapie-positive ewes, followed by several studies that reported vertical transmission of infectious prions in milk prior to clinical symptoms in the ewe[19-22]. These studies also determined that milk is most infectious when ewes also have lentiviral mastitis, although this is not a requirement for successful transmission [21].

More recently, maternal transmission of feline spongiform encephalopathy (FSE) has been implicated in dissemination of FSE-prions. A cheetah cub was born to an FSE-positive cheetah queen, who was euthanized five weeks later due to terminal TSE disease. This study reported that the newborn cub was allowed to nurse on the mother until her euthanasia, and was subsequently held in a prion-free environment and fed a TSE-free diet. The cub's only known exposure to the infectious agent was that associated with her mother pre and post parturition. 7 years later the cub began to develop clinical signs consistent with TSE infection and was found to be FSE positive by postmortem histopathology [24].

Epidemiological findings have indicated a correlation between bovine spongiform encephalopathy (BSE) infected cow: calf pairs, although the assays used at the time did not detect PrP^{res} in maternal or fetal tissue harvested from BSE-positive cows [25-27]. However, it is important to note that these studies were conducted in the 80's with conventional assays that have been shown to have less sensitivity than current in-vitro methodologies that are used today [28, 29]. A more recent study performed in bovidized transgenic mice suggests that vertical transmission of BSE does occur when females are bred at late-timepoints in BSE infection. However, IC-inoculations of milk into BoTg mice failed to show infectivity [30].

Our laboratory has recently reported maternal transmission in experimentally-inoculated Reeves muntjac deer [31] and in free-ranging elk that are naturally-exposed to CWD [32]. In addition, we reported in Chapter two of this thesis that maternal transmission of CWD occurs in low levels using cervidized transgenic mice Tg (CerPrP-E226) [33] as an experimental model. The aim of this work is to further characterize the role of maternal transmission in a transgenic host with a hemochorial placental structure, by analyzing tissues collected *in utero* at different timepoints in CWD-infection and gestation, as well as milk collected from CWD-positive dams.

Materials and Methods

Study design

Transgenic females (TgCer(PrP-E226)) were intracranially inoculated with known CWD positive material, and bred to CWD-naïve males at various timepoints in infection. Depending upon their respective cohorts, dams were sacrificed at different timepoints in gestation (early [post E7] or late [post E14]) and infection (early [45 dpi] or late [120 dpi]), or were repeatedly bred up to terminal clinical disease to facilitate serial milk collections ranging across various clinical stages of TSE disease. In both scenarios, terminal tissues, including brain, spleen, reproductive (vagina, uterus, and ovaries) and mammary tissue were collected at necropsy for analysis by ultrasensitive amplification/conversion assays (PMCA/RT-QuIC). (Figure 3.1.)

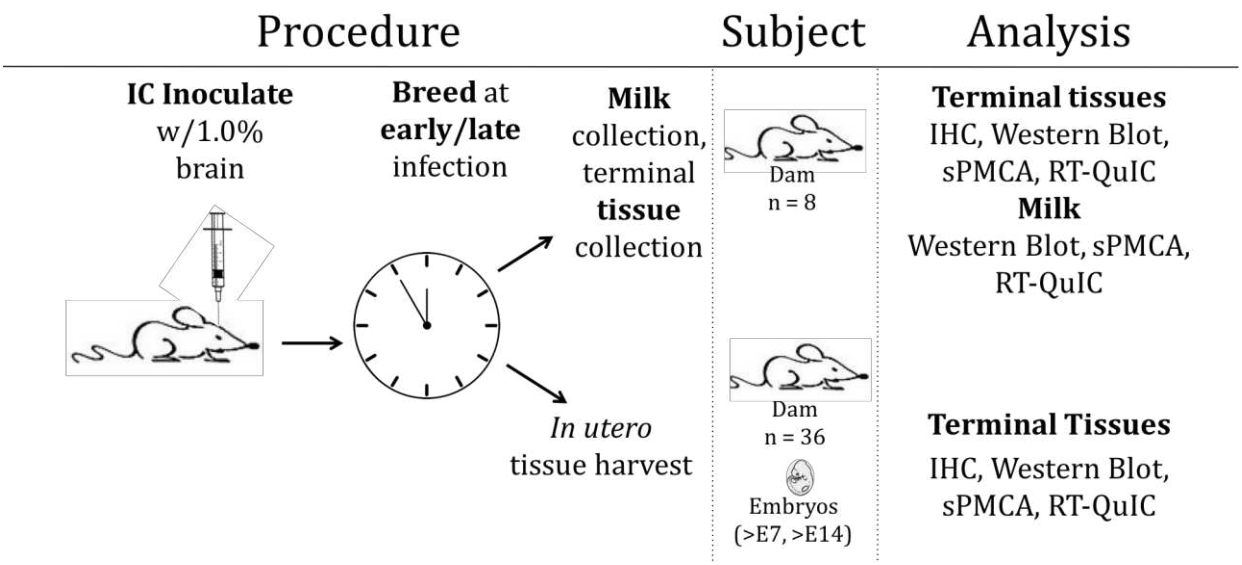

Figure 3.1 Experimental design.

Source of Tg(CerPrP-E226) 5037$^{+/-}$

Tg(CerPrP-E226) mice, developed by our long time collaborator Dr. Glenn Telling [34], were made to express the elk PrP coding sequence via site directed mutagenesis at codon 226 of the deer PrP gene (*PRNP*) via a Q to E mutation. This coding sequence was inserted into a MoPrP.Xho expression vector, followed by microinjection of the transgene into pronuclei of fertilized PrP knockout oocytes. PCR screening of genomic DNA identified three Tg(CerPrP-E226) founders: Tg5029, Tg5034, and Tg5037. Each was mated to PrP knockout mice to produce hemizygous transgenic lines. PrP expression levels were estimated using immuno-dot blotting and Western blotting using the monoclonal antibody (MAb) 6H4 [33, 34].

Genotypic confirmation of elk PRNP insert

DNA was isolated from tail tips collected at weaning (Qiagen), and amplified using Polymerase Chain Reaction with the primer pair 158F TCATGGTGAAAAGCCACATAGG and 159R CATCCTCCTCCAGGTTTTGG [31].

Tg(CerPrP-E226) mouse breeding scheme:

CWD positive IC-inoculated female mice and CWD negative IC-inoculated female mice were bred at specific timepoints (before and after 120 dpi) by exposure to intact naïve Tg(CerPrP-E226) males.

Tg(CerPrP-E226) cohorts:

IC-inoculated females were split into cohorts 1-5. Cohorts 1-4: *in utero* study. These mice were divided into cohorts based on time of conception post inoculation, and the week in gestation at which the mouse was sacrificed. Cohort 1: n= 7 mice bred at early timepoint, average 82 dpi and sacrificed during the 2nd week of gestation. Cohort 2: n=10 mice were bred at early timepoint, an average of 73 dpi, and were sacrificed during the 3rd week of gestation. Cohort 3; n= 9, bred at an average of 144 dpi, and sacrificed during the 2nd week of gestation, and cohort 4: n= 9 mice were bred at an average of 190 dpi and sacrificed during the 3rd week of gestation. All cohorts were matched with negative control counterparts (Table 3.1.) Cohort 5: milk study. N is variable, milk was collected from various CWD-inoculated mice and from negative mice of our breeding colony, as well as sham-inoculated mice. These dams were bred continuously from 45 days post inoculation (dpi) until they reached clinical terminal disease and thus were not split into timepoint-based cohorts. (Table 3.2.)

Inoculation

Tg(CerPrP-E226) adult female mice were IC- inoculated under isofluorane anesthesia with a 30 ul solution composed of 1% brain homogenate in 1X phosphate buffered saline (ThermoFisher Scientific) and a 2% penicillin/streptomycin (Gibco) antibiotic mixture. The source of inoculum was brain material collected from previously inoculated muntjac deer that were confirmed positive (MJ 11/15) or negative (MJ 62/64) for CWD prions using western blot.

Breeding practices

Two to three Tg(CerPrP-E226) females were housed together without the presence of males for several weeks prior to breeding in order to stimulate the influence of pheromones and social factors on the estrous cycle. This process prolongs the diestrus cycle and suppresses estrus a phenomenon known as the Whitten effect [35]. Two females were each placed with one male to restart the estrus cycle at the specific breeding time-point associated with each cohort. Intact males used in the milk study remained with the females during pregnancy and parturition until weaning of the mice at 21 days post birth, whereas males bred with females that would be euthanized at set timepoints during pregnancy were removed once the timed-pregnancy was confirmed.

Milk collection

We collected milk at various timepoints throughout the course of CWD disease, ranging from 45-200 dpi. These milk samples were collected from CWD-inoculated mice, as well as sham-inoculated and naïve Tg(CerPrP-E226) females from our breeding colony. Milking procedures were followed from our previously published methods [36], with the exception that we used isofluorane gas as an anesthetic instead of the ketamine/xylazine mixture described in the publication. In short, we dosed the females with oxytocin (VETone), anaesthetized them using isofluorane gas, and manually collected milk using a P-200 pipette (Eppendorf).

Milk optimization in RT-QuIC

Prior to this study, our laboratory had not analyzed milk via RT-QuIC. In order to ensure that the assay would behave properly in the presence of an opaque, somewhat viscous fluid, we surveyed naive milk in various conditions before testing milk collected from a CWD-positive animal. The conditions included seeding the reaction with 2 ul milk alone, or at varying ratios (1:3, 1:1, 3:1) with known positive or negative brain homogenate from experimental deer and mice. (Figure 3.5 A-C.) All other conditions of the assay are as previously described.

Table 3.1. *In-utero* harvest cohorts.
Blue = sham-inoculated. Pink = CWD-inoculated.

Cohort 1

# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding	# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding
3	2nd week	42	4	2nd week	82

Cohort 2

# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding	# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding
4	3rd week	58.5	6	3rd week	73

Cohort 3

# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding	# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding
3	2nd week	162.33	6	2nd week	144

Cohort 4

# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding	# Tg(CerPrP-E226)	Gestation at *in-utero* tissue harvest	Average DPI at breeding
6	3rd week	163.17	3	3rd week	190

Table 3.2. Milk collection cohort organization.

Cohort 5

# Tg(CerPrP-E226)	Milk source	# Tg(CerPrP-E226)	Milk source
Variable (>4)	Sham-inoculated and/or naïve females	4	CWD-inoculated females

Euthanasia/tissue collection

Cohorts 1-4 were sacrificed according to their placement in early or late infection/gestation timepoints. Cohort 5 was sacrificed at terminal disease as characterized by a 20% weight loss, or inability to reach water or food due to hind limb ataxia. All mice were euthanized by carbon dioxide inhalation (flow rate of 1.3L/min) (IACUC Protocol #14-4890A.)

Immediately upon euthanasia, blood samples were collected from the dam using cardiac puncture. Brain, salivary gland, tongue, heart, spleen, gastrointestinal tract, muscle, kidneys, adrenal glands, mammary, and reproductive tissues (uterine horns, ovaries, and vagina) were collected from each Tg(CerPrP-E226) dam. Each tissue was divided, half for frozen storage and half for fixation in a periodate-lysine-formaldehyde solution.

In utero necropsy procedures were modified slightly to avoid potential contamination of fetal materials. Following sagittal sectioning of the uterine horns, a separate pair of surgical scissors and forceps was used to collect embryos and placentas encased in their individual yolk sacs and immediately transferred to a sterile surface for dissection. Our ability to dissect these tissues and adequately separate them from other tissues differed across timepoints in gestation. We were able to collect a more consistent set of tissues from embryos greater than E13. Overall, tissues include yolk sac, placenta, and whole fetuses. Half of the number of placentas and fetuses were kept for frozen storage, and the other half for fixation in a periodate-lysine-formaldehyde (PLP) solution. Fetal sacs were pooled and frozen. In cases where the total mass of an individual fetus or placenta was less than 0.4 grams, tissues were pooled. Fixed tissues were moved to 60% ethanol after 48 hours in PLP.

Analysis of tissues for CWD prions

Tissues analyzed for PrP^{res} deposition and/or amplification competent prions include brain, reproductive (ovary, vagina and uterus), mammary tissue, and spleen of Tg(CerPrP-E226) dams

and placenta, fetal tissue, and fetal sac from *in utero* by conventional and amplification methods described below.

Western blot

Brain homogenates were made at a 10% (w/v) concentration in 1X phosphate-buffered saline (ThermoFisher Scientific) using a bead ruptor (Omni). Homogenates were mixed with proteinase K (PK) (Invitrogen) at a final concentration of 1 mg/ml and incubated at 37°C for 30 minutes, followed by 10 minutes at 45°C in a shaking thermomixer (Eppendorf). Samples were mixed with Reducing Agent (10X)/LDS Sample Buffer (4X) (Invitrogen) at a final concentration of 1X, heated to 95°C for 3 minutes, then run through a NuPAGE 10% Bis-Tris gel at 135 volts for 1 hour. Proteins were transferred to a polyvinylidene fluoride (PVDF) membrane with a Trans-Blot Turbo™ blotting system for 7 minutes. The membrane was loaded into a pre-wetted SNAP i.d. blot holder (Millipore) then placed in the SNAP i.d. system (Millipore). Blocking buffer (Blocker Casein in TBS [Thermo Scientific] with 0.1% Tween 20) was added to the blot holder for 3 minutes followed by vacuum removal. Primary antibody BAR224 (Cayman Chemical) conjugated to horseradish peroxidase (HRP) was diluted in blocking buffer to 0.2 ug/ml and added to the blot holder for a 12-minute incubation. The antibody was pulled through the membrane via vacuum and was followed by three washes with 30 ml volumes of wash buffer (50% Blocker Casein in TBS, 50% 1X Tris-buffered saline (TBS), 0.1% Tween 20 (Sigma) each). Proteins were visualized by using ECL Plus™ (GE Healthcare) and a digital Gel-Doc™ capturing chemiluminescent signals using ImageGauge™ software.

Immunohistochemistry

Tissues were stored in a Periodate-Lysine-Paraformaldehyde (PLP) solution for a minimum of 48 hours, and switched to 60% ethanol prior to paraffin embedding. Paraffin-embedded tissues were cut into 6um sections and transferred to positively charged glass slides (Unifrost.) Slides were incubated at 37°C for one hour to remove paraffin which was followed by graded alcohol-

rehydration and a 40-minute immersion in 88% formic acid to digest cellular PrP. The slides were then subjected to heat-induced epitope retrieval in a 2100-Retriever (Prestige Medical) in sodium citrate buffer (0.01 M sodium citrate, 0.05% Tween 20, pH 6.0) followed by incubation with primary antibody BAR224 (Cayman Chemical) at 1 mg/ml* and Envision+™ anti-mouse HRP labeled polymer (Dako) and rinsed twice with TNT (0.1M Tris (pH 7.5) + 0.15M NaCl + 0.05% Tween-20). Slides were incubated with AEC (3-Amino-9-Ethylcarbazole) Substrate-Chromagen (Dako), counterstained with hematoxylin and bluing reagent (0.1% sodium bicarbonate), and coverslipped with an aqueous mounting medium (Vector Laboratories) [37].

*In cases where detection of PrP^{res} was not achieved using the protocol above, a Tyramide Signal Amplification (TSA) kit (Fisher Scientific) was used to enhance signal potentially missed by the conventional IHC methodology. This differs from the standard protocol in that a 30-minute PK-digestion at 1 mg/mL in a CaCl/Tris buffer was added following rehydration of the slides in combination with a shorter (5 min) 88% formic acid treatment. Following addition of anti-mouse-HRP (secondary antibody) as described above, all slides were treated with a Dinitrophenyl (DNP) Amplification Reagent (stock solution diluted 1:50 in amplification diluent), washed twice in TNT buffer, and incubated for 30 minutes in anti-DNP-HRP diluted 1:100 in TNB. These additional steps are then followed by incubation with AEC and counterstained with hematoxylin and bluing reagent as described above.

Preparation of normal brain homogenate (NBH)

An 8.5% normal brain homogenate (NBH) in 0.1 M PBS buffer (pH 7.5, with 1 % Triton X-100) was prepared from whole brains collected from naïve Tg(CerPrP-E226) mice no older than 4 months of age. These homogenates were then frozen at -80C, serving as a substrate for protein misfolding cyclic amplification (PMCA) experiments.

Serial protein misfolding cyclic amplification (sPMCA)

The initial round of sPMCA incorporated 30 ul of 10% tissue homogenate sample plus 50 ul of 8.5% w/v normal Tg(CerPrP-E226) brain homogenate (NBH) placed in individual 0.6ml thin-walled PCR tubes (USA Scientific) containing two 2.38 mm and three 3.15 mm Teflon beads (McMaster-Carr). The PCR tubes were sealed with parafilm, vortexed for 6 seconds and subjected to one round of PMCA. Each round of PMCA is equal to 288 cycles of sonication (Misonix) (10 seconds separated by 5 minute incubations) at 37°C over 24 hours. Seven total rounds of sPMCA were completed in duplicate per tissue by transferring 30 ul from the previous round into 50 ul fresh NBH (1:1.6 dilution). Standard CWD positive and negative assay controls, consisted of 10% homogenates made from brain harvested from Tg(CerPrP-E226) mice IC-inoculated with CWD-positive or negative muntjac brain. These samples were incorporated into the first run at a different ratio than samples, 2 ul sample to 98 ul NBH, and subsequently treated like a sample in the following runs. The seventh round of sPMCA was analyzed by conventional PK digestion and western blot analysis as above.

Sodium phosphotungstic acid precipitation (NaPTA)

10% (w/v) tissue homogenates were added to 90ul of 0.1%SDS/1X PBS buffer, followed by a 7ul addition of a NaPTA (Sodium phosphotungstic acid + $MgCl_2$ 6-hydrate) solution [38]. This mixture was incubated while shaking for 1 hour at 37°C in a thermomixer (Eppendorf), and spun at 14,000 x rpm for 30 minutes in a Beckman centrifuge at room temperature. The resultant pellet was diluted into 10 ul of 0.1%SDS/1X PBS buffer prior to RT-QuIC analysis.

Ethanol precipitation

10 ul of undiluted milk was added to 90 ul 100% ethanol, followed by a 5-minute incubation at room temperature and centrifugation at 14,000 x rpm for 5 minutes to form a pellet. The resulting supernatant was removed, and the pellet diluted into 100ul 1X PBS (ThermoFisher

Scientific). This mixture was then centrifuged at 14,000 x rpm for 5 minutes to form a pellet, and resuspended in 100 ul 0.1% SDS/1X PBS (Hoover C. et al. 2016, manuscript in preparation).

Real-time quaking induced conversion assay (RT-QuIC)

Recombinant truncated Syrian hamster prion protein (rPrP) containing amino acid sequences 90-231 was prepared as previously described [9]. RT-QuIC premixed reaction buffer containing 20 mM $NaPO_4$, 1 mM ethylenediaminetetraacetic acid (EDTA), 320 mM sodium chloride (NaCl) was mixed with the 0.1 mg/ml rPRP, and 10 μM thioflavin T (Sigma). 95 ul of this mixture was added to each well of a 96-well plate. Plates were shaken in a microplate fluorometer (BMG) for 1 min (700 rpm, double orbital) followed by 1 min of rest. Fluorescence (450-nm excitation and 480-nm emission, 20 flashes/well) was recorded every 15 min using a gain of 1,700.

Rate calculations in RT-QuIC

In order to determine the rate at which a given sample is able to convert rPrP, the threshold in which a sample is deemed positive is calculated by the following equation:

Threshold = average baseline fluorescence + 5 X the standard deviation of baseline fluorescence

The rate of conversion is defined by *1/time* at which a positive sample crosses this threshold. When possible, we took this a step further, normalizing samples to a plate control. This extra method is used in order to account for any differences caused by tissues being tested across several plates, fluorometers, and recombinant protein batches. Finally, either the raw rate or normalized rate was calculated for each sample and statistically compared to negative tissue-matched controls (when available) using the Mann-Whitney test.

Results

Clinical status of Tg(CerPrP-E226) females does not affect breeding patterns.

We bred Tg(CerPrP-E226) at both early and late timepoints associated with CWD infection, and regardless of clinical disease progression, the mice had no problems establishing normal pregnancies.

In addition to CWD prions being localized to the brain of IC-inoculated Tg(CerPrP-E226) dams, conversion competent prions are also found in their spleens.

Dams were inoculated through the intracranial route with a 1.0% homogenate of brain collected from muntjac deer (MJ 11/15) confirmed CWD-positive by western blot [31]. We detected amyloid seeding activity as early as 72 dpi in mice from Cohort 2, by RT-QuIC (Figure 3.2.) While it is possible that those prions are from the original inoculum, we were also able to identify conversion-competent prions in the spleens of the same females, indicating uptake of prions by peripheral lymphatic tissue. (Figure 3.3.)

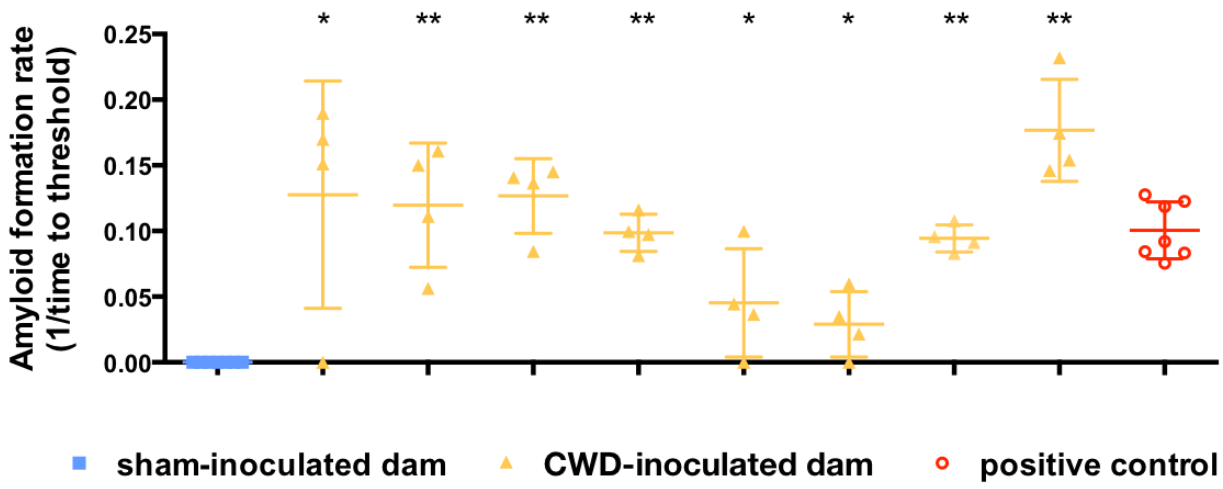

Figure 3.2. Detection of amyloid conversion activity (CWD prions) in brains of IC-inoculated dams as early as 72 days post inoculation.

Each group indicates a sample taken from one individual animal. P values: **** ≥ 0.0001, *** ≥ 0.001 ** ≥ 0.01 * ≥0.05.

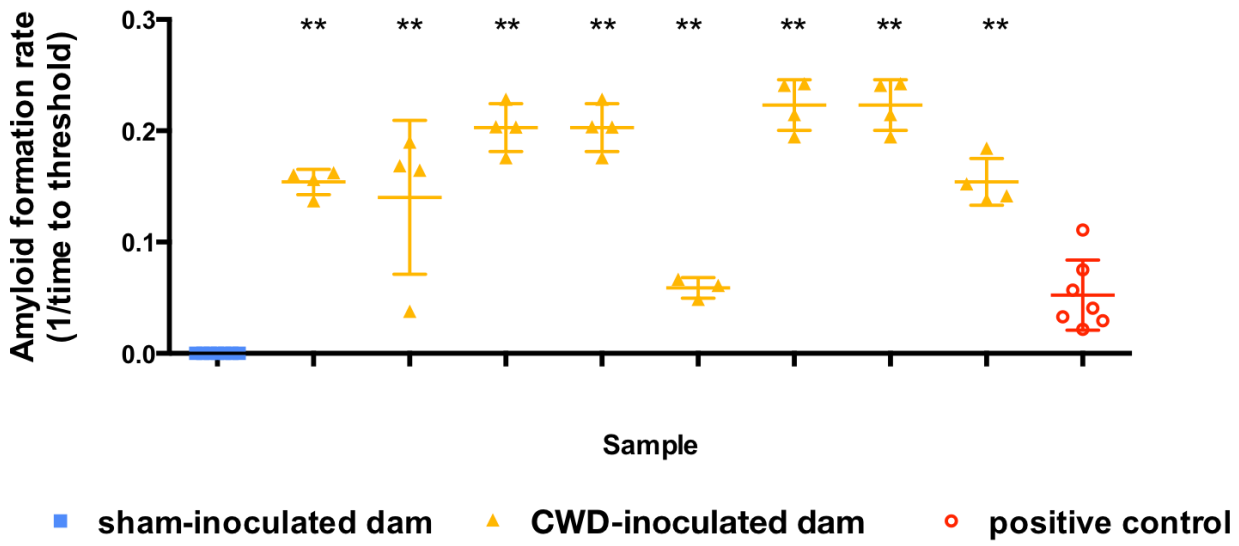

Figure 3.3. Detection of amyloid seeding activity (CWD prions) in spleens of IC-inoculated dams as early as 72 days post inoculation.

RT-QuIC rates of conversion in 1.0% spleen homogenates collected in dams as early as 72 dpi. Each group indicates a sample taken from one individual animal. P values: **** ≥ 0.0001, *** ≥ 0.001 ** ≥ 0.01 * ≥0.05.

PrP^{res} detected in reproductive and mammary tissue of IC-inoculated dams via amyloid seeding assays.

We and others have shown that amplification methods such as PMCA and RT-QuIC are more sensitive in detecting minute quantities of prions; therefore, our inability to detect PrP^{res} in reproductive or mammary tissues by conventional methodologies led us to analyze them using these highly sensitive amplification assays. We identified amplification competent prions in reproductive tissue by PMCA (Figure 3.4A) and RT-QuIC (Figure 3.4B and C). We also identified amyloid conversion activity by PMCA in mammary tissue (Figure 3.4A) but this particular tissue displayed high rates of spontaneous conversion in RT-QuIC (data not shown), complicating final analysis.

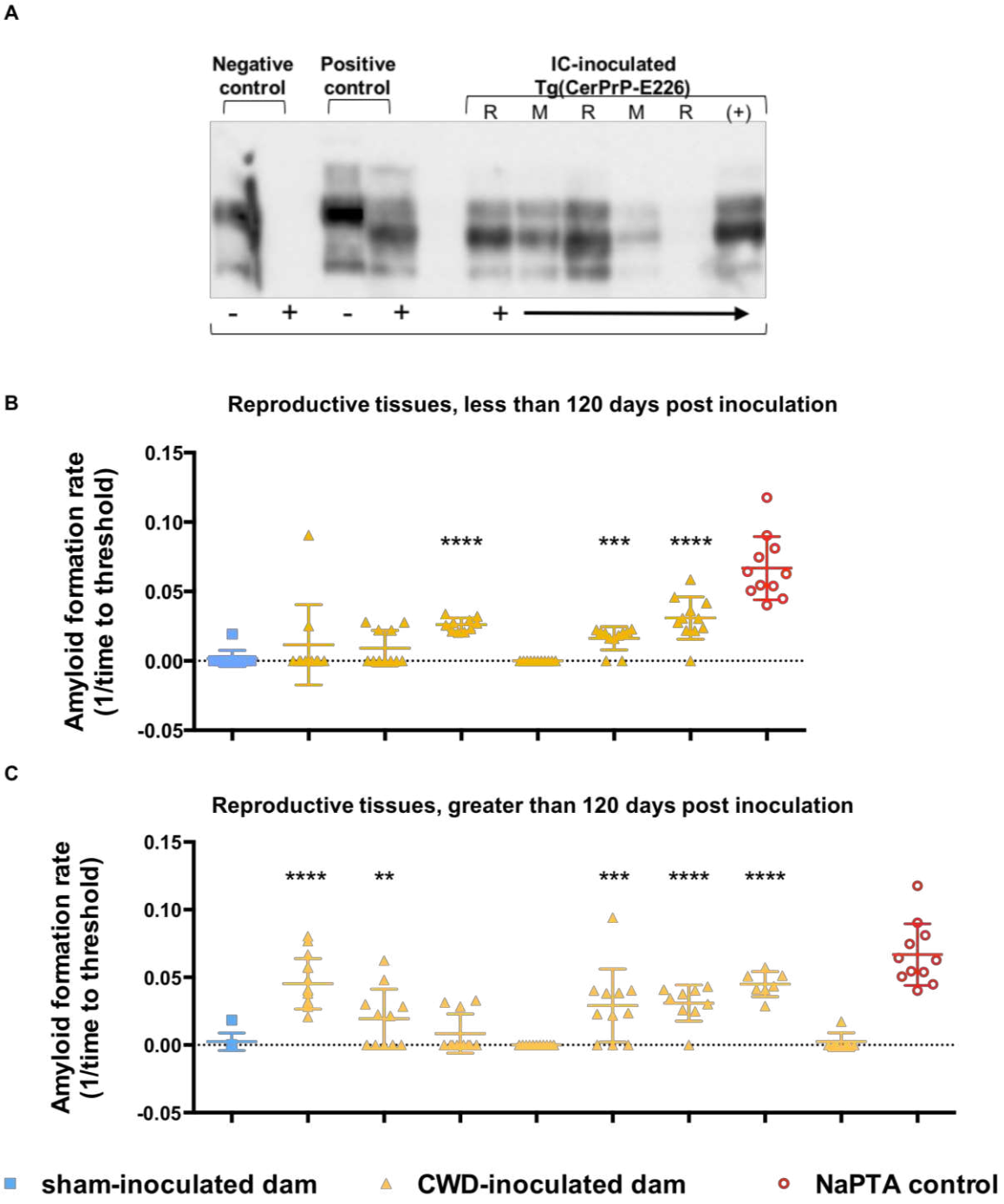

Figure 3.4. Detection of amyloid-conversion (CWD prions) in reproductive and mammary tissue collected from CWD-positive dams at various timepoints post inoculation.

A) Western blot following 7 rounds of PMCA (+) denotes PMCA-amplified positive control. R=repro. M=mammary. B) RT-QuIC following NaPTA precipitation in reproductive tissues collected from dams as early as 72 dpi. C) RT-QuIC rates of conversion following NaPTA precipitation in reproductive tissues collected at > 120 dpi. P values: **** $\geq$ 0.0001, *** $\geq$ 0.001 ** $\geq$ 0.01 * $\geq$0.05.

Conversion competent (CWD prions) were not found in the milk of CWD-infected cervidized mice.

We analyzed milk that was collected from mice at various timepoints in clinical infection, from approximately 45 to 190 dpi. Because we hypothesized that prion load would increase over time [9], we elected to first analyze milk collected at periods of time when the dams demonstrated overt clinical TSE disease. We previously demonstrated that milk collected from negative control mice neither inhibited or caused spontaneous conversion in the RT-QuIC assay. (Figure 3.5.) Undiluted milk collected from CWD-infected dams did not elicit seeding activity. (Figure 3.6A.) To enrich prion content in milk samples we preprocessed milk samples by PTA (phosphotungstic acid) precipitation [9, 10]. (Figure 3.6B), sodium phosphotungstic acid treatment (NaPTA) (Figure 3.6C.) and ethanol precipitation. From these experiments we determined that milk does not harbor amplification/conversion competent prions.

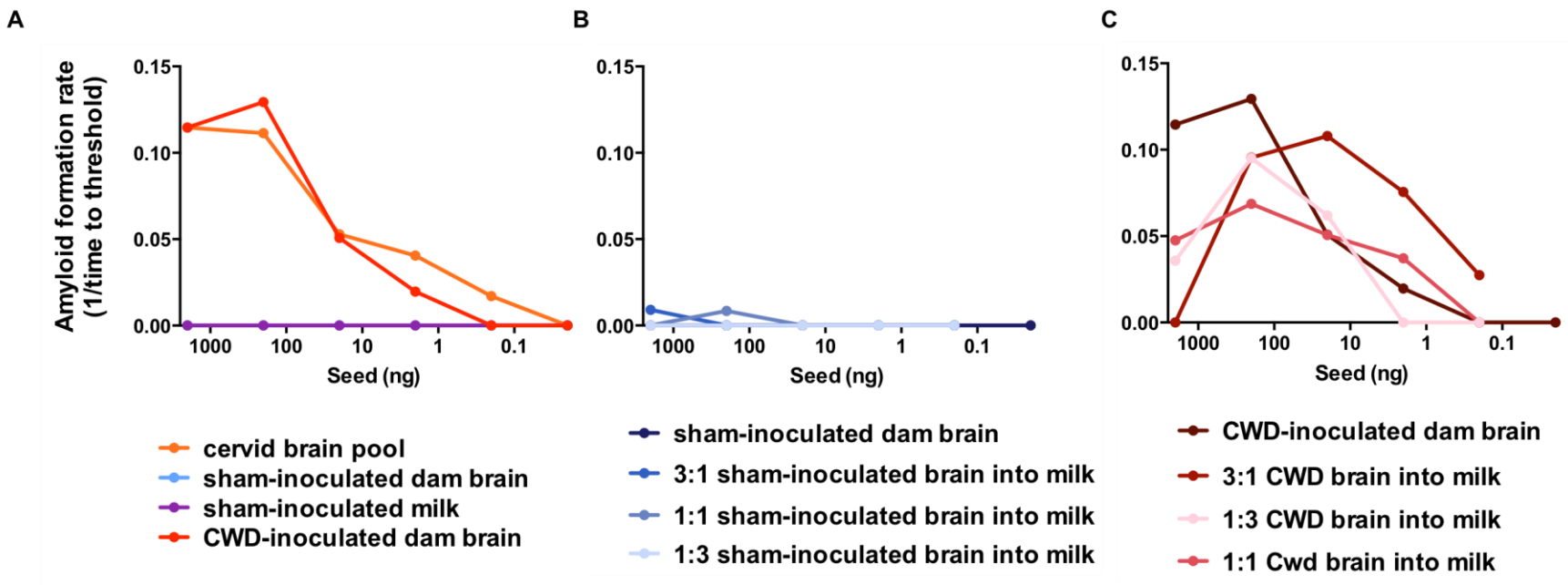

Figure 3.5. Optimization of milk for RT-QuIC.

A) Seed behavior in RT-QuIC, including milk. B) Varying ratios of sham-inoculated brain spiked into milk collected from sham-inoculated dams. Negative milk does not illicit spontaneous conversion in the presence of brain material. C) Varying ratios of CWD-inoculated brain spiked into milk collected from sham-inoculated dams. Negative milk does not inhibit the RT-QuIC reaction.

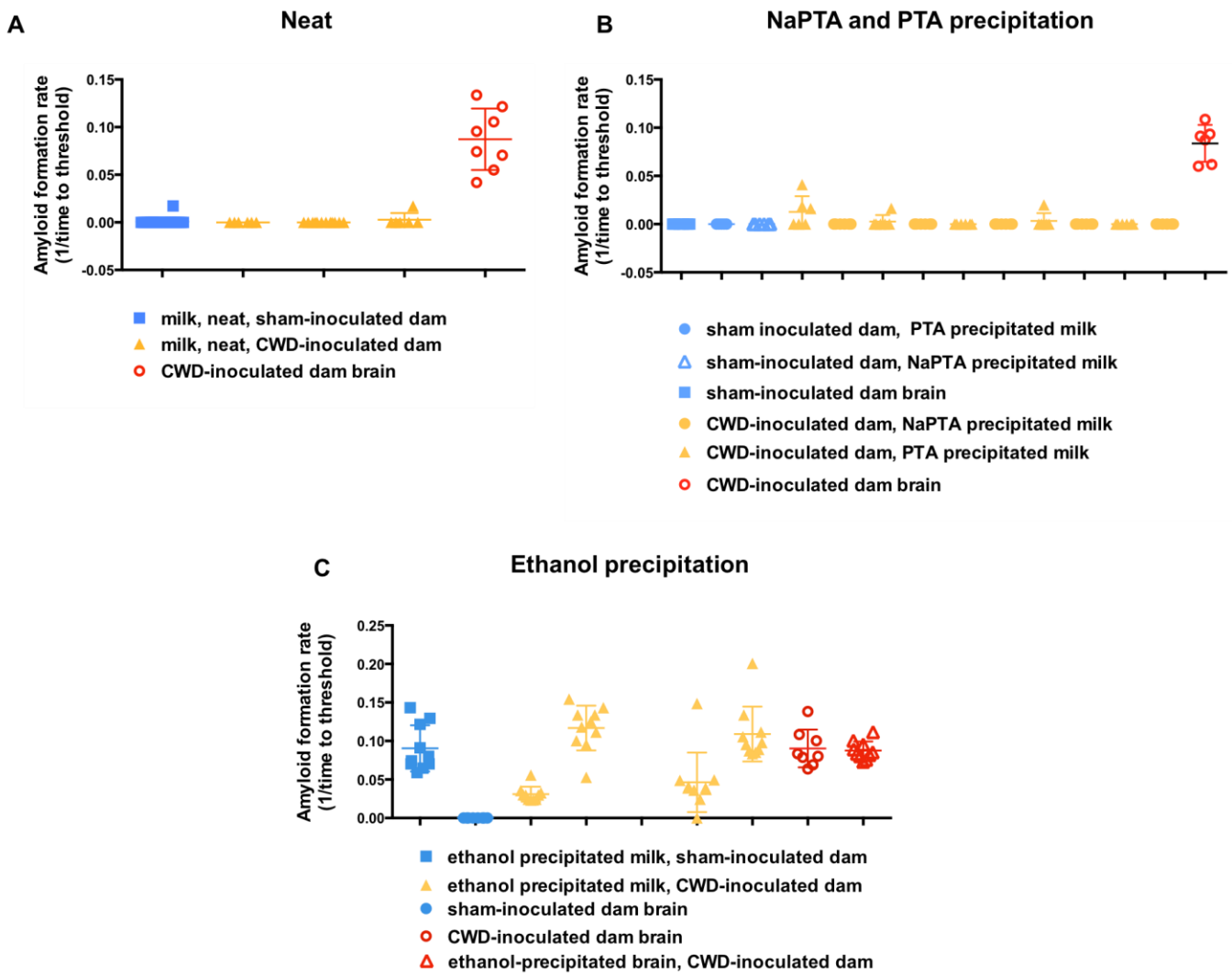

Figure 3.6. Lack of amyloid conversion activity (CWD prions) in milk collected from CWD-positive dams.

A) Undiluted milk added to the RT-QuIC reaction does not elicit amyloid seeding behavior. B) Neither NaPTA nor PTA precipitation changes the amyloid seeding capacity of milk collected from CWD-positive dams.
C) Ethanol precipitation of milk results in amyloid seeding of all samples, including negative controls.

Limited amyloid seeding activity is detected in tissues collected in utero at various timepoints in CWD infection and gestation.

Our previous work with amyloid seeding reactions led us to the conclusion that RT-QuIC is more sensitive than PMCA. Due to this finding, we evaluated tissues collected from *in utero* using RT-QuIC only. We detected amyloid seeding activity in fetal tissues harvested from 1/29 dams at >120 day post infection during the 2nd week of gestation (Figure 3.7).

Placental tissue displayed lower amyloid seeding capacity than fetal tissue. One tissue collected out of 21 tested had a statistically significant rate of amyloid conversion compared to the negative control (in some cases placenta was pooled if necessary to make a 10% homogenate.) This placenta was collected from a different dam than that of the offspring that showed positive seeding activity. (Figure 3.8.)

We also analyzed pooled fetal sac tissue collected from *in utero* harvests. The majority of this tissue was collected from pregnancies in the 3rd week of gestation. Amyloid seeding activity seen was not higher than negative control conversion (Figure 3.9).

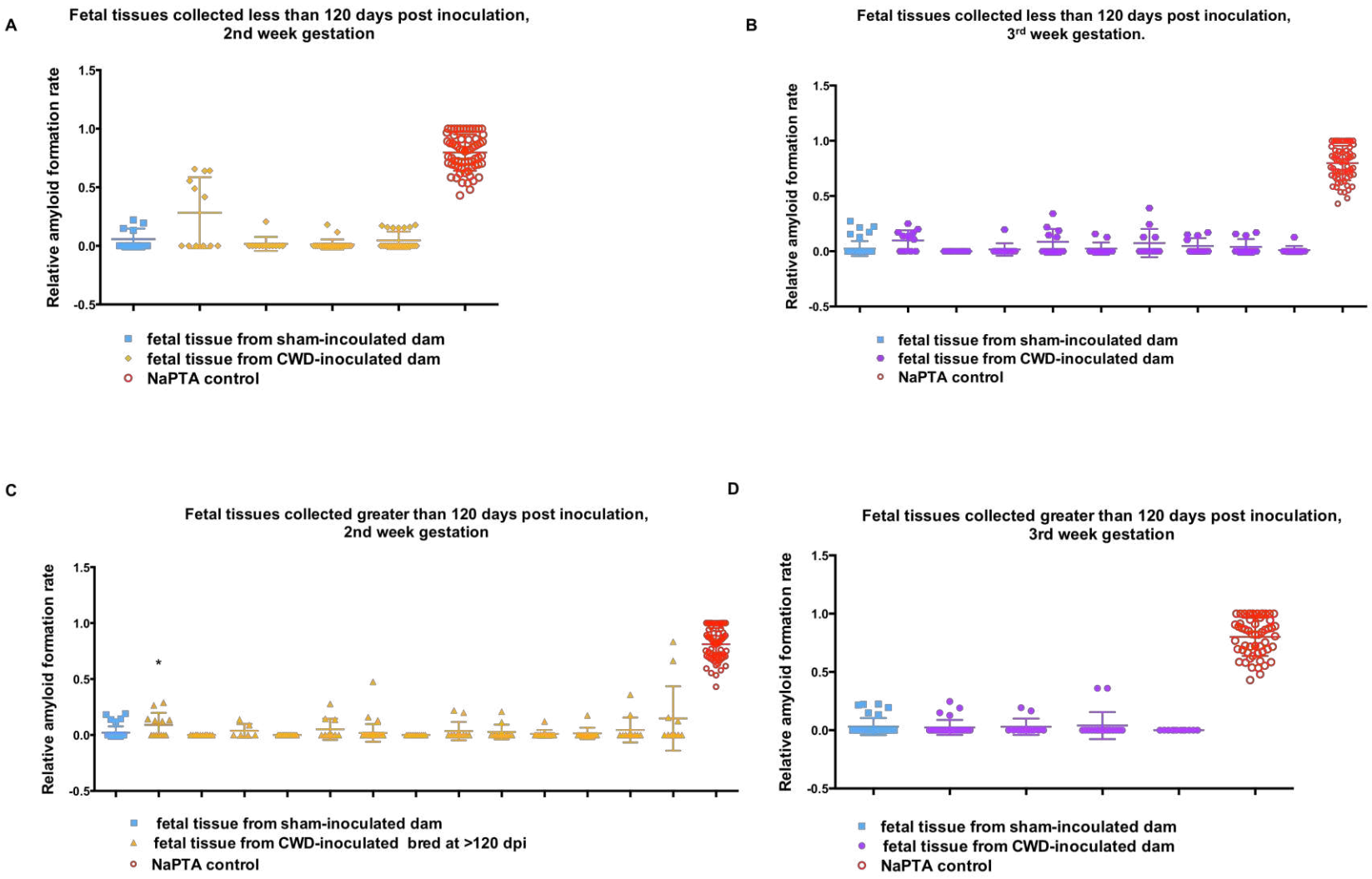

Figure 3.7. Limited amyloid conversion activity (CWD prions) in fetal tissue collected from CWD-positive dams.

A-B) Fetal tissues collected at late-timepoints in CWD infection, 2nd and 3rd week gestation, respectively. 1/17 tissues were determined statistically significant from the negative control. C-D) Fetal tissues collected less than 120 dpi, 2nd and 3rd week gestation, respectively. P values: **** $\geq$ 0.0001, *** $\geq$ 0.001 ** $\geq$ 0.01 * $\geq$0.05.

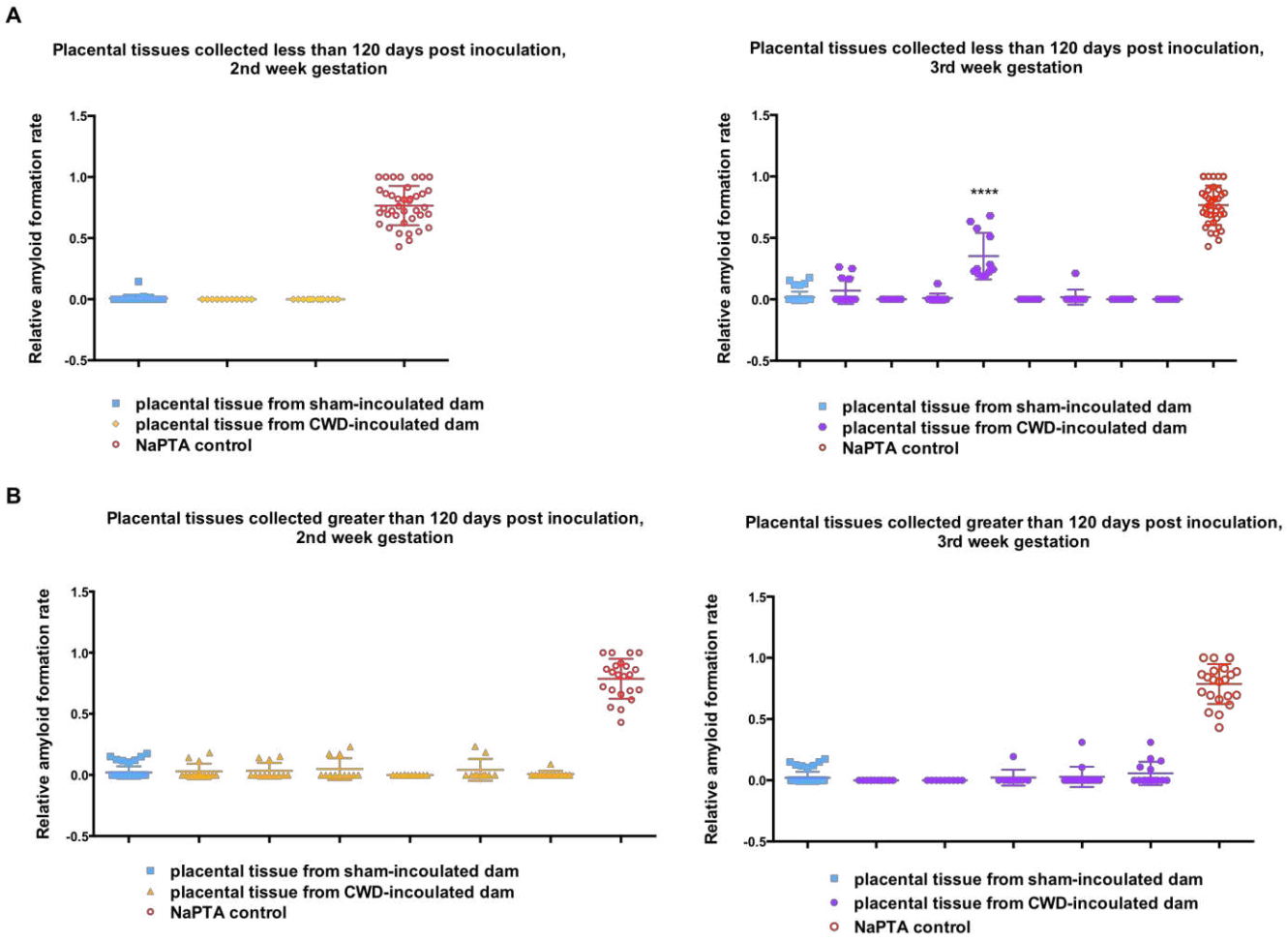

Figure 3.8. Low levels of amyloid conversion (CWD prions) in placental tissue collected from CWD-positive dams.

A) RT-QuIC analysis following NaPTA precipitation of placental tissue collected from dams at late-timepoints in CWD-infection. B) RT-QuIC analysis following NaPTA precipitation of placental tissue collected at early-timepoints in infection. 1/10 tissues tested was determined statistically significant from the negative control. P values: **** $\geq$ 0.0001, *** $\geq$ 0.001 ** $\geq$ 0.01 * $\geq$0.05.

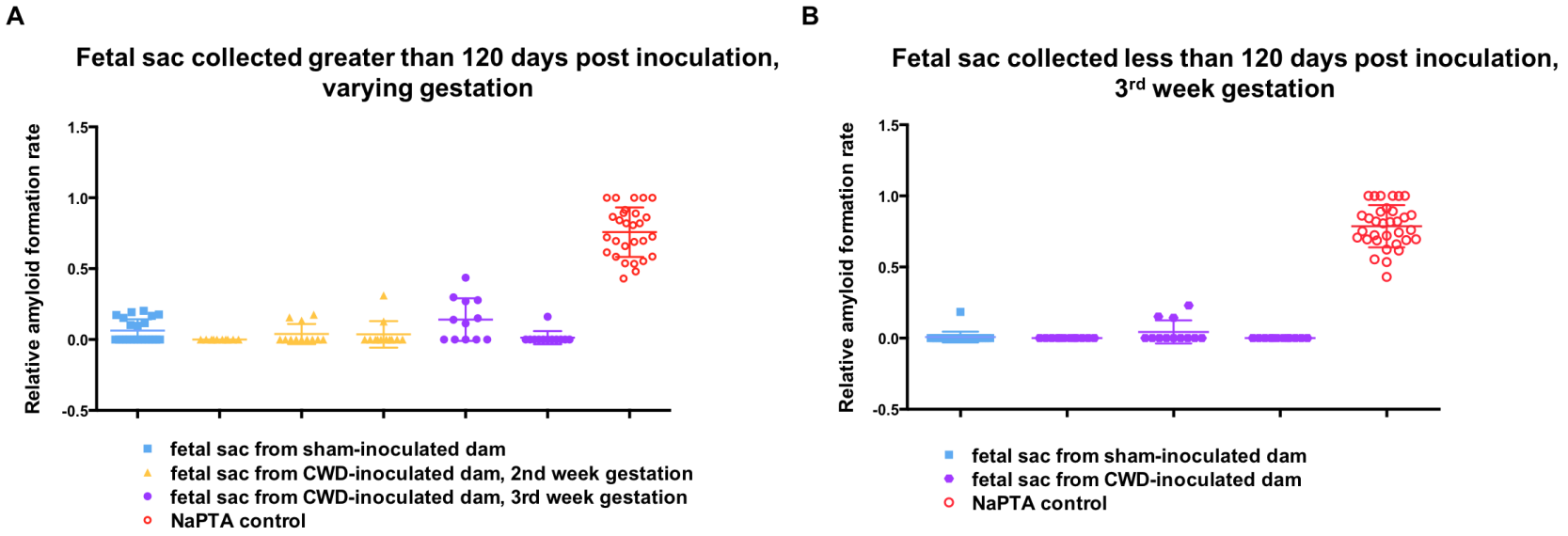

Figure 3.9. Absence of amyloid conversion activity in fetal sac collected from CWD-positive dams.

A) RT-QuIC rate analysis following NaPTA precipitation of fetal sac collected at late timepoints in CWD-infection. B) RT-QuIC rate analysis following NaPTA precipitation of fetal sac collected at early-timepoints in CWD-infection.

Discussion

A notable characteristic of chronic wasting disease is its high transmission efficiency in nature. Being the only prion disease in a wildlife population, efforts to control its spread have been sought. Before prevention will be successful, it is necessary to further understand how the disease is spread from one susceptible individual to another. Recent work has reported maternal/vertical transmission of CWD under experimental conditions —analysis of experimentally-inoculated muntjac indicated that transmission of prions occurs *in utero,* as demonstrated by PMCA amyloid seeding activity in tissues of unborn fawns [31]. Similarly, a study conducted to analyze cow: calf pairs in elk localized to Rocky Mountain National Park not only reported *in utero* transmission, but indicated that the incidence of CWD in this area may be much higher than previously thought, emphasizing the importance to identify the route by which maternal transmission is occurring [32].

Here we sought to establish an experimental model to further studies to assess the efficiency of prion maternal transmission via a hemochorial placental structure, as well as to assess the likelihood of prion transmission post birth through milk.

Previous work implicating infectious milk as a potential route by which scrapie is spread to lambs from their scrapie-positive mothers led us to assess the likelihood of this occurring in CWD. We opted to analyze milk collected from CWD-positive females at timepoints spanning the duration of CWD disease. In this study we were unable to identify prions in milk. There are several explanations as to why this may be, including 1) Aberrant prion conversion requires normal cellular prion protein substrate for conversion [39]. The level of PrP^{c} expression in mammary tissues of Tg(CerPrP-E226) compared to wild type mice is currently unknown**.** If PrP^{c} is in minute levels within this tissue, this may explain why PrP^{res} is not passed to offspring in milk. 2) The mice in our studies did not experience lentiviral mastitis, which is thought to contribute to the infectious potential of prions in milk due to their lymphoreticular association[19, 20, 22]. PrP^{res} identified in scrapie-infected mammary tissue was localized using IHC, and in our hands PrP^{res} is below the limit

of detection by this method. Therefore, minute levels of prions *without* lentiviral infection to exacerbate the prion load may not be sufficient to cause disease.

Our original hypothesis with regard to *in utero* transmission of prions is that the infectious proteins are transported to the fetus through the placenta. We hypothesized that placental trafficking of prions would be associated with fetal-derived, highly mobile trophoblast cells that make up part of the placenta, and are responsible for nutrient and waste exchange between the mother and fetus [40]. Prions have been identified feto-maternal interface of scrapie-positive ewes [15], specifically localized to trophoblast cells, [41] and placental tissue has been shown to be infectious [23]. The trophoblasts' role in nutrient delivery requires their ability to invade tight junctions between cells to travel through the maternal-fetal interface. In hemochorial placental structure there is a direct bathing of fetal chorionic epithelium in maternal blood [40] and it has been well established that prions are present in blood of TSE infected humans and animals [11, 13, 42-46]. Therefore, it may be possible that prion infected blood cells are phagocytized by and travel with trophoblasts where they are delivered to the fetus. That being said, the probability of this occurrence may be dependent on the disease status of the mother, i.e., prion load in her blood, and whether the fetus is genetically susceptible to prion disease.

One of the goals of this study was to identify similarities/differences in maternal transmission via use of a mouse model as opposed to the native host. Maternal/vertical transmission has been identified in ruminants containing epitheliochorial placentation, such as deer, elk, and sheep. We originally hypothesized that hemochorial placentation of mice, which contains less layers of epithelium (three total) and direct bathing in blood, vs. ruminant epitheliochorial placentation (six total layers) with less direct contact to maternal blood, would provide a *more efficient* environment for transmission of prions across the feto-maternal interface. The results of this current study indicate that changing a variable such as placental structure

significantly lowers the probability of maternal transmission; therefore this research does not support our hypothesis.

While we were surprised by this result, what we have found in mice corroborates with what is known so far in other species with hemochorial placentation, i.e., humans. For instance a study published in 2010 identified PrP^{res} in ovary and uterus in a woman who died of terminal variant Creutzfeldt-Jakob disease (CJD) [47]. Similar to what we show in this report, this discovery relied on techniques to increase detection of PrP^{res} (NaPTA precipitation.) Additionally, a bioassay study published in 1992 identified infectious prions in placental tissue and colostrum from a CJD-positive woman with clinical signs through the duration of her pregnancy [48]. In both of these studies, there were low levels of infectivity carried within pregnancy-related tissues, much like we have shown in this work.

There are several aspects that may explain this. According to "Host Defense and Tolerance: Unique Challenges in the Placenta" a layer of the placenta called the syncytium (possessed by both humans and mice) that protects the unborn fetus from pathogens [49]. In general, vertically transmitted human pathogens have found some way to evade this syncytium and/or breach it in some way. For example, the histopathology of *Plasmodium falciparum*, the causative agent of malaria shows syncytiotrophoblast degradation [50]. This leads us to believe that perhaps the prion protein is reaching offspring via some other route. Additionally, it is possible that the short gestation in mouse negatively impacts the potential for maternal transmission of prions. The murine placenta is not fully developed until day 10 of pregnancy [51], and gestation is only 19-21 days, providing very little exposure time to the prion pathogen *if* the prion were indeed capable of breaching the protective placental barrier.

In summary, we have identified prions in brain, reproductive, mammary, and spleen tissue collected from experimentally inoculated Tg(CerPrP-E226) dams. It appears that milk does not carry infectious prions, and most of the tissues collected *in* utero did not exhibit seeding activity

higher than that of negative control conversion. This indicates that prions are likely in low levels in these tissues. However, our previous work shown in Chapter two provides proof that repeated exposure to low levels of prions over time may contribute to the number of asymptomatic, silent-carriers of prions within a given population. Whether these silent carriers are capable of shedding prions, thus further perpetuating the cycle of prion transmission, remains to be seen. The facile transmission of CWD would indicate that this is a definite possibility.

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CHAPTER FOUR:
MILK COLLECTION METHODS FOR MICE AND REEVES' MUNTJAC DEER[1]

Introduction

Animal models are commonly used throughout research laboratories to accomplish what would normally be considered impractical in a pathogen's native host. Milk collection from animals allows scientists the opportunity to study many aspects of reproduction including vertical transmission, passive immunity, mammary gland biology, and lactation. Obtaining adequate volumes of milk for these studies is a challenging task, especially from small animal models. Here we illustrate an inexpensive and facile method for milk collection in mice and Reeves' muntjac deer that does not require specialized equipment or extensive training. This particular method requires two researchers: one to express the milk and to stabilize the animal, and one to collect the milk in an appropriate container from either a Muntjac or mouse model. The mouse model also requires the use of a P-200 pipetman and corresponding pipette tips. While this method is low cost and relatively easy to perform, researchers should be advised that anesthetizing the animal is required for optimal milk collection.

Background

Animal models provide insight into disease pathology that cannot be gained by *in vitro* analysis. To provide the most efficacious results, it is important to use an animal model that is closely related to the disease and species of interest. For example, the Reeves' muntjac (*Muntiacus reevesi*), a small Asian deer [1, 2], and transgenic mice expressing the cervid prion protein (CerTgPrP) are useful animal models for cervid species [3]. Both species are polyestrous, allowing year-round breeding, and therefore a consistent source for pregnancy-related tissues and fluids to

[1] Kassandra Willingham, Erin McNulty, Kelly Anderson, Jeanette Hayes-Klug, and Candace Mathiason. *Milk Collection Methods for Mice and Reeves' Muntjac Deer*, in *Journal of Visual Experiments*. 2014.

study specific mechanisms in cervid biology. Studies of milk have a vast array of applications that are more simply (and inexpensively) accomplished in animal models than in humans. Researchers could investigate milk and colostrum as a potential source of 1) infectious disease transmission, 2) immunoglobulins transferred from mother to offspring in the development of passive immunity [4] and 3) lactoferrin, a protein found in human breast milk involved in passive immunity that researchers are currently attempting to commercially produce [5].

Collecting a substantial amount of milk from small animals can prove to be a difficult task. Rodgers proposed an approach to collect milk from rats [6], which was subsequently used in mice. DePeters and Hovey proposed two methods for milk collection, one using a manually-generated vacuum produced by a rubber pipette bulb attached to a Pasteur pipette, and a second requiring the construction of a milking unit, which is then attached to a vacuum source (such as a faucet) to harvest mouse milk [7]. Here we propose a simple, low cost method for collecting milk from both mice and Reeves' muntjac deer, which requires only readily available laboratory equipment and basic technical skills. Our method yields sufficient volumes of milk for various applications.

Materials and Methods

Mouse

Separation of the dam from offspring

1.1.1 Select the dam to be milked. Choose a dam with a litter of 4 or more pups that is 8-12 days post parturition to provide maximal milk collection— although collection is possible at any time point post parturition up to 21 days.

1.1.2 Separate the dam from her litter at least 2 hours before milking.

Note: Should one desire to milk more than one dam in a given time period, it is acceptable to house the separated dams in the same cage with adequate food and water supply.

Administration of oxytocin, anesthesia and eye lubricant

1.2.1 Administer 2 IU/ kg of oxytocin intraperitoneally (IP). Oxytocin is a hormone that acts on the mammary glands of lactating females to stimulate the release of milk. Oxytocin can be acquired from a veterinarian.

1.2.2 Administer an anesthetic mixture containing 80-100 mg/kg ketamine in combination with 5-10 mg/kg xylazine IP. For example, a 10 ml stock anesthetic mixture could be prepared in advance for mice weighing approximately 30 g and would include 0.1 ml xylazine, 8.9 ml H_2O, and 1.0 ml ketamine. 0.25 ml total anesthetic will sedate a mouse of this size for approximately 20-30 minutes, which should provide adequate time for milk collection.

Note: To determine that the animal has been successfully anesthetized, gently place her on her stomach on a solid surface; she should not be moving her feet or attempting to rise to her feet if she is fully anesthetized. In the case of a mouse not being fully anesthetized with the original dosage, the researcher may administer another ¼ or ½ of the original dose of the anesthetic mixture depending on the size of the mouse and/or level of sedation that the mouse has already achieved. However, this could increase the chance of an adverse reaction to anesthesia, and also may result in the mouse being more deeply anesthetized and/or anesthetized for a longer period of time.

Note: If milking more than one dam, it is necessary to stagger oxytocin and anesthetic injections due to the short half-life of oxytocin and sedation times for ketamine and xylazine.

1.2.3 Apply a small amount of eye lubricant to the corner of each eye and spread to prevent the mouse's eyes from drying out while under anesthesia.

Milk collection

Note: The milk collection is most easily performed with two researchers: one researcher to hold the anesthetized mouse while manually expressing the milk (referred to as **R1**,) and one researcher to collect the milk (**R2**.) This method can also be performed with one person, if the mouse is secured on a flat surface to avoid harm.

1.3.1 **R2** should be equipped with sterile alcohol prep pads, a P-200 pipetman, a clean pipette tip for each mouse, and a container to hold the milk, such as a 1.5 ml eppendorf tube or a 1.2 ml cryovial.

1.3.2 As the oxytocin begins to take effect, milk letdown will become visible in the mouse's mammary area (see Figure 4.1A). **R1**: Unwrap a sterile alcohol prep pad and gently wipe the mammary area of the mouse to clean the area prior to milking. Manually express milk from the teat by using the thumb and forefinger to gently massage and squeeze the mammary tissue in an upward motion until a visible bead of milk begins to form at the base of the teat.

1.3.3 **R2**: Press the P-200 pipetman plunger to its first stop to release air out of the pipette tip and prepare for milk collection.

1.3.4 **R2:** Position the pipette tip at the top of the drop of milk, and gently pull the milk into the pipette tip by slowly releasing pressure on the plunger. Take care not to put the tip too close to the teat or the skin (see Figure 4.1B). It is not necessary to completely release the plunger as this will pull the milk all the way to the top of the pipette tip and will make it difficult to expel the often viscous milk into the holding container.

1.3.5 **R2:** Expel the milk into the container by using the thumb to press the plunger past the first stop (see Figure 4.1c). To facilitate expulsion of small volumes, press the pipette tip against the side of the tube so that no liquid is lost.

1.3.6 **R1:** Continue to manually express milk from each teat, moving in either a clockwise or counterclockwise direction so as not to skip any teats. It is both acceptable and useful to come back to and continually express milk from the same teat should it produce more milk than another.

Anesthetic Reversal in the Dam

1.4.1 In most cases, it is common for the dam to begin to begin to wake up during the milking process. If this is not the case, place the dam in a cage indirectly underneath a heat source, such as a heated circulating blanket, until she begins to wake up.

Note: As the dam begins to wake up, keep close observation of her activity until she is able to walk about her cage on her own.

1.4.2 Once the dam is moving about the cage without struggle, place her back into her original cage with her litter. No ill effects have been noted with respect to the pups' ability to feed after milk collection.

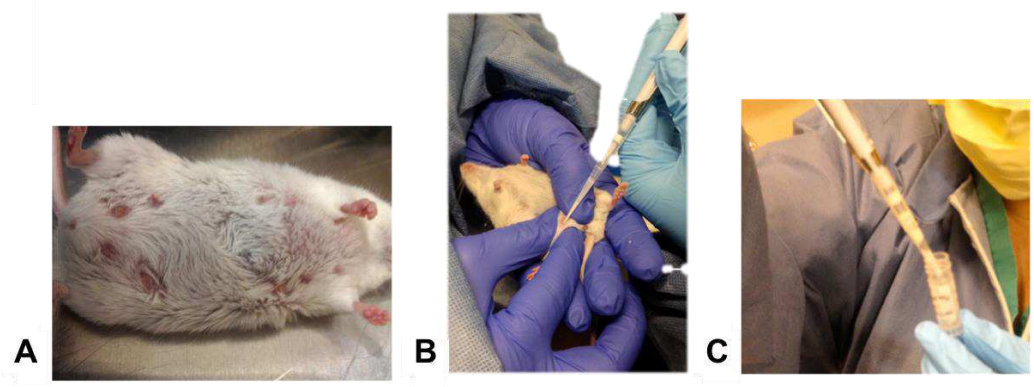


Figure 4.1. Milk collection in the mouse.

A) The dam's teats become engorged with milk upon oxytocin injection. B) Collection of milk from an anesthetized dam using a P-200 pipetman. C) Ejection of milk into a 1.2 ml cryovial.

Muntjac

Separation of the doe from offspring

2.1.1 Select the doe to be milked. Reeves' muntjac deer generally give birth to one fawn (2). Milk the doe the day after birthing to allow the fawn opportunity to gain access to essential nutrients in colostrum.

Note: The fawn usually hides and does not reappear during the procedure; separation of the mother-offspring pair is not necessary.

Administration of oxytocin, anesthesia and eye lubricant

2.2.1 Administer 10 IU/kg of Oxytocin intramuscularly (IM). Oxytocin is a hormone that acts on the mammary glands of lactating females to stimulate the let-down of milk. Oxytocin can be acquired from a veterinarian.

2.2.2 Administer the BAM anesthetic IM[11] containing butorphanol 0.45 mg/kg, azaperone 0.25 mg/kg, medetomadine 0.07 mg/kg. One dose of the BAM anesthetic mixture will sedate a muntjac for approximately 20-30 minutes, which provides ample time for complete milk collection. Another option for anesthetic is midazolam at 1-2 mg/kg, which will sedate a muntjac for approximately 10-15 minutes. While this provides adequate time for milk collection, this anesthetic is metabolized more quickly than BAM and the animal may attempt to escape at the end of the shorter time period.

Note: Minimal response to pain, minor reflexes upon touching the ears, followed by the ability of R1 to hold the MJ without struggle indicates that the animal has been successfully anesthetized.

Note: In the case of a muntjac not being fully anesthetized with the original dosage, the researcher may administer another ¼ or ½ of the original dose of the anesthetic mixture depending on the size of the animal and/or level of sedation that has already been achieved. However, this could increase the chance of an adverse reaction to anesthesia, and also may result in the muntjac being more deeply anesthetized and/or anesthetized for a longer period of time.

2.2.3 Apply a small amount of lubricant to the corner of each eye and spread to prevent the muntjac's eyes from drying out while under anesthesia.

Milk Collection

Note: The milk collection is most easily performed with two researchers; one researcher to hold the anesthetized muntjac in the lap while manually expressing the milk (Referred to as **R1**), and a second researcher to keep the muntjac's head in a comfortable and safe position and to collect the milk (**R2**).

2.3.1 Depending upon the anesthetic used, milking should be performed either 1) in their pens to provide a safe place for them should they wake up during the procedure (midazolam-induced) or 2) in a bedding-free anteroom to maintain cleanliness (BAM-induced).

2.3.2 **R1** and **R2** should be equipped with ethanol-soaked gauze and several 15 ml conical polypropylene tubes to hold the milk.

2.3.3 As the oxytocin begins to take effect, milk letdown will become visible in the muntjac's mammary area. **R1**: Sanitize gloved hands and fingers, as well as the entire udder and each individual teat of the animal with the ethanol-soaked gauze. **R2:** Hold the muntjac's head in a safe and comfortable position to allow ease of breathing and help prevent rumen regurgitation. Hold the collection tube close to the teat being milked without touching it to prevent potential contamination.

2.3.4 **R1**: Express milk from one teat by using the thumb and forefinger to gently squeeze the mammary tissue in an upward motion until the teat is engorged with milk, and gently roll the milk out of the teat and into the tube that R2 is holding (see figure 4.2).

Note: The milk comes out in a stream, so it is important for R2 to have the collection tube positioned so that little to no milk will be wasted.

2.3.5 The muntjac's udder is divided into quadrants. **R1:** Continue to express milk from each quadrant until the milk no longer flows easily. If milk cannot be expressed from one teat, express from all other teats before returning to the first. It is both acceptable and useful to come back to and continually express milk from the same teat should it produce more milk than another.

Note: It is common for midazolam-induced doe to begin to wake up during the milking process. In most cases, the doe will attempt to escape before all the milk has been expressed. Minor restraint is used in an effort to increase the milk yield; however the safety and comfort of both the researchers and the doe are equally important.

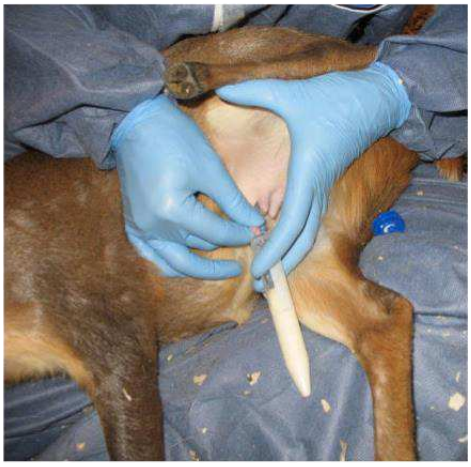


Figure 4.2. Muntjac milk is collected into a 15 ml conical tube.

Anesthetic reversal in the doe

2.4.1 Administer atipamezole at 2.5 mg/kg medetomadine subcutaneously for rapid reversal of BAM anesthetized doe.

Note: With both types of anesthetic, it is necessary to maintain close observation of the doe until she is able to walk on her own without stumbling or falling, and is able to eat and drink without fear of aspiration or choking. No ill effects have been noted with respect to the fawn's ability to nurse after milk collection.

Results

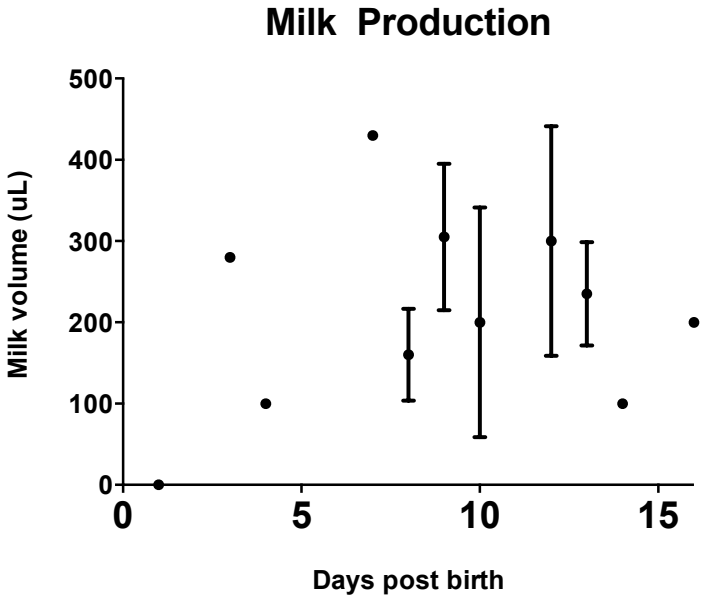
Mouse

From our experiments, we have determined that it is possible to collect approximately 100-400µl) of milk from one laboratory mouse, dependent on several variables. These variables include 1) the amount of time set aside for collection, 2) the dose of oxytocin administered, 3) how many pups the dam is currently nursing and 4) the amount of time post parturition. Our studies have shown that the highest yield is obtained when the dams are separated from their offspring for at least two hours, and at least 45 minutes to an hour is set aside per mouse for milking. Maximum milk volumes were harvested 8-12 days post parturition using 2 IU/kg of oxytocin. Our results are

mostly in accordance with other studies where litter size was positively correlated with milk yield [8]. (Figure 4.3.)

Muntjac

Although the amount of milk harvested from the muntjac was variable from day to day, we were able to collect 5 to 30 ml milk/dam/session, depending on the anesthesia used and/or time post parturition. Muntjac deer are polyestrous; milk can be collected at various time points throughout the year [2] (see Table 4.1.) Midazolam-induced doe remained under anesthesia for as little as ten minutes, allowing for smaller volume collections (typically 5-30 ml), whereas BAM-induced doe sustained a longer period of anesthetic effect (20-30 minutes), allowing complete milk expression (15-130 ml). Milk collections were discontinued when milk was no longer being produced in sufficient amounts by the doe, and the corresponding fawn was being nourished on a diet of mostly hay forage.



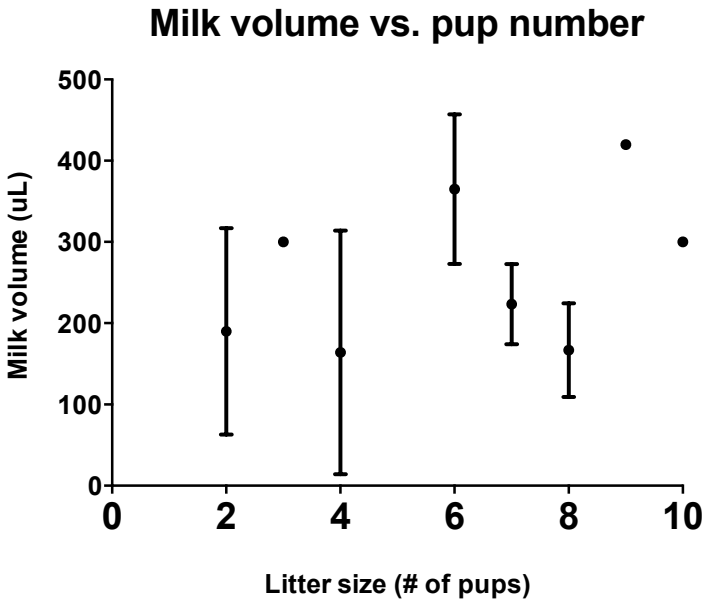


Figure 4.3. Approximate milk yield based on litter size, and age of pups post parturition.

Table 4.1. Milk collection start and finish dates for lactating Reeves' muntjac dams.

Muntjac #	Date started	Date Finished	Total days
19	06.10.08	11.06.08	150
16	07.02.08	11.06.08	128
11	05.05.09	06.25.09	52
19	04.01.10	05.20.10	50
44	04.06.10	04.23.10	18
45	04.06.10	05.20.10	44
77	01.05.12	04.19.12	106
75	01.03.12	04.19.12	108
73	01.03.12	04.19.12	108
76	02.08.12	04.19.12	71
111	09.13.12	10.30.12	48
		Average	**80.27**

Discussion

Mouse

There are several factors to take into consideration when collecting milk from a mouse, including 1) the amount of time set aside for collection, 2) the dose of oxytocin administered, 3) how many pups the dam is currently nursing and 4) the amount of time that has passed since parturition at the time of collection. Using previous studies as guidelines, we set out to optimize conditions for milk yield.

Previous research has directly correlated milk yield with litter size [9]. Unfortunately, litter size is a variable that cannot be controlled. However, the time of milking post parturition can be controlled. According to *The Laboratory Mouse* maximum milk secretion is at 8-10 days post parturition [8]. Our experience is in congruence with this statement.

It has been shown that oxytocin plays an irreplaceable role in milk production post parturition [10]. Oxytocin doses ranging from 0.1 IU/kg (9) to 4 IU/kg (6) have been reported for

collection of milk from rodents [6, 7, 9]. We began our study by administering the lowest dose suggested, 0.1 IU/kg. Using this dosage, the amount of milk produced by the mouse was too small to measure or collect. Upon increasing the oxytocin dose to 2 IU/kg, milk yield was considerably higher at approximately 120 µl. Because we wanted to use the lowest dosage of oxytocin to achieve optimal milk collection, we did not administer doses higher than 2 IU/kg.

Milk yield is also dependent upon the amount of time set aside for collection. We have determined that a minimum of three hours is necessary for optimal collection—two hours for the pups to be separated from the dam and one hour for material preparation and collection. Collecting milk from several dams in one session will decrease the overall time necessary to harvest a specific volume of milk i.e., two hours to separate pups, 30 minutes to setup materials and 20-30 minutes to collect milk from each dam. Each dam must be monitored until they are awake before being placed back with their pups. We have noted that the more time set aside for milking leads to higher milk yields, because the mouse can be milked until she regains consciousness.

Instances do arise with animal models that will affect milk yield. One common issue is the presence of blood or exudate in the milk of older dams that have been bred repeatedly and have large litters, or in dams that are nursing their first litter. Should this occur, we suggest discontinuing milk collection from that particular teat. It is advisable to switch pipet tips at this time, and discard any exudate or blood.

Researchers carrying out this method should also be aware that, as is often the case when using animal models, results vary (Figure 4.3). If a less than optimal amount of milk is collected in one session, it is preferable to repeat the procedure at a later time (e.g. 24-48 hours) rather than alter the experimental conditions to force milk release.

Muntjac

The volume of milk collected from Reeves' muntjac dams was dependent upon the anesthetic used. Our initial anesthetic of choice was midazolam. Midazolam provided sufficient

sedation to allow restraint for short periods of time—i.e. if R1 had a calm disposition and could keep the muntjac calm, R2 could take over the milking to increase the yield before the muntjac would awaken— the safety and comfort of both animal and handler were of utmost importance and we abandoned the milking effort if we felt either was compromised. We discovered that muntjac doe quickly become tolerant to midazolam and that higher doses would be necessary after repeated exposure, making milk collection twice weekly increasingly difficult.

The shortage of midazolam, coupled with a building tolerance, led us to research an alternative anesthetic. A mixture of Butorphanol, Azaperone and Medetomadine (BAM) is effectively used for the immobilization of white tail deer [11]. In our hands, the BAM cocktail provided sufficient anesthesia to allow collection of acceptable volumes of milk in a safe manner, with less drug tolerance after repeated use, and with better control in reversing the effects of the anesthetic.

Some muntjac dams suffered from sensitive udders and/or were first-time mothers. These muntjac developed bruising on the teats during the milking process, and the milk flow slowed or stopped completely, prompting us to discontinue the ongoing session. Should this occur, one should use a more gentle milking technique in an attempt to prevent discomfort or bruising in the teats.

Milk production appeared to wax and wane on several occasions. Low milk harvests may be caused by 1) the fawns having nursed just prior to collections or 2) a decline in milk production due to the natural process of fawn weaning.

While it can be a trying process to obtain a sizeable volume of milk from small animal models, our work illustrates that it is indeed a possibility for both mouse and muntjac models; neither requiring extensive skill nor deep pockets, simply perseverance.

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CONCLUSION

Through this work, we have gained valuable insight into the transmission of prion diseases, not only for chronic wasting disease, but into other prion diseases as well. Specifically we have learned that—1) infectious prions do not negatively impact the ability of dams to breed with naïve intact males nor the outcome of their offspring with regard to litter size and survival; 2) offspring born to CWD-positive dams at early and late timepoints in disease do not exhibit clinical signs throughout their lifetime but may harbor conversion competent prions in their brains and spleens, contributing to a silent carrier state in asymptomatic populations of prion-impacted individuals; 3) CWD-positive dams at clinical stages of infection do not pass infectious prions into their milk during suckling periods; and 4) Low levels of prions are present in fetal sac, placenta, and fetus collected *in utero* from CWD-positive dams.

It has long been speculated that maternal/vertical transmission accounts for the high transmission efficiency of CWD throughout populations. Our previous studies in CWD-transmission from cervid mothers with epitheliochorial placentation showed decreased viability of offspring, accompanied by a gradual onset of recognizable symptoms that eventually resulted in terminal clinical disease [1]. The work of this thesis shows that the placental structure of the infected individual may be an important factor in maternal transmission of prions: we report that in a CWD model with *hemochorial* placentation, very low levels of prions are detected in otherwise healthy, asymptomatic offspring born to CWD-positive mouse mothers. Correlated to our findings, infectious prions have been identified in pregnancy-related tissues, such as uterus, ovary, and placenta of CJD-positive women [2, 3](who also possess hemochorial placentation), yet there has been no diagnosis in children born to symptomatic CJD-positive mothers [4]. Although there are obvious differences between mouse and human aside from their placental structure, there is one message that is completely clear from this correlation: diagnosis of TSE disease cannot rely on clinical symptoms

alone, as covert prion transmission may result in a small population of asymptomatic, silent prion carriers. These silent carriers may or may not influence the increase of prion-positive species of any given population, but this information does stimulate new questions regarding the ever-evolving knowledge of prion diseases. For one, can we still define prions as uniformly fatal if there is a population of individuals carrying prions that may never succumb to clinical disease? This remains to be seen.

FUTURE DIRECTIONS

Naturally, the insight that we have gained through this work opens the door for a new subset of questions, providing new ways to assess and potentially elucidate these mechanisms once and for all. The recent advent of a gene-targeted mouse species by our collaborator, Glenn Telling (unpublished) may prove to be a more suited model for the experiments conducted in this study. In order to discern the potential for use in our future studies, our most immediate "future" work entails characterization of cellular prion protein expression in several tissues including reproductive, mammary, spleen and brain collected from this gene-targeted mouse, and comparing that back to expression patterns in FVB wild type, PrP-knockout, and transgenic mice, including the Tg(CerPrP-E226) mouse used in this study.

We will also continue to assess and characterize maternal transmission in the native host, focusing on localization and infectivity of PrP^{res} in tissues collected from cervid mother:offspring pairs, as well as gaining insight as to the culprit behind decreased offspring viability. Additionally, we aim to elucidate novel mechanisms in transmission of CWD, including transmission through the germ line from a mother and father to their offspring.

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