THESIS

THE ROLE OF PLANTS AS AN ENVIRONMENTAL RESERVOIR OF CHRONIC WASTING DISEASE PRIONS

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ABSTRACT

THE ROLE OF PLANTS AS AN ENVIRONMENTAL RESERVOIR OF CHRONIC WASTING DISEASE PRIONS

Transmissible Spongiform Encephalopathies (TSEs) are a group of diseases caused by an abnormal version, PrP^{RES}, of the normal cellular host protein prion protein (*Prnp*) termed PrP^{C}. Disease is fatal resulting in amyloid deposits and spongiform degeneration in the brain in most but not all cases. Clinical signs can include wasting, increases in salivation, and general motor impairment but many other clinical signs exist and can vary between TSEs. PrP^{RES} is incredibly resistant to inactivation and can withstand radiation, formalin treatment, and autoclaving to name a few tried decontamination methods whereas PrP^{C} is degraded normally. This difference in degradation allows for differentiation between the two protein forms as PrP^{RES} is resistant to degradation by Proteinase K. In the early 1980s this abnormal protein was discovered to be the sole causative agent of the various TSEs which at the time was a novel finding and a novel method of disease transmission. It is thought that slightly misfolded forms of PrP^{C} occur which can then misfold further eventually forming PrP^{RES}. PrP^{RES} then has the ability to act as a template for conversion, converting PrP^{C}. Numerous TSEs exist that affect both humans and a variety of animals. One of the animal TSEs is Chronic Wasting Disease (CWD) which affects cervids such as elk, deer, and moose (*Cervus candensis, Odocoileus hemionus, Alces alces*) and has become endemic in both free-ranging and captive herds. The exact mechanisms behind spread of CWD are unknown but research has shown that environmental reservoirs play a role in transmission dynamics. We chose to explore whether PrP^{RES} can be detected on or inside grasses and plants naturally exposed to prions in CWD endemic areas by use of Protein Misfolding Cyclic Amplification (PMCA). Here we present novel environmental evidence showing that PrP^{RES} can be found on the surface of multiple plants from Rocky Mountain National Park and mice inoculated with these samples are showing clinical signs of disease.

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CHAPTER 1 – INTRODUCTION

Prion Diseases

Identification and Classification of Transmissible Spongiform Encephalopathies

Prion diseases comprise a wide class of diseases affecting both humans and animals. Often, prion diseases are referred to as Transmissible Spongiform Encephalopathies (TSEs) named largely due to similarities in neuronal degeneration, astrocytic gliosis, and vacuolation, or spongiform degeneration, seen in all diseased brains (Collinge, 2001; Beck & Daniel 1987; Gajdusek & Zigas, 1959; Hadlow, 1959, 1999; Pattison, 1957; Pawel, 2004). The story of how individual, seemingly disparate diseases became linked and identified as having the same causative agent is quite interesting. Initially the connection was based on clinical observations and postulations. William Hadlow, a United States Department of Agriculture employee studying pathology at the time, made the astute observation in 1959 that many disease hallmarks were shared between an animal TSE, Scrapie (affecting sheep), and a human TSE called Kuru. In the very same communication Hadlow expressed uncertainty if these two TSEs were even infectious considering the inability to identify a causative agent (Hadlow, 1959). Unbeknownst to Hadlow, researchers elsewhere were already trying to elucidate the very same question of whether the diseases they were seeing were infectious or transmissible. Demonstration of infectivity or transmissibility would imply the presence of an infectious agent. At this point in time it was postulated to perhaps be due to a slow virus (Sigurdsson, 1954). In 1936 Scrapie was shown to be transmissible to goats and again in 1954 when positive sheep brain homogenate was used to inoculate naïve goats and 100% of the animals inoculated developed disease proving transmission of an infectious agent (Cuille & Chelle 1936; Pattison, 1957). Brain material from Scrapie positive animals upon inoculation into mice also caused hallmark encephalopathy (Chandler, 1961). The ability to transmit a human TSE, Kuru, to three chimpanzees solidified the evidence that these diseases were at least transmissible even though

no infectious causative agent could be isolated (Gadjusek D. C, 1966). Multiple other human TSEs were shown to also be transmissible in a variety of animal hosts (Gibbs, Gajdusek, Asher, Alpers, & Beck, 1968; Manuelidis, Kim, Angelo, & Manuelidis, 1976; Masters, Gajdusek, & Gibbs, 1981). Around this time researchers started to link these diseases together under the term "transmissible dementias" (Collinge, 2001).

The Prion Protein

The causative agent of all TSEs was not identified as a virus or microorganism; rather, a misfolded isoform of a normal cellular host protein called the prion protein - PrP^{C} (S B Prusiner, 1982). The identification of this protein as the causative agent of TSEs was quite a challenge mainly because scientists had never considered the idea that something lacking nucleic acid could be infectious. There were many indications that the agent was unusual. In scrapie, lack of inactivation by numerous methods including but not limited to boiling in water for hours, treatment with chloroform for hours, treatment with formalin, freezing and thawing, and treatment with ultraviolet radiation was shown (T Alper, Cramp, Haig, & Clarke, 1967; Tikvah Alper, Haig, & Clarke, 1966; I. H. Pattison & Millson, 1961a, 1961b; IH Pattison, 1965). These observations led to some researchers to speculate the agent was devoid of nucleic acid even though this was a preposterous albeit novel idea (T Alper et al., 1967). By utilizing sedimentation techniques it was determined that the agent causing Scrapie was independent of cell membranes (S B Prusiner, Hadlow, Eklund, & Race, 1977). These techniques allowed for partial purification of the Scrapie agent, identification of hydrophobic domains, and observation of aggregation of proteins contained therein (S B Prusiner et al., 1978). By 1981 researchers were getting very close to determining that the causative agent was protein only. Prusiner demonstrated partial inactivation of the agent when treated with proteinase K (PK) confirming that a protein was part of the agent. However, the researchers still continued to look for evidence of nucleic acid,

"Does a genome coding for the scrapie agent protein reside within the infectious agent itself or is the genome part of the cellular genetic material? Will the unusual properties of the scrapie agent, which distinguish it from conventional viruses, reveal unprecedented mechanisms of replication?" (S B Prusiner et al., 1981).

A year later in 1982 Stanley Prusiner published seminal findings illustrating a wide range of evidence over the years concluding that protein was the only causative agent. He coined the term prion derived from proteinaceous infectious particles (S B Prusiner, 1982). Further work identified that a PK resistant form of the protein termed PrP^{RES}, around 27,000 – 30,000 Daltons, was only seen in infected brains when purified and then visualized using electrophoretic gels and radioautographic exposure. Intermediate isoforms of PrP have been found that are insoluble but sensitive to PK (Gabizon, 1996). Previous PK sensitivity was most likely due to extremely long treatment times (Bolton, McKinley, & Prusiner, 1982; S B Prusiner et al., 1982). This group went on to determine that PrP was the only protein and structural component of Scrapie (Michael P. McKinley, Bolton, & Prusiner, 1983). A new purification protocol decreased loss of infectivity previously seen when purifying PrP and showed evidence of PrP oligomers when visualized using electron microscopy. These rods weren't seen in purifications made from healthy animals. Additionally, structures showed positive Congo Red staining and green birefringence indicative of amyloid (Bhaduri, Nogami, & Van Dijk, 1962; G. G. Glenner, Eanes, Bladen, Linke, & Termine, 1974; Stanley B. Prusiner et al., 1983). Amyloid is composed of protein that is filamentous and has a tendency to aggregate and deposit in tissues causing destructive pathology (Eanes & Glenner, 1968; Virchow, 1854). X-ray diffraction and infrared spectroscopy studies performed on amyloid filaments showed a structure that was composed of β-pleated sheets (Eanes & Glenner, 1968; Termine, Eanes, Ein, & Glenner, 1972). The presence of amyloid, which had been previously reported in some of the other TSEs, added to the growing pile of evidence of the causative agent being a prion (Gajdusek & Zigas, 1959; G. G. Glenner et al., 1974; G. Glenner, 1980; Masters et al., 1981; Stanley B. Prusiner et al., 1983; Elizabeth S Williams & Young, 1980). Further proof that PrP^{RES} was, at the very least, the Scrapie agent was confirmed when purified protein was injected into rabbits and immune

serum showed an antibody reaction. No antibody reaction was seen when immune serum and purified protein from uninfected brains were placed together. Immunocytochemistry was also performed with PrP serum allowing for visualization of amyloid plaques seen only in the brain of hamsters infected with Scrapie. These amyloid plaques were composed of PrP fibrils upon further observation (P E Bendheim, Barry, DeArmond, Stites, & Prusiner, 1984; Dearmond et al., 1985). In 1985 it was determined that PrP was encoded by a cellular gene found in both normal and infected animals. A larger polypeptide PrP 33-35 was found and determined to be the precursor to PrP 27-30 (PrP^{RES}) prior to PK digestion. In normal brains purifications this precursor protein, PrP 33-35 (PrP^{C}), shows complete sensitivity to PK. PrP DNA sequences were also identified in goats, rats, *Drosophila*, and the nematode. (Oesch et al., 1985; Westaway & B.Prusiner, 1986). Resulting data determining that PrP^{RES} could be purified from the brains of Creutzfeldt-Jakob Disease (CJD) sufferers linked Scrapie and CJD together. From there it was just a matter of determining that the remaining TSE's had the same causative agent, forever linking these diseases together (Paul E Bendheim, Bockman, McKinley, Kingsbury, & Prusiner, 1985; Bockman, J, 1985; S B Prusiner, 1986).

To fully understand TSE's a more thorough explanation of the PrP^{C} and PrP^{RES} proteins is needed. The prion gene complex, *Prn*, includes the prion protein gene, *Prn-p*. Originally it was thought to also include a separate gene with direct effects related to incubation time of disease – *Prn-i* but later it was shown that *Prn-i and Prn-p* are the same gene (Carlson et al., 1986; Richard C Moore et al., 1998). PrP^{C} is membrane associated protein with a glyco-phosphatidylinositol anchor whereas PrP^{RES} 33-35 is found within the cell (Borchelt, Scott, Taraboulos, Stahl, & Prusiner, 1990; Meyer et al., 1986; Price, Sisodia, & Borchelt, 1998; Stahl, Borchelt, Hsiao, & Prusiner, 1987). PrP^{C} mRNA can be found in hamsters as early as one day after birth after which transcript levels increase and remain steady state. mRNA from the protein is found in highest concentrations in the brain with expression primarily found in neuronal cells

(Kretzschmar, Prusiner, Stowring, & DeArmond, 1986; M P McKinley, Hay, Lingappa, Lieberburg, & Prusiner, 1987; Oesch et al., 1985). Neurodegeneration is a downstream effect of the high levels of PrP^{C} protein found in the brain that serve as a major site for PrP^{RES} to seed, misfold, and replicate eventually forming amyloid plaques. The exact mechanisms of how PrP^{RES} seeds conversion and propagates itself are still unclear but it's thought that post-translational modification plays a role in initial derivation of PrP^{RES} (Borchelt et al., 1990; B. Caughey & Raymond, 1991). The current mechanistic hypothesis postulates that intermediate or heterodimeric forms of PrP^{C} occur. These then misfold further into PrP^{RES} which acts as a template causing other PrP^{C} molecules to misfold (Chothia & Janin, 1975; Collinge, 2001; Jarrett & Lansbury, 1993; S. Jones & Thornton, 1996; Stanley B. Prusiner et al., 1990). This model makes sense for numerous reasons which include: 1) High concentrations of PrP^{RES} form in the brain where the highest amounts of PrP^{C} are found and 2) Lack of PrP renders animals resistant to disease (Büeler et al., 1993; Sailer, Bueler, Fischer, Aguzzi, & Weissmann, 1994). In a cell-free assay, conversion of PrP^{C} to the misfolded form occurs when PrP^{RES} is introduced. Recombinant *Escherichia coli*-derived PrP^{C} was also able to be converted in the same assay. Many other studies also illustrate propagation of PrP^{RES} independent of nucleic acid (Bessen et al., 1995; B Caughey, Kocisko, Raymond, & Lansbury, 1995; Nathan R Deleault, Harris, Rees, & Supattapone, 2007; Kirby, Birkett, Rudyk, Gilbert, & Hope, 2003; Kocisko et al., 1994; Legname, Baskakov, & Nguyen, 2004; Sparrer, 2000). The origin of PrP^{RES} in each TSE can vary. The main routes of acquiring the disease causing agent involve inheritance of a mutation in *Prnp* (all autosomal dominant), spontaneous generation, or contact with an infectious form (McKintosh, Tabrizi, & Collinge, 2003)

Animal TSEs

To date, the animal TSEs include Scrapie, Bovine Spongiform Encephalopathy (BSE), Chronic Wasting Disease (CWD), Transmissible Mink Encephalopathy (TME), and Feline Spongiform Encephalopathy

(FSE). All of these diseases present with the characteristic pathologies of prion disease including spongiosis, neuronal degeneration, with or without a vacuolated appearance, and lastly an increase in astrocytes and increased abnormalities of these cells. Ultimately at terminal stages of disease evidence of PrP^{RES} is found accumulated in the brain (Hadlow, 1999).

Scrapie was the first animal TSE to be described and was the model disease for many of the initial TSE studies. Sheep present with numerous clinical signs including but not limited to scraping of the skin, hyper-activity, and weight loss. Further examination of terminal disease shows astrocytosis followed by neurodegeneration (Abinanti, 1967; McGowan & Scott, 1922). It's been shown that genetic differences play a role in resistance or susceptibility to infection in certain breeds of sheep (Hunter et al., 1997). Transmission of Scrapie was proven, by accident, in 1937 when formalin-treated sheep brain tissue meant to provide protection against a common virus unknowingly contained PrP^{RES} (Cullie & Chelle, 1939). In 1986 BSE was first reported and immediate similarities to Scrapie and other human TSEs were noted (Wilesmith, Wells, Cranwell, & Ryan, 1988). Fibrils found in the brain contained bovine PrP^{RES}. BSE spread throughout cattle in Great Britain and had a wide economic impact. Cattle are thought to have been exposed to PrP^{RES} through contaminated feed purported to come from a scrapie infected sheep offal (Collinge, 2001; Hadlow, 1999; G. A. H. Wells et al., 1987; G. A. Wells, Wilesmith, & McGill, 1991). Across the pond in the United States a TSE affecting deer and, later shown to affect elk, was being described in 1980. Clinical signs included severe weight loss, or wasting, listlessness, increased salivation, and urination. The hallmark neurological pathology was seen when histological sections of affected brains were analyzed (Elizabeth S Williams & Young, 1980). CWD is the only TSE to affect both free-ranging and captive populations (Spraker et al., 1997). TME seems to be a result of the commercialization and ranching of mink for their pelts. Due to the increase in farming mink feed was developed and sold to many farms. Occurrences of TME were occurred at random and isolated with the

only constant factor being the mink feed. Clinical signs in mink seem to differ from the other TSEs in that the mink initially tended to become more hyperactive and aggressive (Hadlow, 1999; Harstough & Burger, 1965; McKenzie, Bartz, & Marsh, 1996). Lastly, FSE, a TSE affecting felines was identified in 1990 (Gruffydd-Jones, Galloway, & Pearson, 1991).

Human TSEs

Human prion diseases are as fatal as their animal counterparts. They fall under three categories: sporadic, inherited, or iatrogenic (Collinge, 2001; Soto, 2006) Creutzfeldt-Jakob Disease (CJD) was remarked to be a new disease due to distinct differences seen during post-mortem examination compared to other neurological or late stage dementias. It is one of the earliest human TSEs to be reported. The disease is found in middle to late age adults and was proven to be transmissible, confirming it as a TSE in 1968 (Creutzfeld, 1920; Gibbs et al., 1968; Jakob, 1921; May, 1968; Soto, 2006). Kuru reported in 1957, was one of the first human TSEs described as having similarities to an animal TSE – Scrapie (Hadlow, 1959). Like many other TSEs it was characterized by degeneration of the central nervous system. To date this is the only prion disease spread by cannibalism (Brown, 1990; Collinge, 2001; Gadjusek D. C, 1966; Gajdusek & Zigas, 1959; Hadlow, 1959; Haïk & Brandel, 2014). Mutations in the *Prnp* gene are the cause of Gerstmann-Straussler-Scheinker syndrome (GSS) which was named for the researchers who discovered it. Traditionally it has a prolonged disease course, much longer than CJD, but it still occurs late in life (Collinge, 2001; Hsiao et al., 1989; Soto, 2006). While amyloid plaques are a hallmark of disease they are seen more frequently in GSS versus CJD patients (Kitamoto, Tateishi, & Tashima, 1986; Kübler, Oesch, & Raeber, 2003). Fatal Familial Insomnia (FFI), is another autosomal dominant *Prnp* disorder. As the name suggests patients experience a progressive inability to sleep, which ultimately results in fatality (Lugaresi, Medori, Montagna, Baruzzi, & Cortelli, 1986; Montagna, Cortelli, Gambetti, & Lugaresi, 1995). Variant-Creutzfeldt-Jakob Disease (vCJD) presents as an atypical

variant of CJD with early onset seen in teenagers and young adults. This variant form is postulated to have occurred due to BSE transmission to humans. (Bruce et al., 1997; Diack et al., 2014; M. R. Scott et al., 1997, 1999). A distinctive florid plaque is noted in vCJD. This was also noted when BSE was inoculated into macaques supporting the idea that BSE prions are the probable cause of vCJD (Lasmezas, Deslys, & Demalmay, 1996).

TSE detection assays

Detection of a TSE is quite challenging. Clinical signs, family history, and surgical records are often the first paths to detection. Confirmation is still done post-mortem in both humans and animals. Because PrP^{RES} is derived from the host, it isn't recognized as a foreign agent and an antibody response isn't mounted. This eliminates the possibility of screening humans and animals for titers to the abnormal protein. While terminal disease often results in massive accumulation of PrP^{RES} in brain tissue many other tissues and body fluids often lack any indication of abnormal prion accumulation (Kübler et al., 2003). In terminal cases, gold standards for detection are visual observation of the 'TSE Triad, Immunohistochemistry (IHC), Western Blotting (WB), and bioassay of brain material into a susceptible host to show the presence of infectious transmittable particles (Hadlow, 1999; Hnasko, Serban, Carlson, Prusiner, & Stanker, 2010; MacGregor, 2001; Pawel, 2004). IHC allows for visual confirmation of PrP^{RES} and overall distribution throughout the brain and has been utilized to analyze lymphoid tissues in TSE's with known lymphotropism (Kübler et al., 2003; Sigurdson et al., 1999). Because the sequence of PrP^{C} and PrP^{RES} are identical it was difficult to develop antibodies that only recognize one form of the protein. The development of monoclonal antibodies that recognize PrP^{RES} specific antigen and species specific antigen have been very beneficial to the field as they allow for detection of disease (Furuoka et al., 2007; Jeong et al., 2012; Korth et al., 1997). In order to distinguish between the disease-associated form we take advantage of the resistance to PK and the resulting shift in size on an SDS-PAGE gel when

western blotted (P E Bendheim et al., 1984; Michael P. McKinley et al., 1983). Western Blot detection is limited by the amount of protein present in the sample (MacGregor, 2001; Natallia Makarava, 2008). In CJD cases, other proteins indicative of disease (14-3-3 for example) can be screened for in cerebrospinal fluid. Other neurological screening methods and accumulation of clinical signs also help support diagnosis (Diack et al., 2014; Kübler et al., 2003; Poser et al., 2000; Zerr & Poser, 2002). Animal bioassays were and still are an essential part of the field. Initially they were used to quantify titer, prove transmission, and infectivity. In the early 1980's animal bioassays were used repeatedly to generate large amounts of infectious brain tissue which eventually lead to the identification of PrP (Bolton et al., 1982; Michael P. McKinley et al., 1983; S B Prusiner, 1982; S B Prusiner et al., 1981, 1982; S B Prusiner, Groth, Bolton, Kent, & Hood, 1984). Limitations with animal bioassay mainly involve the amount of time it takes for clinical signs and terminal disease to occur, but other factors include species barriers between original inoculum and animal care and cost (Kübler et al., 2003). The use of genetic engineering has helped to bridge species barriers, slow incubation hurdles, and reduce the cost of animal handling. Mouse models are used frequently having been genetically modified to include different species PrP as well as varying amounts of expression levels for a more rapid course of incubation and endpoint of disease. Development of knockout mice allowed for better understanding of the cellular form of PrP and its role in PrP^{RES} propagation (Büeler et al., 1992, 1993; Kübler et al., 2003; Lledo, Tremblay, DeArmond, Prusiner, & Nicoll, 1996; Manson et al., 1999; R. C. Moore et al., 1995; Sailer et al., 1994; M. Scott et al., 1989).

However, in suspected cases, instances lacking the stereotypical accumulation in brain tissue, asymptomatic cases, population monitoring, diagnostic development, and pre-clinical shedding detection, the gold standards don't work. The amount of abnormal protein is often too low to be detected in the brain prior to terminal disease but tissues may be infectious at this point (by bioassay).

Clinical disease signs don't always present even when detectable levels of PrP^{RES} occur (Bueler et al., 1994; Frigg, Klein, Hegyi, Zinkernagel, & Aguzzi, 1999; Hill & Collinge, 2003; R. Race, Raines, Raymond, Caughey, & Chesebro, 2001; Thackray, Klein, Aguzzi, & Bujdoso, 2002). Trying to analyze other tissues or body fluids for accumulation can be as challenging as hunting for a needle in a haystack despite the fact that accumulation occurs in skeletal tissue, muscle, and lymphoreticular sites of mice and hamsters both orally and intra-cranially inoculated (Bosque, Ryou, & Telling, 2002; Bueler et al., 1994; Kübler et al., 2003; Rubenstein et al., 2010; Thomzing, Kratzel, Lenz, Kruger, & Beekes, 2003). Inhibitors in blood products and lack of findings in spinal fluid also make early detection near impossible (Brown et al., 1965, 1999; Chang et al., 2007; Edgeworth et al., 2011; Rubenstein et al., 2010; Tattum et al., 2010; Wadsworth et al., 2001).

These lapses in detection ability put more lives at risk. Blood donation, reuse of surgical tools, and hormones derived from humans are all ways transmission of human TSEs have occurred in the past (Hewitt, Llewelyn, Mackenzie, & Will, 2006; Llewelyn et al., 2004; Peden, Head, Ritchie, Bell, & Ironside, 2004; Wroe et al., 2006). The latter issues in conjunction with caveats listed in the former paragraph resulted in the development of a number of assays capable of increased sensitivity and the ability to not only detect but amplify low level samples. For this body of work, I will focus on two *in*-vitro amplification assays: protein misfolding cyclic amplification (PMCA) and quaking-induced conversion (QuIC).

Protein Misfolding Cyclic Amplification

In the late 1980s through the 1990s multiple studies showed evidence of amyloid fibrils self-seeding further amyloid formation and sped up formation *in-vitro* in the presence of preformed fibrils (Ganowiak, Hultman, Engstrom, Gustavsson, & Westermark, 1994; Jarrett, Berger, & Lansbury, 1993; Johan et al., 1998; Niewold, Hol, van Andel, Lutz, & Gruys, 1987; Snow & Kisilevsky, 1987). Development

of amyloid is often impeded by a rate-determining step often referred to as nucleus formation. Once the nucleus is formed, monomer additions occur quite rapidly due to favorable thermodynamics and multiple sites for growth; however, lag times still occur and are dependent on protein concentration and initial nucleus size. Addition of a preformed nucleus 'seed' bypasses some of the lag period especially when saturated and occurs both *in-vitro* and *in-vivo* (Jarrett & Lansbury, 1993). PMCA results in amplification of previously undetectable samples *in-vitro*. First described in 2001, large amounts of PrP^{C} from brain tissue serve as substrate for undetectable amounts of PrP^{RES} in biological or experimental (spiked) samples. The excess substrate gets converted into infectious protease resistant PrP^{RES} aggregates. When sonicated the infectious aggregates break apart and new seeds for conversion are formed continuously expedites amplification. Repetition of this process allows for the previously undetectable samples to accumulate to a level detectable by WB. The original development of this method reported that roughly 97% of PrP^{RES} detected after 5 rounds of amplification was protein misfolded due to the assay. Sensitivity allowed for detection of 6-12 picograms of PrP^{RES} in a sample. Later findings reported the ability to detect 1.3 attograms, about 26 PrP molecules, after seven consecutive rounds of PMCA. The particles generated *in-vitro* retained their infectious ability and conferred disease when inoculated into new hosts intra-cranially (Joaquin Castilla, Saa, Hetz, & Soto, 2005; Joaquín Castilla et al., 2008; Gonzalez-Montalban et al., 2011; Morales, Duran-Aniotz, Diaz-Espinoza, Camacho, & Soto, 2012; Saa, Castilla, & Soto, 2006; Saá, Castilla, & Soto, 2006; Saborio, Permanne, & Soto, 2001a). Amplification of previously undetectable levels of PrP^{RES} revolutionized the field by decreasing the amount of time needed to wait for bioassay and increased sensitivity in diagnosis. After the method was developed and refined; detection increased and was made possible for a variety of samples in which detection was previously unsuccessful. (Joaquin Castilla, Saa, Hetz, et al., 2005; Joaquin Castilla, Saa, & Soto, 2005; Joaquin Castilla et al., 2006; Haley et al., 2013; M. Jones et al., 2007; Kurt et al., 2007; Murayama et al., 2007; T. a Nichols et al., 2013; Pritzkow et al., 2015; Pulford et

al., 2012; Rubenstein et al., 2010; L. Thorne & Terry, 2008). PMCA was used to further study the mechanisms of conversion of PrP^{C} to PrP^{RES} and components required for conversion. Unintentionally, the use of PMCA led to de-novo generation of infectious and transmissible PrP^{RES} in the absence of a starting seed which further solidified the prion protein hypothesis and provided a possible mechanism of sporadic prion disease etiology (N R Deleault, Lucassen, & Supattapone, 2003; Nathan R Deleault et al., 2007; Saa et al., 2006). This spontaneous generation of PrP^{RES}, albeit a rare occurrence, is one limitation of the assay. Other limitations include the continuous need for PrP^{C} substrate and the time to get necessary amplification. Five to six rounds, corresponding to five to six days, of PMCA is faster than traditional bioassay however another amplification assay with an even more rapid amplification exists.

Quaking-Induced Conversion

Perhaps one of the more surprising findings in favor of the prion protein hypothesis were the findings showing that recombinant PrP^{C} (rPrP) has the ability to successfully form amyloid fibrils independent of seeding. rPrP also has the ability to convert into PrP^{RES} when seeded, has been shown to be slightly transmissible, and for all intents and purposes is a good model for understanding biochemical mechanisms of conversion (Baskakov & Breydo, 2007; Baskakov, 2004; Byron Caughey, 2001; Kirby et al., 2003; Legname et al., 2004; Swietnicki, Petersen, Gambetti, & Surewicz, 1997). The ability of rPrP to form amyloid was utilized in development of another amplification assay – Quaking Induced Conversion (QuIC) (Atarashi et al., 2008a). QuIC was originally termed rPrP-PMCA. The rapid amplification and detection technique utilized sonication and other methods analogous to PMCA. The main difference was the use of rPrP which was easier to manipulate and produce high yields of in comparison to PrP^{C}. The assay was able to detect PrP^{RES} from cerebrospinal fluid collected from scrapie infected hamsters and produced consistent sensitivity as low as 50 attograms. While spontaneous generation of amyloid occurred in the absence of PrP^{RES} seeded reactions, these were able to be differentiated from amyloid

formed in seeded reactions by difference in fragment size when visualized by WB after treatment with PK (Atarashi et al., 2007, 2008a). A year later, in 2008, Atarashi not only renamed the method to QuIC but also departed from sonication in favor of shaking. Shaking samples seemingly has the ability to agitate the samples to cause aggregation and denaturation due to liquid-solid interface interaction. Additionally, PrP^{C} and PrP^{RES} are more likely to come into contact by continuously being mixed leading to monomeric addition to fibril ends followed by fragmentation and more seeds for conversion (Collins, Douglass, Vale, & Weissman, 2004; Toth, Smith, & Ahmed, 2009). The presence of other denaturants, compounds, and PrP^{RES} seed in the buffer also encourages amyloid formation. This lead to increased sensitivity and a faster rate of PrP^{RES} amplification – 25-50 attograms were able to be detected after one round. Rate of amplification was also increased at higher temperatures but spontaneous generation of PrP^{RES} occurred more frequently at these higher temperatures as well (Atarashi et al., 2008a, 2008b). The method was further refined (RT-QuIC) to eliminate the need for running a WB by incorporating Thioflavin-T (ThT) in the reaction tube. ThT intercalates into and stains amyloid and the resulting fluorescent excitation shift can be selected for at 450nm. Fluorescence can then be read at periodic time points to track the rate and kinetics of conversion. Due to multiple binding sites on amyloid fibrils for ThT and fluorescence being dependent on binding site number amongst other factors standard curves are used to normalize data (Atarashi et al., 2007; H LeVine, 1993; Harry LeVine, 1999). It was noted that guanidine-HCL (GdnHCL), a denaturant, greatly increased de-novo fibril formation dependent on the concentration used. Denaturing PrP^{C} in the presence of infectious seeds seems to encourage the protein to refold in the misfolded state at a much faster rate; however, addition of too high a concentration of GdnHCL eliminates formation of misfolded protein entirely (Atarashi, Satoh, et al., 2011; Kocisko et al., 1994; Toth et al., 2009). RT-QuIC has since been used for detection of low levels of abnormal prion protein in a variety of samples, quantification of prion seeding dose amounts without needing to perform animal bioassays, better understanding of species barriers, and ante-mortem detection of prion

disease (Atarashi, Sano, Satoh, & Nishida, 2011; Atarashi, Satoh, et al., 2011; Davenport et al., 2015; Haley et al., 2013, 2014; Davin M. Henderson et al., 2015; Orrú et al., 2009, 2014; Orrú, Groveman, Hughson, Zanusso, & Coulthart, 2015; Orrú, Wilham, & Raymond, 2011; Wilham et al., 2010). A few downsides to RT-QuIC include the time it takes to optimize the assay, the generation of seeding proteins versus infectious proteins indicating some modification from the original infectious form, and the fact that substances have been shown to have an inhibitory effect unless diluted to lower concentrations (Wilham et al., 2010).

Chronic Wasting Disease

CWD History

The discovery of CWD happened slowly over time. A population of captive deer being held in wildlife facilities in Colorado were noted to have been experiencing the same group of symptoms from 1967-1979. Clinical signs included weight loss, listlessness, polydipsia, polyuria, low urine specific gravity and hypotonia. Histopathological changes were noted in the brain tissue and included spongiform degeneration and vacuolization (E. S. Williams & Young, 1980). Two years later Williams and Young reported similar findings in Rocky Mountain elk and later noted disease in free ranging animals as well (Spraker et al., 1997; E S Williams & Miller, 2002; E S Williams & Young, 1982; E. S. Williams & Young, 1992). In both papers similarities to other TSE's both human and animal were noted; however, at the time there was no evidence for transmission except some indirect fence contact between animals. This quickly changed when Williams intra-cranially inoculated deer and ferrets with brain-suspensions from affected deer. After an incubation time of 17-21 months' disease followed. Amyloid plaques were also noted in the brains of affected deer (Bahmanyar, Williams, Johnson, Young, & Gajdusek, 1985). An anti-scrapie amyloid antibody that recognized PrP 27-30 immunolocalized to amyloid found in the brains of CWD affected elk and deer providing evidence of CWD sharing the same causative agent as other TSEs

(Guiroy, Williams, Yanagihara, & Gajdusek, 1991). To this day the origin of CWD is still widely contested. It's unclear whether this occurred as a sporadic incident in one or a few free-ranging or captive deer that then spread further. There's been no evidence of this being caused by infected feed or feed products. One line of thought assumes scrapie crossed the species barrier from sheep to deer when animals were housed concurrently in Colorado facilities. This may have then spread to free-ranging animals when they came into contact through fence-to-fence contact with captive animals. (E. S. Williams & Young, 1992). To look for increased susceptibility or resistance to disease in deer and elk numerous studies have been done to look for mutations or variations in the host *Prnp* gene. In mule deer multiple polymorphisms have been identified. The main polymorphisms have been characterized according to frequency in deer populations. The wild-type genotype at codons 95,96, and 138 respectively are glutamine, glycine, and serine (QGS), and other genotypes include QSS, QGN, and a rare allele HGS. A methionine to leucine substitution at codon 132 was also found in Rocky Mountain elk (C. Johnson, Johnson, Clayton, McKenzie, & Aiken, 2003; O'Rourke et al., 1998, 2004; Raymond et al., 2000). A study performed on genotype comparisons between free-ranging and captive Rocky Mountain elk showed a linkage between susceptibility to CWD when homozygous for methionine at codon 132 (O'Rourke et al., 1999). When negative deer from Wisconsin were genotyped the majority of them had genotypes similar to the CWD-positive deer indicating that they would be susceptible to spread of disease (C. Johnson et al., 2003). Evidence that resistant genotypes provide delayed onset or prolonged incubation periods has been shown however these genotypes are rarely found in large populations (Jewell, Conner, Wolfe, Miller, & Williams, 2005; C. Johnson et al., 2006; Kelly et al., 2008). These data allow for surveillance and other wildlife management authorities to be aware of the susceptible populations in their areas and if at all possible try to curtail continued spread based off of this information.

Risk to Humans

Such high prevalence and continued spread of CWD causes public health concern about possible transmission to humans especially after transmission of BSE to humans in the form of vCJD (Bruce et al., 1997; M W Miller et al., 2000; M. R. Scott et al., 1999). Free-ranging infected animals, deer and elk hunting, and consumption of deer and elk meat are all factors that increase risk of exposure to CWD. Thus far there has been no evidence of transmission of CWD to humans, but on a molecular level it seems as though the species barrier may be slight or can be overcome after adaptation. The propensity for molecular conversion of PrP^{C} to PrP^{RES} in a cell-free assay using CWD prions as a seeding source for human, bovine, and ovine PrP^{C} showed slight ability to convert to PrP^{RES} in all three species; however, the conversion was inefficient compared to cervid-to-cervid conversion (Raymond et al., 2000). Initial serial PMCA experiments using CWD PrP^{RES} as inoculum to induce conversion of human PrP^{C} were unsuccessful even when trying known conversion-efficient strategies. However, after passaging CWD PrP^{RES} inoculum by sPMCA using deer PrP^{C} as substrate, the resulting infectious CWD PrP^{RES} was able to convert human PrP^{C} into PrP^{RES} and this form was termed CWD-human PrP^{RES}. PK resistance was seen and this form was confirmed by use of a human specific antibody (with slight cat and hamster reactivity), 3F4, confirming a human rather than cervid protein. To ensure this wasn't a random sPMCA specific event CWD inoculum was passaged in transgenic cervid mice containing deer PrP^{C}. The resulting prions were also able to convert human PrP^{C} into PrP^{RES} indicating an ability for the species barrier to be overcome (Barria et al., 2011). RT-QuIC has also been used to show successful conversion of recombinant human PrP^{C} to PrP^{RES} when seeded with CWD prions. Interestingly the conversion was more efficient than conversion of recombinant human PrP^{C} to PrP^{RES} when seeded with BSE prions - the purported cause of vCJD (Davenport et al., 2015). Human brain, collected at autopsy, and humanized transgenic mouse PrP^{C} were also able to be converted to PrP^{RES} by CWD seed in PMCA. Conversion efficiency was reduced when the human *Prnp* sequence coded for valine at codon 129 versus

methionine (Barria et al., 2014). In an *in-vitro* fibrillization assay CWD seeds were unable to convert human PrP^{C} (Luers et al., 2013). Bioassays with humanized transgenic mice conflict with what is seen molecularly. Cervidized transgenic mice came down with CWD after being inoculated intra-cranially with CWD positive brain homogenate whereas two lines of humanized transgenic mice showed no clinical, histopathological, or immunohistochemistry evidence of PrP^{RES} (Kong, 2005). Studies repeated using different transgenic mouse lines show similar results – no amplification of CWD PrP^{RES} in humanized mice (Sandberg et al., 2010; Tamgüney et al., 2006; Wilson et al., 2012). To date, the best evidence illustrating a barrier of CWD transmission to humans is work done by Kurt et al. in 2015. Chimeric human transgenic mice with elk amino acid substitutions at specific codons were generated and showed the ability to develop PrP^{RES} compared to normal humanized transgenic mice illuminates the importance of *Prnp* sequence for transmission. The main factor hindering transmission seems to be the β2 – α2 loop of the human PrP^{C} protein. Interaction between human protein residues and those of CWD PrP^{RES} are energetically unfavorable impeding formation of more PrP^{RES} (Kurt et al., 2015). While data may be contradictory, it's important to note these are drawbacks to molecular *in-vitro* assays and models. Routes of exposure, titer of infectivity, and repeated exposure all may influence transmission of disease and all of those factors are hard to model in a laboratory experiment. There is compelling evidence both supporting and repudiating CWD transmission to humans but it seems that time may be the most telling factor.

Transmission of CWD

By 1992 CWD had been reported in captive facilities only – 4 in Colorado, 1 in Wyoming, and a zoological park in Canada (E. S. Williams & Young, 1992). Detection in free-ranging populations occurred as early as 1981 but was monitored for some time and not reported until 1997 (Spraker et al., 1997). Currently CWD can be found in 24 states, many of them non-contiguous, 2 Canadian provinces, Korea, and

Norway (Haley & Hoover, 2015; Sohn et al., 2002). The rapid and continuing spread is very disconcerting. There's a high possibility that in 1992 there were cases of CWD outside of the disease epicenter that went unrecognized and unreported. At the time CWD was still a new emerging disease and it is unclear how many animals may have been subclinical. Coupled with the high levels of susceptible populations, it's evident that introduction of the disease into a naïve population would quickly spread. In the middle to late 1990s deer and elk ranches, mainly used for hunting, started to detect CWD in their animals upon screening. Cervid transport between ranches is thought to have contributed to non-contiguous spread and the detection of disease in previously CWD negative areas. State regulations were variable and not all ranchers were required or inclined to keep records of animals and disease. Oftentimes animals would be transported to a new ranch and remaining herd individuals would come down with disease but that information wasn't passed along to the new ranch (Knight, 2002). Control of free-ranging animals is more difficult. While most adult animals remain in their designated home-range, younger males travel further and often away from their initial home-range to establish a new home-range. This could also be contributing to spread of CWD (Clements et al., 2011). Crows have also been posited to play a role in translocation of CWD to non-contiguous sites as they can excrete CWD positive material after ingestion. The excreted material retains its infectivity as was seen when mice were intraperitoneally inoculated with infected feces collected from crows that had been previously orally-gavaged with CWD positive material (Fischer, Phillips, Nichols, & Vercauteren, 2013; VerCauteren, Pilon, Nash, Phillips, & Fischer, 2012). Management solutions include selective culling of animals to reduce population and density in the hopes of slowing transmission, bans on transporting animals, and continued surveillance to monitor spread (Barlow, 1996; Manjerovic, Green, Mateus-Pinilla, & Novakofski, 2014; E S Williams & Miller, 2002).

As CWD continues to spread there's been concern and speculation of transmission to other non-cervid species. CWD transmission to other species has occurred experimentally in cattle, sheep, goats, squirrel monkeys, domestic cats, voles, and ferrets, but it hasn't been observed in any natural setting (A. N. Hamir et al., 2005; Amir N Hamir et al., 2006; Kurt, Telling, Zabel, & Hoover, 2009; C K Mathiason et al., 2012; B. Race et al., 2009). When cattle were intra-cranially inoculated with CWD the abnormal protein, PrP^{RES}, was able to be found in 38% of animals. Cattle that were positive for PrP^{RES} had varying incubation times with the shortest being 23 months and the longest 63 months (A. N. Hamir et al., 2005). 2 of 8 sheep intra-cranially infected with CWD prions had detectable levels of PrP^{RES} in analyzed central nervous system tissues (Amir N Hamir et al., 2006). In both of the previous experiments animals that developed prion disease lacked the stereotypical clinical and in some cases neuropathological changes normally associated with BSE and Scrapie respectively. Squirrel monkeys have been shown to contract CWD after both oral and intra-cranial inoculation (B. Race et al., 2009). Transmission of CWD to cats occurred only when the animals were inoculated intra-cranially; however, no disease developed after oral inoculation. Upon a secondary passage of infected material to subsequent cats the animals had a shortened incubation time and more widespread histopathologic findings suggesting adaptation to the host (C K Mathiason et al., 2012). *In-vitro* passage of CWD by PMCA also provides evidence that prions can adapt to a new host environment upon repeated passage (Meyerett et al., 2008a). All of this indicates CWD transmission to other animals in a natural setting may not be likely to occur. However, the disease is still relatively new and as it continues to spread frequency of contact with other animals may increase. Repeated passage through cervids and other animals could lead to development of a new TSE which may not be immediately recognized due to host-adapted changes (Barria et. al, 2011).

Transmission dynamics of CWD sustain the disease in both wild and captive cervid populations. It's often hard to pinpoint how transmission between cervids is occurring because of the multiple transmission

routes that exist. CWD can be spread horizontally through direct contact with animal byproducts including saliva, blood, urine, carcasses, and feces. PrP^{RES} is shed prior to terminal disease and in asymptomatic animals (Haley et al., 2011, 2013, 2014; Davin M. Henderson et al., 2015; Candace K Mathiason et al., 2006; Pulford et al., 2012; Seeger et al., 2005). This was shown in multiple cervid co-housing studies, oral inoculations of deer with PrP^{RES}, oral shedding, and presence of PrP^{RES} in palatine tonsil and lymphoid tissue prior to accumulation in the central nervous system (Fox, Jewell, Williams, & Miller, 2006; M W Miller, Wild, & Williams, 1998; Michael W Miller & Williams, 2003a; Sigurdson et al., 1999; Spraker et al., 2002). Rutting may be a time when contracting CWD is increased due to a higher frequency of contact between deer. Direct contact between deer also occurs at wallowing sites where animals urinate, ejaculate, and mark territory. When wallowing sites were monitored for 3 months multiple incidences of urination and naso-oral contact between animals and the wallowing site were observed (Vercauteren et al., 2007). Both nasal, aerosol, and oral lesions and secretions may contribute to spread during these times as well (N. D. Denkers, Telling, & Hoover, 2011; Nathaniel D. Denkers, Seelig, Telling, & Hoover, 2010; Michael W Miller & Conner, 2005). Epidemic models also have provided supporting evidence for horizontal transmission as prevalence, determined from field studies, would've been impossible to reach unless horizontal transmission was included as a transmission route (M W Miller et al., 2000). Vertical transmission of CWD has been shown to occur from CWD infected dams and their offspring; however, it's still unclear as to whether this is a major route of transmission (M W Miller et al., 2000). A recent publication found evidence of PrP^{RES} in 78% of dams (15 of 19) and 80% (12 of 15) of fetal tissues collected from dam-calf pairs in Rocky Mountain National Park when the tissues were assessed by sPMCA. 12 of the 15 dams had previously been determined to be CWD negative by immunohistochemistry and 5 of the sPMCA positive dams showed no indication of PrP^{RES} in central nervous tissue (Selariu et al., 2015). In a different set of studies cervidized transgenic mice orally exposed to a combination of urine and feces didn't show evidence of disease or PrP^{RES} in any tissues.

However, when these tissues were re-analyzed using sPMCA, PrP^{RES} was amplified also indicating another instance of subclinical infection (Haley, Mathiason, Zabel, Telling, & Hoover, 2009). The data indicate there could be large population of subclinical animals still capable of transmitting PrP^{RES} to one another even though clinical disease may never develop. The role of environmental CWD reservoirs and subsequent transmission was first illustrated when CWD negative deer were housed in different environmentally contaminated paddocks. Three different conditions included: paddocks containing CWD-positive deer, an infected deer carcass that had decomposed one year and eight months prior, and a paddock with excreta from two years and two months prior. In all cases previously CWD-negative deer became CWD positive (Michael W Miller, Williams, Hobbs, & Wolfe, 2004).

The Role of Environmental Reservoirs in Transmission of Chronic Wasting Disease

Implications of diseases affecting free-ranging and domestic populations

Any disease affecting wildlife has a large impact on the affected animals, the surrounding ecosystem, and any industrial applications the animals may be used for such as domestic livestock. There is also the added risk of zoonotic or transspecies transmission with new emerging diseases. All of these factors burden management and can have a large economic impact as a result (Dobson & Meagher, 1996; Dobson & Miller, 1989; Gross, John E.; Miller, 2000). Many examples of diseases that have had a major impact on domestic (captive) and wild (free-ranging) populations can be mentioned but a few that have relevance to this body of work include Brucellosis, BSE, and Scrapie. Brucellosis is caused by the microbial pathogen *Brucella abortus*. It is a major disease of domestic livestock, namely cattle, and both elk and bison are reservoirs of *B. abortus*. An endemic area of infected elk is in Yellowstone, Wyoming. Because elk are free-ranging the risk of spreading disease increases both to domestic animals when grazing nearby; as well as to hunters or other humans who come into contact with aborted tissue or sick elk. Elk have been identified as the source for a Brucellosis outbreak in domestic cattle in Northwestern

Wyoming (Boyce, 1989; Dobson & Meagher, 1996; Dobson & Miller, 1989; Meagher & Meyer, 1994; Peterson, 2010; E. T. Thorne, Walthall, & Tebaldi, 1981). Huge economic impacts have been seen in the United Kingdom (UK) and the United States (US) in regards to BSE. In the UK, the peak of disease occurred in 1992 with 36,700 confirmed BSE cases and while this impacted the domestic cattle industry it wasn't until vCJD cases were linked to BSE that the industry suffered tremendously. The amount of money estimated to be lost in the year following this finding is estimated to be around 740 – 980 million pounds which is equivalent to 1-1.3 billion US dollars. Between 1996 and 1999 the total estimated cost spent on BSE was projected to be 3.5 billion pounds which is just shy of 5 billion dollars (Atkinson, 1999). The US government implemented many measures to prevent BSE from affecting the domestic livestock industry in the same manner as the UK. New regulations and increased surveillance came with additional costs which were estimated to be 64.6 million dollars to the beef packing sector alone in 2004. The overall total estimated cost to the beef industry in 2004 alone was estimated to be 200 million dollars (Coffey, Mintert, Fox, Schroeder, & Valentin, 2005). Since 2009 the prevalence of Scrapie in the US is estimated to be 0.05%. While prevalence may be extremely low, costs totaling 10-20 million dollars per year are still being incurred. These costs include loss of sale, breeding profits, and increased production. No purported cases of Scrapie to human transmission have occurred but public concern exists over potential for BSE-sheep or goat transmission and the associated risk for possible transmission to humans. The US government therefore is trying to eradicate Scrapie and as a result increased surveillance is necessitated (Department of Agriculture, 2010). These costs are necessary to ensure safety of the public, but they illustrate the need for proactive disease management and control when possible to prevent future occurrences. In addition to disease management, assurance that the pathogen does not or cannot persist in the environment is also vital to control and effective eradication strategies.

Pathogen persistence in the environment

Perpetuation of disease requires transmission either between susceptible animals, vectors, or a reservoir whether that be an animal or an environmental reservoir. Not only must the pathogen be able to persist in the reservoir, it must be able to transmit disease to a target population (Haydon, Cleaveland, Taylor, & Laurenson, 2002). *Bacillus anthracis*, a bacterial example of an environmental persistent pathogen, causes the bacterial disease anthrax. *B. anthracis* is an endospore forming bacteria rendering it extremely resistant to degradation of any kind including treatment to irradiation, dessication, and chemicals. These properties allow it to survive in the environment for decades if not longer. Dissemination into the environment often occurs when *B. anthracis* sporulates from infected decaying animal carcasses into the underlying soil. Grazing animals can then encounter these spores and after ingestion germination to a vegetative cell and subsequent multiplication of organisms leads to infection of susceptible hosts (Dobson & Miller, 1989; Turnbull, Lindeque, Le Roux, Bennett, & Parks, 1998; Turner et al., 2013; WHO, 2008). A recent study of an anthrax outbreak that occurred in 2005 in West Texas and resulted in the deaths of 48 white-tailed deer showed how spatio-temporal patterns influence spread and dissemination of disease when an environmental pathogenic reservoir exists (Mullins et al., 2015). In 1977, *Vibrio cholerae*, the causative agent of cholera was isolated from water samples collected from four separate sites within Chesapeake Bay. It has since been shown that the aquatic environment plays a major role in sustaining *V. cholerae* populations as well as influencing genetic variance and transmission to humans (Colwell, Kaper, & Joseph, 1977; Huq et al., 2005; Vezzulli, Pruzzo, Huq, & Colwell, 2010). Pathogenic *Escherichia coli* strains have also been recovered from treated wastewater indicating another example of bacterial persistence in the environment (M.A. & A.I., 2016). These are just a few examples of bacterial pathogens that have capability to be environmental reservoirs – other examples include fungi, protozoa, and helminths (Wall, Nielsen, & Six, 2015). Ebola, a filovirus, that causes fatal hemorrhagic fever in humans has a reservoir of unknown etiology. Many lines of

evidence point to a reservoir within bats but at one point plants were considered to be putative filovirus reservoir hosts. An insect that eats sap from plants (*Psammotettix* alienus) was found to have viral particles resembling filovirus in extracts examined by negative-contrast electron microscopy (Lundsgaard, 1997; Monath, 1999). The virus was named the Taastrup virus and since has been classified as a member of the Mononegavirales Order which includes the *Filoviridae* family, and while similarities exist between the viruses it is its own separate entity (Bock, Lundsgaard, Pedersen, & Christensen, 2004). PrP^{RES} is also extremely resistant to degradation and there have been numerous incidences supporting its ability to remain in an infectious form in the environment for both Scrapie and CWD (T Alper et al., 1967; Tikvah Alper et al., 1966; I. H. Pattison & Millson, 1961a; IH Pattison, 1965).

Scrapie provides one of the most compelling examples of environmental pathogen persistence. In the 1940s in northern Iceland the entire sheep population was culled in the effort of eradication of Maedi, a lung disease affecting sheep. The area in which the culling took place overlapped with areas where there was evidence of Scrapie. Healthy sheep coming from an area with no evidence of Scrapie were introduced to this area; however, after three years the new animals became sick with Scrapie (Georgsson, Sigurdarson, & Brown, 2006; Palsson, 1979; Sigurdsson, 1954). Evidence suggested persistence for at least 3 years. Scrapie infected hamster brain homogenate was mixed with soil, placed in petri dishes, and buried in soil filled pots for 3 years outside in Washington, D.C.. Two and three log units of the initial titered material was able to be recovered upon end-point titration with intra-cranially inoculations (Brown & Gajdusek, 1991). Oral inoculations of hamster adapted Scrapie that had been buried in soil for 29 months was also shown to be infectious (Seidel et al., 2007). Again in 1978 Iceland tried to eradicate Scrapie by imposing strict implementations including culling positive and at risk animals, disinfecting or destruction of buildings that housed infected animals, banning translocation of animals and equipment from positive farms, restocking farms after at least a two-year period, and

continued surveillance. Eradication was not successful with new occurrences detected anywhere from one year after restocking to as long as sixteen years in one case. Lambs at this farm were restocked three years after culling and the farmer had destroyed all previous sheep houses at the time of the original culling with the exception of one old building. After 16 years the farmer moved some of the flock to that old building and after two years one animal developed clinical scrapie (Georgsson et al., 2006). One group chose to look at environmental sources of Scrapie prions by swabbing a variety of surfaces at indoor and outdoor locations of an endemic Scrapie farm. Swabs were placed in buffer which was then analyzed using sPMCA and the results showed positive detection at both indoor and outdoor locations of the endemic farm with no positive results seen from swabs collected at a known Scrapie negative farm. At the time of sampling no sheep were present at the farm having been removed 20 days prior indicating environmental persistence for a minimum of 20 days (Maddison et al., 2010).

Similar environmental persistence has been noted with CWD cases. The Foothills Wildlife Research Facility (FWRF) where CWD was originally detected underwent similar cleansing and culling in order to eradicate CWD and establish new CWD free herds. About two years after re-introduction of elk herds to the facilities animals began showing clinical signs of illness. An environmental reservoir is suspected to play a role which would indicate disinfection procedures were inefficient; however, this instance is complicated by the fact that all members of the herd were acquired as calves, from Rocky Mountain National Park, born to wild free-ranging dams when they were less than one week old (Miller, Michael W; Wild, Margaret A.; Williams, 1998). It is likely that reoccurrence of infection could have been from the environment in this case as well as from a few positive calves. As the disease progressed animals could have begun shedding PrP^{RES} leading to accumulation in paddocks and served as a direct contact source between elk. A clear cut demonstration of environmental reservoir transmission was shown in 2004 when uninfected mule deer were housed in paddocks containing either infected deer, a positive

deer carcass decomposed one year and eight months prior, or an empty paddock containing excreta from positive deer housed two years and two months prior. Three separate replicates of these situations were setup and in all situations uninfected deer became CWD positive (2/3 replicates, 3/12 animals, for the carcass paddock; 1/3 replicates, 1/9 animals, for the excreta paddock) (Michael W Miller et al., 2004). It was noted that the deer housed with the carcasses ate around the carcass where there was plentiful vegetation most likely due to increased nutrients from the carcass (Towne, 2000). These findings played a big role for better understanding transmission dynamics of CWD. Deer herds stay together over winter which increases the chance of coming into contact with accumulated positive environmental material (Michael W Miller et al., 2004). It's unclear why winter increases transmission but it may be due to density dependent transmission. When bedding and water was transferred from pens housing CWD positive deer to CWD negative deer it only took 15 months for a tonsil biopsy to show presence of PrP^{RES} in one of two animals. Both animals were CWD positive by solely environmental exposure (Candace K. Mathiason et al., 2009). Since PrP^{RES} has been shown to be shed in excreta, urine and feces of clinical as well as asymptomatic animals it could be leading to accumulation of PrP^{RES} in the environment (Haley et al., 2009; D M Henderson et al., 2015; Pulford et al., 2012; Seeger et al., 2005; Tamgüney et al., 2009). It's been estimated that the infectious dose of prions shed in the environment as a result of defecation, about 391g daily, over a 10-month period is 10.9 log LD_{50} units (Arthur III & Alldredge, 1980; Tamgüney et al., 2009). Seasonal changes in amounts of fecal output have been noted with increases occurring between the spring and fall seasons (Rogers, 1987). The estimated dose shed in urine, daily, is estimated to be 100 LD_{50} units (Davin M. Henderson et al., 2015). It's important to note that these values are only estimates since no study has ever shown the level of infectivity found from collected environmental samples. Even if levels fell below these estimates and are shown to be much lower it they may still be relevant due to repeated exposure increasing likelihood of contracting disease. Repeated oral dosing has been shown to have a higher incidence of causing disease especially when the

doses occur frequently (Diringer, Roehmel, & Beekes, 1998). Prevalence of CWD has been shown to be much higher in samples collected from defined winter ranges which may be due to an accumulation of PrP positive material in these areas (Michael W Miller & Conner, 2005).

Examples of PrP^{CWD} reservoirs

Currently, two environmental reservoirs have been well-established in the prion field - soil and water. Both of these are most likely a result of accumulation of PrP^{RES} material deposited from excreta. Other fomites in the environment such as buildings, salt licks, wallows, bedding sites can also be contributing to the presence of PrP^{RES} in the environment as well (Georgsson et al., 2006; Maddison et al., 2010; Candace K. Mathiason et al., 2009; Vercauteren et al., 2007). Wildlife often ingest soil inadvertently while feeding but they also obtain nutrients essential to their metabolism. The estimates of soil consumption by deer and elk are both low (<2% soil consumption in diet) (Beyer, Connor, & Gerould, 1994). These considerations led researchers to begin looking in soil for PrP^{RES} and to determine whether infectivity would be retained, if the conformation of the protein would change when in a soil environment, and the proteins ability to bind to any soil constituents. One study looked at the potential ability of PrP^{C} to misfold into PrP^{RES} when bound to a soil mineral phase component known as montmorillonite (MTE) (Lavalette et al., 2005). The reasoning behind this experiment stems from previous work showing protein conformational changes, and protein aggregation, as a result of electrostatic and hydrophobic interactions between adsorbed proteins and solid surfaces (Baron, Revault, Servagent-Noinville, Abadie, & Quiquampoix, 1999; Lavalette et al., 2005; Quiquampoix, Servagent-Noinville, & Baron, 2002; Servagent-Noinville, Revault, Quiquampoix, & Baron, 2000). While PrP^{C}-MTE complexes were formed and α to β-like structural changes occurred these were different than pH induced conformational changes and not thought to be infectious (Lavalette et al., 2005). Similar work was performed with PrP^{RES} and the misfolded version of the protein also was found to have the

ability to adsorb to MTE, microparticles of Quartz, kaolinite (another mineral phase soil component), and a variety of whole soils. MTE bound PrP^{RES} so tightly that in order to desorb PrP^{RES} from MTE the use of 10% sodium-dodecyl sulfate was required and resulted in truncation of the N-terminus of the protein. Intra-cranial inoculations of PrP^{RES}-MTE complexes proved to retain their infectivity (C. J. Johnson et al., 2006). Further worked showed that infectivity and oral transmission were increased when PrP^{RES} was bound to soil and soil particles (C. J. Johnson, Pedersen, Chappell, McKenzie, & Aiken, 2007). Repeated intranasal inoculations of deer with MTE coupled PrP^{RES}, essentially dust, resulted in positive transmission of CWD (T. a Nichols et al., 2013). These previous experiments had used purified or recombinant PrP^{RES} as their source of infectious material; however, when prion adsorption to soil was looked at using brain homogenates it was shown to occur but at a much slower rate than seen in previous experiments which most likely mimics environmental contamination. Protein that remained unbound degraded over time while bound protein remained at stable or increasing levels (Saunders, Bartz, & Bartelt-Hunt, 2009). These findings indicate that soil bound PrP^{RES} can remain un-degraded and stable in the environment when bound to soil or soil components and are also more infectious. While PrP^{RES}-soil complexes may be how naïve animals are getting exposed to CWD it was also shown that MTE can be used to prevent disease when pre-adsorbed to PrP^{RES} and similar findings were seen when humic substances and recombinant prion protein were combined (Giachin et al., 2014; Wyckoff et al., 2013). In this instance interaction between PrP^{RES}-MTE complexes must have been so tightly bound that conversion of PrP^{C} was impossible.

While experiments with spiked samples of different types of water indicated it would not be likely for PrP^{RES} to be able to survive in an aquatic water environment, actual environmental sampling has shown differently. It was shown that organic materials when present in water seem to prevent degradation of PrP^{RES} and loss of infectivity (Maluquer de Motes, Cano, Torres, Pumarola, & Girones, 2008; Miles,

Takizawa, Gerba, & Pepper, 2011; T. A. Nichols et al., 2009). In an environmentally endemic area of CWD Nichols et. al found PrP^{RES} in a sample collected from the Cache la Poudre River in Colorado and from samples collected from the Fort Collins, CO Water Treatment Facility. Additionally, in an environmental exposure experiment where water and bedding was transferred from CWD-positive deer pens to CWD-negative deer pens animals developed CWD. While two variables, water and bedding, are present here it's likely that the water contained PrP^{RES} that was able to be transmitted (Candace K. Mathiason et al., 2009). Concern about whether plants, the main food source of deer and elk, were contaminated due to bioaccumulation of shed PrP^{RES} coupled with the knowledge that plants have the ability to take up protein, as a nitrogen source, in their roots, stems, and aerial tissues lead to research on plants and PrP^{RES} (Adamczyk, Godlewski, Zimny, & Zimny, 2008; Gorbatsevich, Sela Saldinger, Pinto, & Bernstein, 2013; Jämtgård, Näsholm, & Huss-Danell, 2008; Paungfoo-Lonhienne et al., 2008; Rasmussen et al., 2015; Tan, Ikeda, & Oda, 2000). Plants could seemingly become surface contaminated through direct-contact, saliva, or indirect excretion of urine and other excreta. An experimental model of this situation was performed by spraying infected brain homogenate onto wheat plant (*Triticum aestivum* L.) leaves and looking for PrP^{RES} binding ability and degradation over time. Not only was PrP^{RES} detected by sPMCA it was detected at a stable level for 49 days. Different plant tissues were also exposed to urine and feces from both CWD positive animals and Scrapie adapted hamsters and the results again showed PrP^{RES} bound to the plant tissues after rinsing and drying (Pritzkow et al., 2015). The question then arose regarding uptake into varying plant tissues, not replication, rather a site in which PrP^{RES} might be protected from degradation. Decaying carcasses of any kind affect the ecosystem around them often leading to higher concentrations of nitrogen and a difference in plant species in the area that may be present for years after the initial decomposition. As a carcass decays, the body fluids released destroy the plants underneath and in the surrounding area creating a zone of disturbance which after time becomes zones of fertility due to nutrients and limited competition from other species (Towne, 2000).

Since CWD-prions have been shown to persist in the environment it's postulated that a decaying CWD-positive carcass could saturate the environment with PrP^{RES} which could then be taken up into plants as growth of new flora occurs. An experimental study contaminated soil with Scrapie adapted hamster brain homogenate and grew Barley plants (*Hordeum vulgare*) in the contaminated soil and then analyzed various tissues with sPMCA. They found PrP^{RES} in the stem and leaves of plants grown in contaminated soil for three-weeks indicating that uptake of PrP^{RES} can occur (Pritzkow et al., 2015). Another study also used wheat plants ability to transport both PrP^{C} and PrP^{RES} and their reported findings show detection of PrP^{C} and PrP^{RES} in the root tissues of the plants, but no transport to stem or other aerial tissues (Rasmussen et al., 2014). While these findings contradict what was shown by Pritzkow et al., it's most likely due to sensitivity of the assays used. No PrP amplification methods were used by Rasmussen et al. but they were used by Pritzkow et al. to detect minute levels of the protein that may have been transported into the aerial tissues. Both wheat and barley are grasses which are relevant environmental models because they are consumed quite readily in the spring; around 4-64% of mule deer diet is composed of grasses while the remainder comes from shrubs and trees (Kufeld, Wallmo, & Feddema, 1973).

Modeling transmission dynamics and support for environmental reservoirs

Many emerging infectious diseases that affect or spill-over into wildlife populations are transmitted through reservoir animals. This isn't the case for CWD, rather an environmental reservoir continues to perpetuate disease. Translocation of animals often influences emergence because it can bring susceptible animals in contact with the reservoir. (Daszak, Cunningham, & Hyatt, 2000). Concern arises for the infected animal's habitat; even more so considering the multitude of transmission routes and differences and dynamics. In density dependent transmission (DDT), spread is increased when host density is increased whereas frequency dependent transmission (FDT) is independent of density. DDT

can be curtailed when the population falls below a set threshold; however, it's not clear which type of transmission dynamic is occurring with CWD. These differences in transmission can affect prevalence of disease as well as routes taken to manage disease. In places like Wisconsin where deer density is believed to be ten times higher than that of Colorado, DDT may be occurring more than FDT. Additionally density can increase not just as a result of herd size but host-range overlap (Bartelt, Pardee, & Thiede, 2003; Habib, Merrill, Pybus, & Coltman, 2011; Wasserberg, Osnas, Rolley, & Samuel, 2009). Prevalence of CWD is seemingly increasing in endemic areas with areas from Wyoming to Wisconsin reporting anywhere from 15-50% of CWD positive cases (Saunders, Bartelt-hunt, & Bartz, 2012). With prevalence increasing the end result may be herd destruction, extinction, or very high prevalence, especially in the presence of an environmental reservoir where culling and predation would only reduce prevalence for a period of time (Almberg, Cross, Johnson, Heisey, & Richards, 2011; Jennelle et al., 2014; Saunders et al., 2012; Wasserberg et al., 2009). Currently, many states implement generalized, hunting, and selective culling methods as well as surveillance for disease control but there is public opposition to culling of animals. In Wisconsin, when management strategies shifted from CWD control culling to hunter only culling, prevalence increased (Bartelt et al., 2003; Manjerovic et al., 2014; Wasserberg et al., 2009). In order to predict or discern transmission dynamics ecological modeling can be used to analyze different parameters of disease dynamics. Wasserberg et al. analyzed and modeled different modes of transmission, DDT versus FDT, as well as CWD prevalence and the effect host-culling may have on both transmission types. Both DDT and FDT fit when modeled according to data collected. When prevalence is looked into for DDT and growth is unrestricted or resource limited stabilization occurs after 11-12 years and 16-18 years respectively. With FDT unrestricted population wasn't limited by disease prevalence whereas the resource limited population showed decline due to high prevalence. Culling reduces prevalence assuming DDT however prevalence decreases may be delayed for a period of time and prevalence is shown to increase until herd extinction occurs even with culling when FDT is assumed

(Wasserberg et al., 2009). When sex-specific differences are looked into it seems FDT better fits to the data set analyzed however no indirect transmission is accounted for in the model which may skew the data dependent on the level of environmental accumulation (Jennelle et al., 2014). A study analyzing the effect of predation on CWD prevalence showed no impact on prevalence levels in the slightest (Michael W. Miller et al., 2008). It seems as if culling is implemented when prevalence is low versus already established it is more likely to have an impact (Gross, John E.; Miller, 2000). Another variable involves social behavioral changes between deer dependent on seasons with less contact occurring during summer seasons and higher density of animals resulting in more contact during winter. This could result in a seasonal shift between DDT and FDT and result in more effective culling management strategies (Michael W Miller & Conner, 2005; Oraby, Vasilyeva, Krewski, & Lutscher, 2013).

A study looked specifically at the role of environmental prion persistence, indirect transmission, on both DDT and FDT assuming that these two transmission dynamic mechanisms occur with both direct and indirect transmission. With these two assumptions met it was impossible to meet reported prevalence levels and transmission dynamics when only direct transmission was modeled indicating that indirect transmission plays a large role in both DDT and FDT models. Including indirect transmission in the modeled data sets resulted mimics of current epidemics seen in Colorado, Wyoming, and Wisconsin. These results were dependent on prion half-life with low half-life implicating a larger role for direct transmission. However, when a longer half-life was assumed, indicative of bioaccumulation or persistence, the role of indirect transmission was much larger in disease dynamics (Almberg et al., 2011).

Introduction to the work presented in this thesis

Project Specific Aims

With the current knowledge of environmental contamination and the recent publication by Pritzkow et al. illustrating ability of uptake of PrP^{RES} by a grass plant when soil was spiked with PrP^{RES} we were intrigued by the possibility that plants could also be an environmental reservoir (Gough & Maddison, n.d.; Candace K. Mathiason et al., 2009; Michael W Miller et al., 2004; Pritzkow et al., 2015; Saunders, Bartelt-Hunt, & Bartz, 2008). We chose to look at plants in a CWD endemic area, Rocky Mountain National Park, for contamination with PrP^{RES}.

We are hypothesizing that PrP^{RES} can be taken up into plants by CWD positive water or soil or be contaminated on their surface or internally and therein the plants can serve as a vector for transmission of CWD. Our first aim was to optimize detection of PrP^{RES} in the presence of plant material using the sensitive prion amplification assay PMCA. We wanted to understand if plant material would inhibit amplification of PrP^{RES}, amplification rate, and determine the sensitivity of the assay with plant material. The second aim was to sample plants from Rocky Mountain National Park and look at both the surface and interior tissues for PrP^{RES}.

CHAPTER 2 - ENVIRONMENTAL PRIONS: DETECTION OF PRP^{RES} ON PLANTS COLLECTED IN ROCKY MOUNTAIN NATIONAL PARK

Introduction

In 1980, Chronic Wasting Disease (CWD) was first characterized as neurodegenerative disease with a characteristic spongiform pathology. CWD was first observed to affect mule deer (*Odocoileus hemionus columbianus)* and was thought to be a spontaneously occurring disease (E. S. Williams & Young, 1980). Since then the causative agent has also been found in elk and moose. CWD was proven to be a transmissible spongiform encephalopathy (TSE), alongside scrapie, bovine spongiform encephalopathy, and transmissible mink encephalopathy (Bahmanyar et al., 1985; Browning et al., 2004; Guiroy et al., 1991; E S Williams & Young, 1982; E. S. Williams & Young, 1980, 1992). The epidemiology of CWD is confounding. It is one of the only TSEs to date that is found in both captive and free-ranging populations (M W Miller et al., 2000). As of 2015, 19 states in the United States of America and 2 Canadian Provinces have positively identified CWD in free-ranging populations (Haley & Hoover, 2015). Like other TSEs, the causative agent of CWD is the abnormal, protease-resistant conformer of the cervid prion protein, PrP^{RES} or PrP^{CWD}. Once introduced PrP^{RES}, has the ability to cause the normal prion protein, PrP^{C}, to misfold into PrP^{RES} (S B Prusiner, 1982). The disease reaches a terminal stage once neurodegeneration occurs, accompanied by the presence of amyloid plaques and astrogliosis (Bahmanyar et al., 1985; Guiroy et al., 1991).

Evidence reported since 1998 points to three possible routes of transmission: horizontal, both direct and indirect; vertical; and environmental (M W Miller et al., 1998). Horizontal transmission seems to play a major role in the continued spread of disease (Michael W Miller & Williams, 2003b). While evidence suggests that maternal transmission can occur, it is not thought to be a driving factor of transmission (Michael W Miller & Williams, 2003b; Nalls et al., 2013). Indirect oral transmission has been shown both

experimentally and naturally. A variety of different sources including saliva, urine, feces, blood, and other fomites have proven to be efficient in transmitting CWD (Haley et al., 2009; C. J. Johnson et al., 2006, 2007; Candace K. Mathiason et al., 2009; Candace K Mathiason et al., 2006; Michael W Miller et al., 2004; Pulford et al., 2012; Schramm et al., 2006; Sigurdson et al., 1999). Environmental sources have been speculated to be involved with transmission as well, though the impact of environmental contamination may be playing in spread of CWD remains unclear (M W Miller et al., 2000, 1998; T. A. Nichols et al., 2009). A recent study by Henderson, et. al estimated that 100 LD_{50} units could be deposited into the environment daily from urine alone (D M Henderson et al., 2015). CWD prions shed into the environment from infectious and sub-clinical animals could be major source of bioaccumulation. Coupled with the fact that deer and elk herd together, one can speculate that this would serve as a hotspot of infectivity (Michael W Miller & Williams, 2003b). Accumulation of PrP^{RES} in the environment dictates that the agent must resist degradation, which has been shown for both Scrapie and CWD. Scrapie, a prion disease affecting sheep, has been found to remain infectious for 3 years experimentally and as long as 16 years in a barn that previously housed positive sheep in Iceland (Brown & Gajdusek, 1991; Georgsson et al., 2006). Not only can CWD persist in the environment for at least 2 years it has also been shown to cause detectable infection after 15 months of environmental fomite contact in an experimental setting (Candace K. Mathiason et al., 2009; Michael W Miller et al., 2004).

The ability of PrP^{RES} to persist in the environment and remain infectious has led to the idea of an environmental reservoir that is contributing to and driving the spread of CWD. Current and past data as well as mathematical models based on these data demonstrate that including indirect environmental transmission parameters fit current transmission dynamics four times better than direct transmission parameters alone (Almberg et al., 2011; Michael W. Miller, Hobbs, & Tavener, 2006). A number of

suspected environmental reservoirs exist, including water, plants and soil. Deer and elk may be ingesting soil deliberately for nutrients or inadvertently while foraging or rutting (Beyer et al., 1994). Experimental data show the ability of PrP^{RES} to bind to soil and other soil components (Wyckoff et al., 2013). Additionally, the oral transmissibility of PrP^{RES} is increased when bound to soil (C. J. Johnson et al., 2006, 2007; Schramm et al., 2006). Alongside soil, water from a CWD-endemic area was shown to contain PrP^{RES} (T. A. Nichols et al., 2009). Recently, plants have been considered as putative environmental prion reservoirs. Since deer and elk graze solely on trees, shrubs, forbs, and grasses, the accumulation of PrP^{RES} on these flora could represent a potential route of infection (Kufeld et al., 1973). Recent experimental data offers some evidence that plants could be an environmental reservoir. While McAllister et.al were unable to detect PrP^{RES} in the stem or leaves of wheat (*Triticum aestivum L.*), they used relatively insensitive PrP^{RES} detection methods (Rasmussen et al., 2014). In contrast, Pritzkow et. al reported that both surface and internal structures of wheat grass plants were able to bind PrP^{RES} and retain the ability to convert PrP^{C} using an ultrasensitive *in-vitro* prion amplification assay, Protein Misfolding Cyclic Amplification (PMCA). Experimentally contaminated plants transmitted prion disease to indicator mice at an attack rate of 100% upon oral inoculation (Pritzkow et al., 2015).

These experimental data support the possibility of contaminated plants as prion vectors, however, the prion titers used in these experiments are likely orders of magnitude higher than what would be expected to be found in the environment. In this study we explored the ability of plants to serve as prion reservoirs in an actual environmental setting within a CWD-endemic area. Here we report the optimization of Protein Misfolding Cyclic Amplification (PMCA) assay utilizing rice plants (*Oryza sativa*) and a CWD prion isolate in a brain sample from a naturally exposed elk, E2, to ensure our ability to detect anticipated low environmental levels of PrP^{RES} (Joaquin Castilla et al., 2006; Morales et al., 2012). We then tested plant samples collected from areas previously reported to have CWD prions. We

detected PrP^{RES} on the surface of six different plants of thirty collected within Rocky Mountain National Park (RMNP). These results support the idea of plants as an environmental reservoir and contribute to the overall understanding of CWD transmission dynamics that may lead to new strategies to impede further environmental spread.

Materials and Methods

Environmental Plant Samples

Test plant samples were collected from three locations within Rocky Mountain National Park (RMNP), Colorado (40°22’ N, 105°, 36’ W). RMNP field guides worked with us to correctly identify plant species and in some cases both species and genus. Control plant samples were acquired inside of exclosures, directly adjacent to test plant samples, built by RMNP as part of the Elk and Vegetation Management Plan (EVMP). The EVMP serves to reduce the impact of elk on the habitat with in the park and allow for regrowth of vegetation. Care was taken to try and match species of plants collected both inside and outside of the exclosures to rule out species specific differences. Samples were collected with clean gloves and placed into plastic bags which were then placed into a cooler for storage and transport purposes until they could be stored at -80°C until use

Negative Plant Samples

Rice leaves (*Oryza sativa)* were collected from the greenhouse of our collaborator at Colorado State University (CSU), Jan Leach. These plants have been grown from seed inside the greenhouse and have had no prior contact with the environment that would render them positive.

Assessing Environmental Plants Surface Contamination

To determine surface contamination of the plants the samples were rinsed with 25mL of phosphate buffered saline (PBS). Two 1.5-mL aliquots of this rinse were collected and stored at -80°C to later assay via Protein Misfolding Cyclic Amplification for presence of PrP^{RES}.

Plant Sample Homogenization

10% weight by volume plant homogenates were made using extraction bags and the homogenizer hand model (BIOREBA Ag). Leaves, roots, or stems were weighed and then placed in the extraction bag and an appropriate amount of PMCA buffer #1 (150 mM NaCl, 4mM EDTA, in PBS) was added. These samples were then ground by hand until they were liquefied. If necessary, samples were further homogenized by pipetting the liquid out of the extraction bags and placed into a 1.5 mL eppendorf tube containing glass beads. These tubes were placed in a homogenizer (BulletBlender, NextAdvance, USA) at max power for 5 minutes followed by 5 minutes on ice. This was repeated a maximum of 5 times. Samples were then pipetted into new 1.5 mL Eppendorf tubes and stored at -80°until use.

Ethics Statement

Mice were bred and maintained at Lab Animal Resources at CSU. This facility is accredited by the Association for Assessment of Lab Animal Care International in accordance with protocols approved by the Institutional Animal Care and Use Committee at CSU (protocol ID14-5009A). Mice were euthanized using CO_2 inhalation.

Mice

Tg(cerPrP)5037 mice were generated in the Telling laboratory as previously described (Browning et al., 2004)

CWD Prions

A CWD prion isolate, E2, from a terminally sick elk from a game farm in northern Colorado was utilized for CWD positive material. Preparation of this isolate was previously described (T. A. Nichols et al., 2009).

Preparation of Normal Brain Homogenate (NBH)

Mice were euthanized and perfused in a similar manner as described (Meyerett et al., 2008b) however the brains were frozen at -80°C in 1.5-mL eppendorf tubes. The brains were then weighed after which PMCA #1 Buffer and 2X Complete Protease Inhibitor Cocktail (Roche) tablets were added making a 20% weight/volume solution. Glass beads were added to the tubes and the brains were then homogenized using the BulletBlender (NextAdvance, USA) at maximum speed for 5 minutes. Samples were then placed on ice for 5 minutes and this process was repeated a total of 5 times. Samples were then pooled and an equal volume of PMCA #2 Buffer (PMCA #1 Buffer plus 2.0% Triton X-100) was added and samples were incubated on ice for 20 minutes. After incubation the samples were centrifuged at 1500 x g for 30s in order to clarify them. Supernatants were then stored at -80°C.

Protein Misfolding Cyclic Amplification (PMCA)

25 µL of environmental plant homogenate was added to 25 µL of NBH in 0.5 mL screw top tubes (VWR). Tubes were placed suspended in a Q700 sonicator (Qsonica, Newton, Connecticut, USA) filled with 300 mL deionized water. Samples were pulsed every 30 minutes at an amplitude of 27 for 40 seconds. This

process is repeated for 24 h at a temperature of 32 °C, a period defined as one round. After each round, 25 µL of each sample is pipetted off and stored, and 25 µL of fresh NBH was added to the remaining 25 µL sample. This was repeated for each subsequent round and a total of 6 rounds were performed unless otherwise indicated. Positive amplification controls (CWD prions - E2) were included with each sample group at a dilution of 1:10,000. Negative controls are run with each sample group and included normal brain homogenate and, in some cases, negative plant samples.

CWD-spiked plant sample detection

25 µL of negative plant homogenate was combined with 25 µL of E2. The samples were vortexed and then 25 µL of the spiked plant homogenate sample was added to 25 µL of NBH and then amplified by PMCA. Various dilutions of E2 were made in order to test specificity and sensitivity of PMCA in the presence of plant material

PK Digestion and Western Blot

Samples were digested with 125 µg/mL proteinase K (PK, Invitrogen) for 45 minutes at 42°C. The reaction was halted by the addition of lithium dodecyl sulfate sample loading buffer (Invitrogen) and incubating at 95°C for 10 minutes. The Western Blots were carried out as previously described (35). In some cases a cervid specific antibody, PRC1, was used at a 1:10,000 dilution followed by an anti-mouse IgG, HRP-linked secondary antibody (Cell Signaling Technology, USA) at a 1:10,000 dilution (Kang et al., 2012).

PMCA Analyses

PMCA results were analyzed with a PMCA scoring system in order to standardize results as was previously described (Pulford et al., 2012). Final PMCA scores are inversely correlated to the first PMCA round that PrP^{RES} is detected.

Results

PMCA Optimization

Serial PMCA (sPMCA) has been utilized by our lab before to amplify and detect low levels of environmental PRP^{RES} material (20,28). However, sPMCA had never been used to amplify samples containing plant material. We optimized our assay to ensure plant material would not cause false positive results or interfere with amplification of PrP^{RES}. We achieved a specificity of 96.38% for our normal brain homogenate (NBH) controls after sPMCA (n = 193, **Fig.1, Tables 1 & 2**). Our PMCA scoring system allowed for us to quantify our results graphically and normalize data into relative PMCA units (rpu). This ensured samples determined to be positive in earlier rounds were weighted higher than samples determined to be positive in later rounds. From our normalized mean NBH control value (2.38 rpu) the standard error of the mean (SEM), deviation between NBH control samples that occurred due to contamination or false positives, was calculated and the 99.9% confidence interval was based off the Student's *t*-table. To ensure positives seen in our sample sets were real we multiplied our normalized mean NBH control rpu value (2.38 rpu) by 3 SEM giving an extremely conservative threshold of detection (5.17 rpu) above the 99.9% CI. Samples with mean rpu scores subtracted from their SEM values falling below this threshold are considered negative.

To determine amplification ability in the presence of plant material we serially diluted CWD positive elk homogenate, E2, into 1% and 10% plant homogenates and then ran sPMCA on these samples for 6

rounds (**Table 1, Fig. 2**). We converted our dilution values into LD_{50} units of PrP^{RES}/g of plant tissue based on a previous quantification of this infected material which allowed us to know the corresponding LD_{50} unit for each dilution factor (28). We were able to get amplification of PrP^{RES} in the presence of plant material (**Fig. 2A,2B**). We also assessed the sensitivity of the assay when running sPMCA with various dilutions of E2 and determined we could reproducibly detect a 4.09×10^{-8}, or 0.81 LD_{50} units/g of plant tissue respectively, 100% of the time (4/4, **Fig. 3A, Table 1**). We were able to detect some lower dilutions; however, based on our inability to detect amplification at the 8.19×10^{-8} dilution, 0.41 LD_{50}

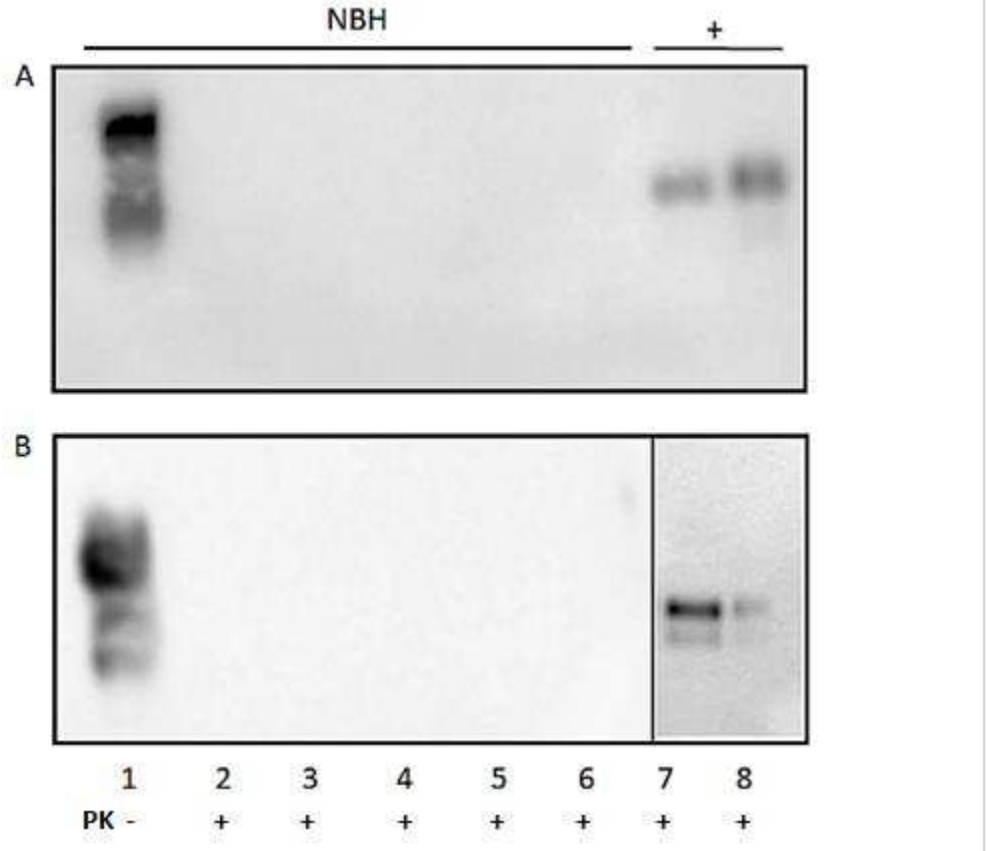


Figure 1. Representative Western Blot A representative of 36 normal brain homogenate negative (NBH) controls of 193 are shown here in A and B. Samples in lanes 2-8 were digested with Proteinase K. Lane 1: NBH. Lanes 2-6: 3 pooled NBH negative controls per lane for a total of 18 per blot. Lanes 7-8: positive 1:10,000 PrP^{RES} amplification control. All samples were negative after 6 rounds of PMCA.

units/g of plant tissue respectively, we do not feel confident saying our lower limit of detection goes below 4.09×10^{-8} (**Fig. 3A**). We then performed 6 rounds of sPMCA on dilutions of E2 only samples in

comparison to E2 diluted samples in the presence of plant homogenate to look for amplification inhibition. We derived the amplification rate of PMCA is slightly inhibited ($p = 0.0114$) in the presence of the plant homogenate (**Fig. 3B**).

Table 1. Summary of sPMCA Optimization Results

Sample	Dilutions	Equivalent LD_{50} Units[1]	sPMCA[2]				
					Tg5037[3]		
PrP^{RES} Dilutions			Tg5037 Negative Control[4]	10% Plant Homogenate Control[4]	E2[4]	E2 + 10% Plant Homogenate[4]	E2 + 1% Plant Homogenate[4]
	no spike		4/38	0/14	ND[5]	ND	ND
	1×10^{-3}	3.33×10^{5}	ND	ND	16/16	11/12	2/2
	1×10^{-4}	3.33×10^{4}	ND	ND	14/14	12/12	2/2
	2×10^{-4}	1.66x104	ND	ND	6/6	2/2	2/2
	5×10^{-4}	6.66×10^{3}	ND	ND	2/2	6/6	2/2
	1×10^{-5}	3.33×10^{3}	ND	ND	2/2	4/4	2/2
	2×10^{-5}	1.67×10^{3}	ND	ND	2/2	4/4	ND
	4×10^{-5}	8.32×10^{2}	ND	ND	4/4	6/6	ND
	8×10^{-5}	4.16×10^{2}	ND	ND	2/2	4/4	ND
	1×10^{-5}	3.33×10^{2}	ND	ND	2/2	4/4	ND
	1.6×10^{-6}	2.08×10^{2}	ND	ND	8/8	10/10	ND
	3.2×10^{-6}	1.04×10^{2}	ND	ND	2/2	4/4	ND
	6.4×10^{-6}	52.031	ND	ND	2/2	4/4	ND
	1.28×10^{-7}	26.016	ND	ND	2/2	4/4	ND
	2.56×10^{-7}	13.008	ND	ND	2/2	4/4	ND
	5.12×10^{-7}	6.504	ND	ND	4/4	6/6	ND
	1.00×10^{-8}	3.335	ND	ND	2/2	2/2	ND
	1.02×10^{-8}	3.252	ND	ND	6/6	5/6	ND
	2.04×10^{-8}	1.626	ND	ND	2/2	2/2	ND
	4.09×10^{-8}	0.81	ND	ND	4/4	4/4	ND
	8.19×10^{-8}	0.41	ND	ND	2/2	0/2	ND
	1.6×10^{-9}	0.205	ND	ND	2/2	1/2	ND
	3.2×10^{-9}	0.103	ND	ND	1/2	1/2	ND
	1×10^{-11}	3.25×10^{-3}	ND	ND	2/2	2/2	ND
	1×10^{-12}	3.25×10^{-4}	ND	ND	4/4	4/4	ND
	1×10^{-13}	3.25×10^{-5}	ND	ND	3/4	4/4	ND

[1]LD_{50} units were calculated in accordance with the dilution of CWD positive material;[2]Serial PMCA was performed - the number of positive samples/total samples are shown;[3]normal brain homogenate used for sPMCA substrate;[4]negative control, plant homogenate control, CWD infected material, CWD infected material with plant homogenate;[5]ND,no data.

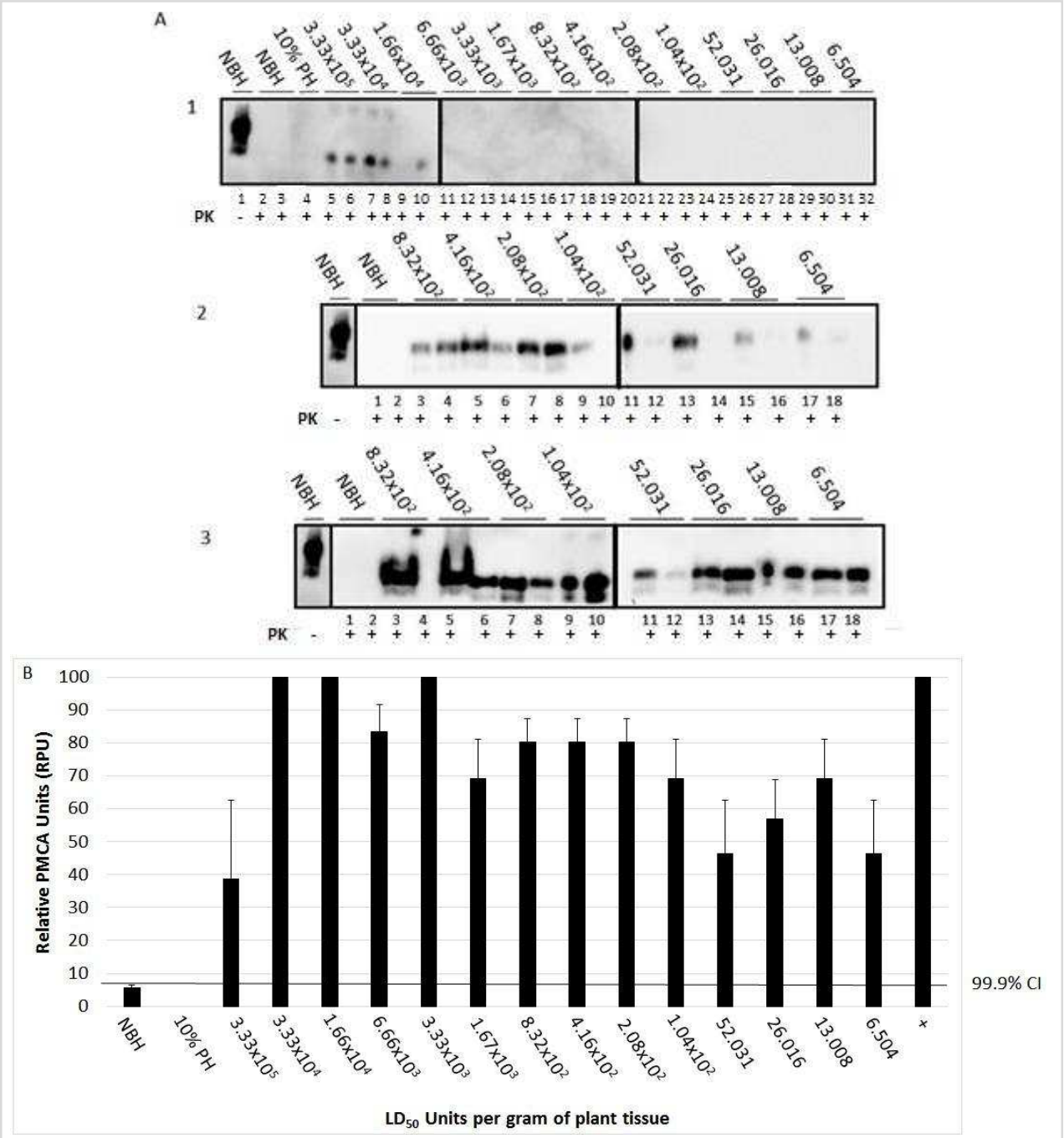


Figure 2. Optimization of Plant PMCA Amplification of PrP^{RES} in the presence of 10% plant homogenate. **(A)** Western blot (WB) 1 corresponds to sPMCA round 3; WB 2, round 4; WB 3, round 6. All samples were digested with Proteinase K except the normal brain homogenate (NBH) in lane one blot 1 and the first sample in blots 2 and 3. Blot 1, lanes 2-4 show amplified negative NBH controls and plant homogenate only controls. WB 2 and 3, lanes 1 and 2 show amplified negative NBH controls. All other lanes in each 3 blots show amplification of PrP^{RES} at different dilutions of E2 represented by the equivalent LD_{50} units/g of plant tissue. **(B)** Qualitative analysis was performed, samples were normalized, averaged, subtracted from their SEM, and given a relative PMCA unit (RPU) value determined by our PMCA scoring system. Error bars indicate individual SEM values after data has been averaged.

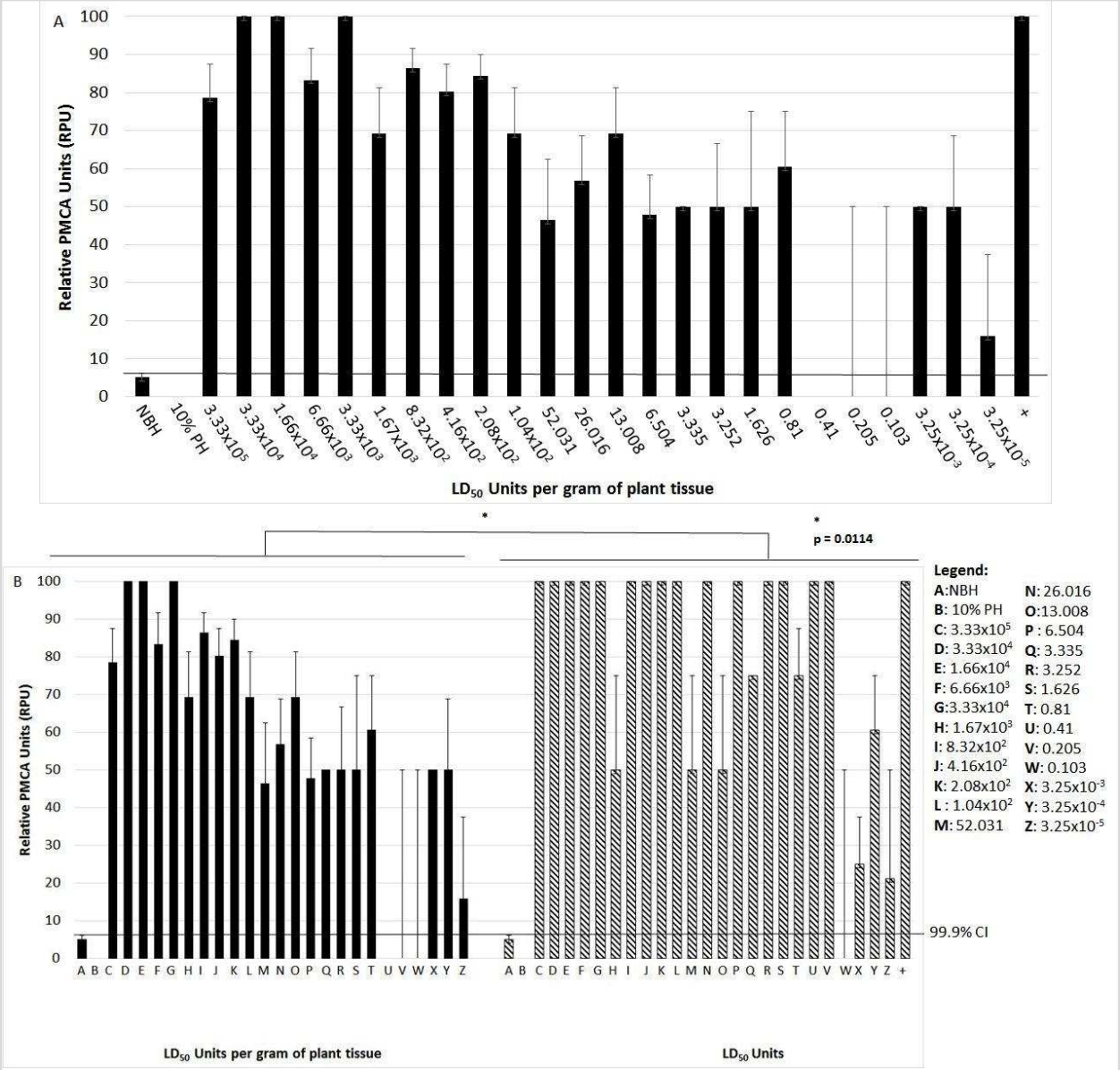


Figure 3. Plant PMCA Sensitivity Lower limit of Detection of PrP^{RES} in the presence of plant homogenate. **(A)** The lowest dilution that could be successfully reproduced was 0.81 LD_{50} units/g of plant tissue which corresponds to a dilution of 4.09 x 10^{-8}. **(B)** When looking at differences between E2 samples in the presence of plant homogenate (in black, on left) compared to E2 only samples (striped, on right) there was a significant difference in amplification when averaged data was compared between the two groups (p =0.0114) in the plant homogenate samples. As indicated in the previous figure the error bars indicate SEM values for averaged data.

Detection of PrP^{RES} on the surface of environmental samples from Rocky Mountain National Park

We decided to look for environmental evidence of PrP^{RES} in Rocky Mountain National Park (RMNP) an area endemic for CWD. Plant samples were collected in early May 2014 from sites where we've

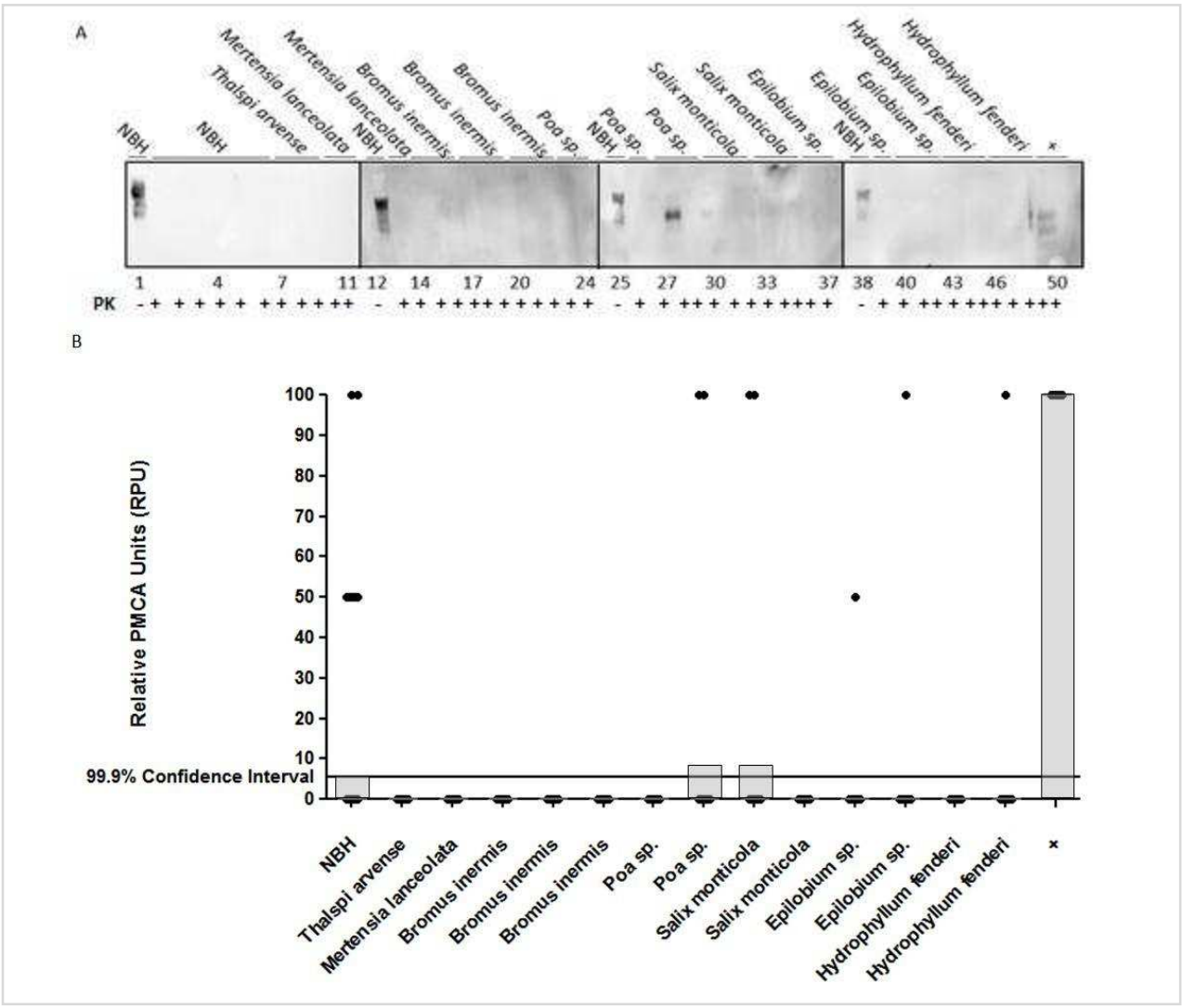
previously collected CWD positive material (20,28). A total of 30 samples were collected at random from both the inside and outside of plant management exclosures. These samples were homogenized (**materials and methods**) and then assayed by sPMCA (**Table 2**).

We rinsed the plants and assayed the rinse by sPMCA on the plants collected from all three locations in order to assess surface contamination of the plants. Triplicate samples from plants collected at each location were assayed in three independent sPMCA runs. We determined that six plants out of the thirty collected show evidence of PrP^{RES} on their surface (**Table 2, Fig. 4**). Western Blots (WB) were run on rounds five and six; however, we rarely saw positivity before round six indicating that the amount of PrP^{RES} on the surface of these plants is at a very low level. Based on our PMCA optimization and sensitivity we're estimating this amount to be around 1 LD_{50} unit. We utilized our PMCA scoring system to quantify our data to ensure the positive samples we detected were above our detection threshold of 5.17 rpu. A total of five samples collected from location 1 showed presence of PrP^{RES} on WB after sPMCA. Of those five, three had means above the threshold but when those values were subtracted from their SEMs they fell below the threshold, resulting in two samples determined to be truly positive after PMCA scoring analysis (**Fig. 4B**). Only two other plants, collected from locations two and three, showed evidence of surface contamination with PrP^{RES} but they did not meet our conservative threshold cutoff (**Fig 4C,D**). It is possible that with additional replicates of independent sPMCA the SEMs could decrease resulting in samples above the threshold. Both positive samples were collected outside of the exclosures; however, some of our suspected positive samples were collected inside the exclosures (**Table 2**). We have concluded this occurred due to sampling too close to the edge, in the range of interaction with deer and elk, when collecting plants from inside of the exclosures.

Table 2. Summary of Environmental Plant Samples Tested for PrP^{RES}

Sample	Location	Inside or Outside of Exclosure	sPMCA[1]				
					Tg5037[2]		
Environmental Material			**Surface Rinse[3]**	**Roots[4]**	**Stem[4]**	**Aerial Tissues[4]**	**Brain[4]**
Thalspi arvense	1[5]	Inside[6]	0/9	ND[7]	ND	ND	ND
Mertensia lanceolata	1	Inside	0/9	ND	ND	ND	ND
Bromus inermis	1	Outside	0/9	ND	ND	ND	ND
Bromus inermis	1	Outside	0/9	ND	ND	ND	ND
Bromus inermis	1	Inside	0/9	ND	ND	ND	ND
Poa sp.	1	Inside	0/9	ND	ND	ND	ND
Poa sp.	1	Outside	2/9	0/9	09	0/9	ND
Salix monticola	1	Outside	2/9	ND	0/9	0/9	ND
Salix monticola	1	Inside	0/9	ND	ND	ND	ND
Epilobium sp.	1	Outside	1/9	ND	ND	ND	ND
Epilobium sp.	1	Inside	2/9	ND	ND	ND	ND
Hydrophyllum fenderi	1	Outside	0/9	ND	ND	ND	ND
Hydrophyllum fenderi	1	Inside	1/9	ND	ND	ND	ND
Hesperostipa comata	2[8]	Outside	0/9	ND	ND	ND	ND
Hesperostipa comata	2	Inside	0/9	ND	ND	ND	ND
Bromus inermis	2	Outside	0/9	ND	ND	ND	ND
Bromus inermis	2	Inside	0/9	ND	ND	ND	ND
Poa sp.	2	Outside	0/9	ND	ND	ND	ND
Poa sp.	2	Inside	0/9	ND	ND	ND	ND
Aspen shoots	2	Outside	0/9	ND	ND	ND	ND
Aspen shoots	2	Inside	1/9	ND	ND	ND	ND
Agrostis sp.	2	Outside	0/9	ND	ND	ND	ND
Agrostis sp.	2	Inside	0/9	ND	ND	ND	ND
Thermopsis aivaracarpa	2	Outside	0/9	ND	ND	ND	ND
Thermopsis aivaracarpa	2	Outside	0/9	ND	ND	ND	ND
Populus termuloides	3[9]	Outside	0/9	ND	ND	ND	ND
Populus termuloides	3	Inside	0/9	ND	ND	ND	ND
Salix monticola - willow	3	Outside	0/9	ND	ND	ND	ND
Heracleum sphondylium	3	Inside	0/9	ND	ND	ND	ND
Bromus inermis	3	Outside	1/9	ND	ND	ND	ND
Negative Control[10]	NA	NA	ND	ND	ND	ND	3/155

[1]Summary of sPMCA results on environmental plant samples showing number of positive samples/total samples;[2]normal brain homogenate used for sPMCA substrate;[3,4]Material assayed from each sample;[5]An exclosure from Location 1,[6]Samples were collected from inside and outside of the exclosures;[7]ND, No data;[8,9]An exclosure from location 2 and location 3 areas.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NBH
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Thalspi arvense
Mertensia lanceolata
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Bromus inermis
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Bromus inermis
Poa sp.
Poa sp.
NBH
Poa sp.
Salix monticola
Salix monticola
Epilobium sp.
Epilobium sp.
NBH
Epilobium sp.
Hydrophyllum fenderi
Hydrophyllum fenderi
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1 4 7 11 12 14 17 20 24 25 27 30 33 37 38 40 43 46 50
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Relative PMCA Units (RPU)
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Mertensia lanceolata
Bromus inermis
Bromus inermis
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Poa sp.
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Salix monticola
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Epilobium sp.
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Hydrophyllum fenderi
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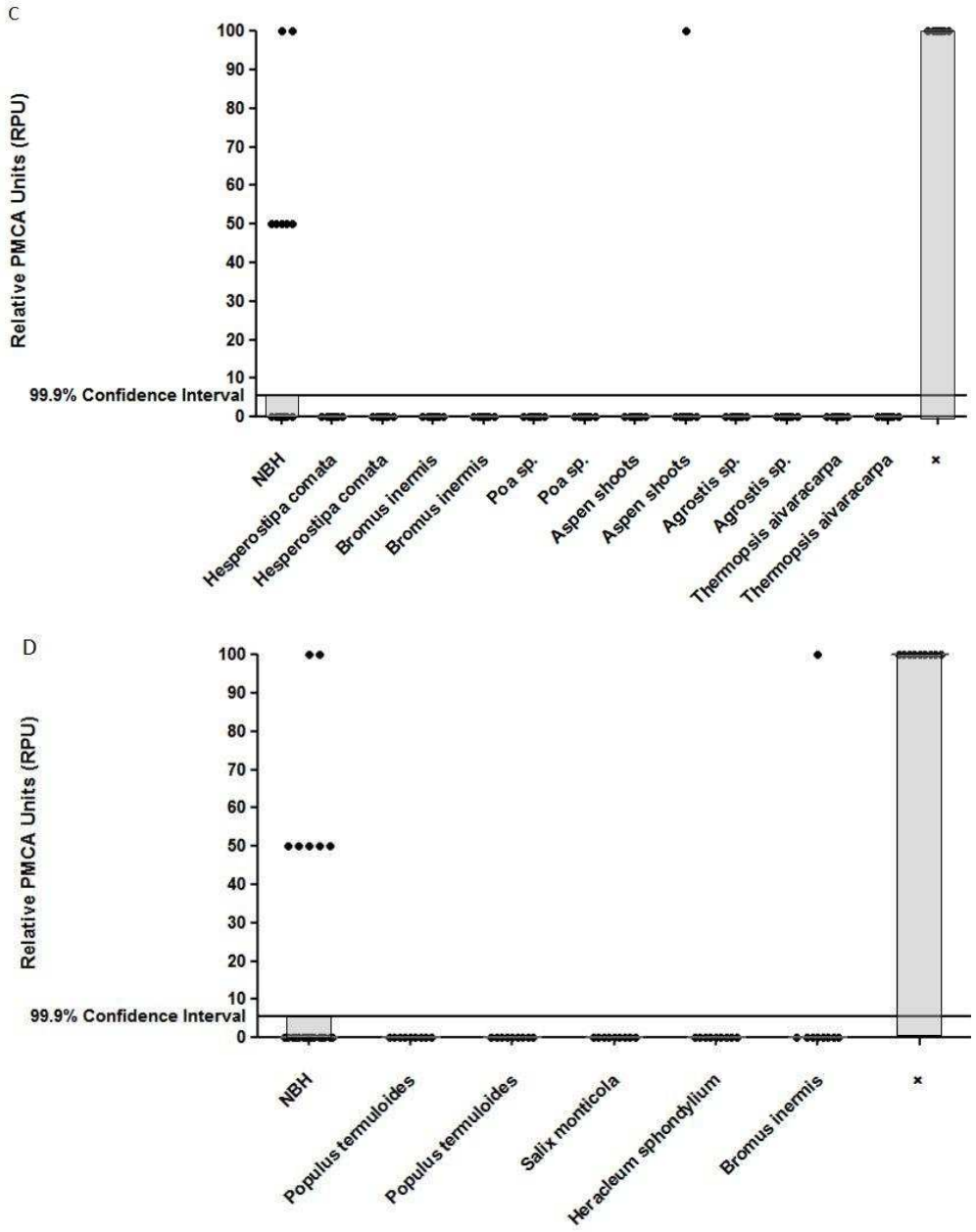

Figure 4. Surface Rinse PMCA Surface contamination of environmental plant samples collected from three separate locations. **(A)** Representative Western Blot (WB) on the 6th round of sPMCA performed on location 1 plants. All sampled were digested with Proteinase K except for the normal brain homogenate (NBH) in lanes 1, 12, 25, and 38. Lanes 2-6: 3 pooled NBH negative control samples per lane. Lanes 49-50: CWD-positive E2 control. All remaining lanes show plant samples from location 1 in triplicate. In this representative 2 of the 12 plants assayed showed the presence of PrP^{RES} (lanes 27,30). **(B)** Further testing of location 1 samples culminated in a total of 5 positive plants with PrP^{RES} seen via WB. Scatterplot points show individual relative PMCA units (RPU) for each sample run (NBH: n=193, Samples:n=6). Columns indicate the mean RPU value comprised of 3 triplicates and 3 rounds of PMCA for each sample minus the SEM resulting in 2 positive plants. **(C,D)** One plant from locations 2 and 3 both showed presence of PrP^{RES} but this was not above our conservative threshold after normalization.

Detection of PrP^{RES} in the roots, stems, and aerial tissues of environmental samples from Rocky Mountain National Park

The two plants from location 1 that had detectable PrP^{RES} contamination on their surface were further analyzed to detect internal contamination of PrP^{RES}. Plants were sectioned into roots (when collected), stem, and aerial tissue and then homogenized (**materials and methods**). These various sections were then assayed by sPMCA after which WB, and the PMCA scoring system were used in order to quantify the results (**Table 2**). Triplicate samples from the plants and each of their respective tissues were assayed in three independent sPMCA runs. We found no evidence of PrP^{RES} accumulation in any of the tissues in these samples (**Fig 5**).

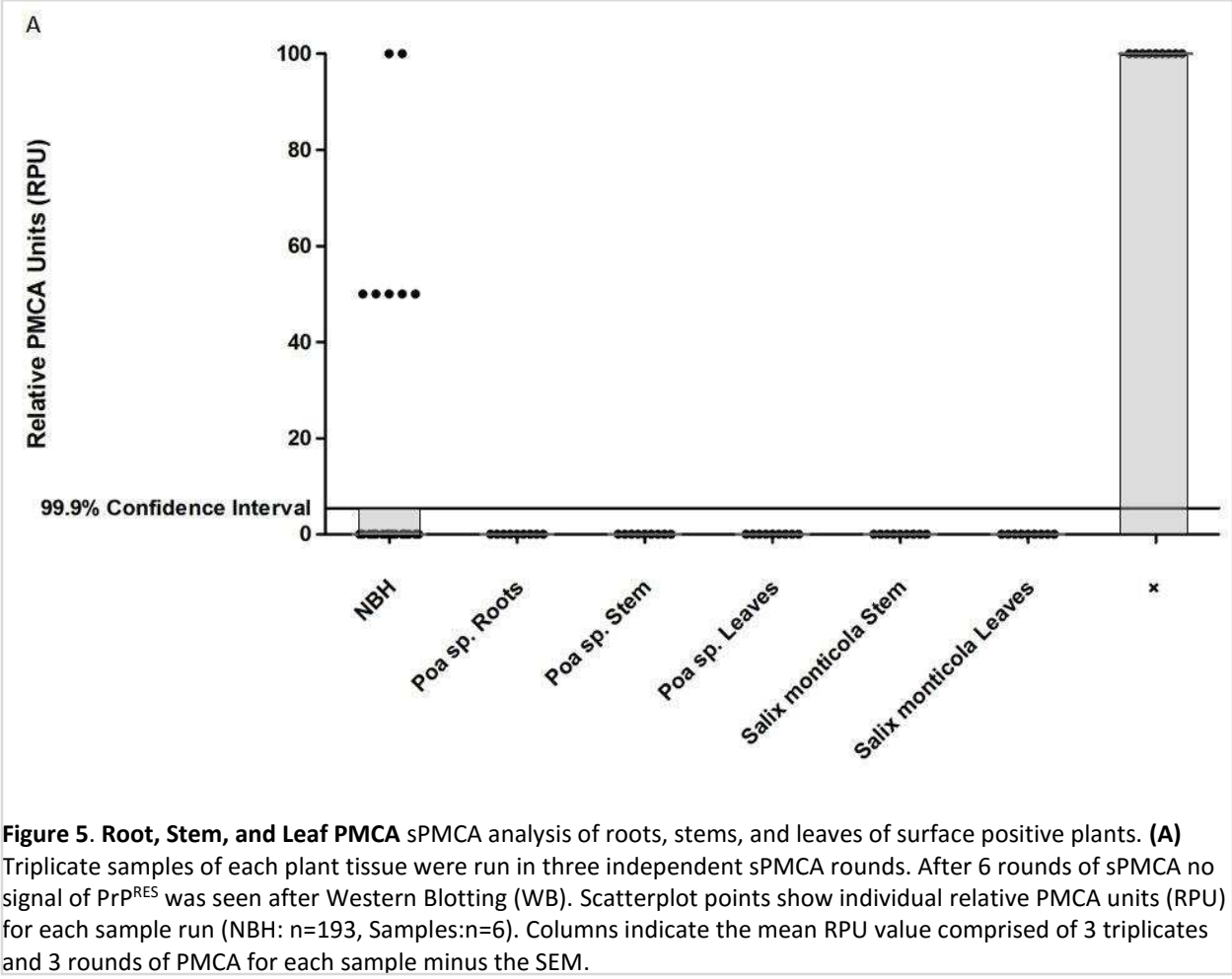

Figure 5. **Root, Stem, and Leaf PMCA** sPMCA analysis of roots, stems, and leaves of surface positive plants. **(A)** Triplicate samples of each plant tissue were run in three independent sPMCA rounds. After 6 rounds of sPMCA no signal of PrP^{RES} was seen after Western Blotting (WB). Scatterplot points show individual relative PMCA units (RPU) for each sample run (NBH: n=193, Samples:n=6). Columns indicate the mean RPU value comprised of 3 triplicates and 3 rounds of PMCA for each sample minus the SEM.

Discussion

In recent years more work has gone into demonstrating and quantifying the presence of PrP^{RES} in the environment utilizing *in-vitro*, *in-vivo*, modeling, and field sampling methods (Almberg et al., 2011; Brown & Gajdusek, 1991; Georgsson et al., 2006; Haley et al., 2009; Davin M. Henderson et al., 2015; C. J. Johnson et al., 2006, 2007; Candace K. Mathiason et al., 2009; Candace K Mathiason et al., 2006; Michael W Miller et al., 2004; Morales et al., 2012; T. A. Nichols et al., 2009; Pritzkow et al., 2015; Pulford et al., 2012; Rasmussen et al., 2014; Schramm et al., 2006). Our study shows the first evidence of environmental contamination of plants with PrP^{RES} from a CWD endemic area. For this study plants were randomly collected proximal to inside exclosure areas within the Beaver Meadows and Moraine Park valleys in RMNP. These sites were chosen for multiple reasons: 1) These areas have been previously surveyed for elk tissue, feces and water samples that were determined to be positive for CWD prions were collected here (T. A. Nichols et al., 2009; Pulford et al., 2012). 2) In 2008, 13 deer captured of 136 from Beaver Meadows and Moraine Park tested positive for CWD (Elk and Vegetation Management Plan Fact Sheet, RMNP, August, 2012). 3) The exclosures keep ungulate species out while allowing for regrowth of vegetation. These sites can therefore serve as negative environmental controls.

We used PMCA for detection due to its ability to amplify low levels of PrP^{RES} (Joaquin Castilla et al., 2006; Morales et al., 2012; Saa et al., 2006; Saborio, Permanne, & Soto, 2001b). The assay was optimized with control plant samples grown in a greenhouse at Colorado State University with no previous exposure to CWD. We were able to amplify CWD prions in the presence of plant material and determine that we can detect 0.81 LD_{50} units/g of plant tissue. We also determined that the amplification rate of PMCA was slightly inhibited in the presence of plant material in comparison to amplification of CWD positive material only. Eight of thirty plants collected from RMNP showed the presence of PrP^{RES} after sPMCA when visualized by Western Blot and before quantifying the data with our PMCA scoring system. The

PMCA scoring system allows us to normalize our data but also estimate prion load based on LD_{50} units that were previously determined (T. A. Nichols et al., 2009). After analyzing the sPMCA data with our PMCA scoring system only six of the eight plants were above our NBH negative control threshold, and only two received mean scores that, when their SEM was subtracted, were still above our 99.9% confidence threshold. The two positive samples were collected outside of the exclosure, but some of the samples below our confidence threshold that showed WB positivity were collected inside of the exclosures. Based off of our assay specificity of 96% we do not think these are false positives but true positive samples. Sampling inside of the exclosure was meant to serve as an environmental negative control, but the small size of the exclosures and plant growth inside growing through the fencing may have limited the 'sterility' of the exclosures. We believe these samples may have been contaminated by an animal grazing or urinating nearby.

We analyzed the roots, stems, and leaf tissues of the two positive surface contaminated plants and none of the tissues showed any signs of positivity after 6 rounds of sPMCA and WB. This doesn't necessarily rule out the possibility that the internal tissues of the plants are contaminated. PrP^{RES} may be in a truncated or altered form within the plants and this form may not be able to be amplified let alone amplify to a level of visible detection by WB. Formation of a truncated protein could occur during osmotic uptake or proteolytic degradation by plant enzymes when taking up nutrients from the surrounding environment. Alternatively, the plants may truly lack any internal presence of PrP^{RES} which could be due to low level of surface contaminant, lack of uptake of PrP^{RES} through roots or hydathodes, or tight binding of soil particles to PrP^{RES} inhibiting uptake.

Previously we have estimated 1 million kg of feces are shed in the environment from cervids and of that 2-10% contains CWD positive material which would contribute about 2-500mg of PrP^{RES} being shed (T. A.

Nichols et al., 2009; Pulford et al., 2012). Estimates have also been made about the amount of PrP^{RES} shed in urine (Haley & Hoover, 2015; Davin M. Henderson et al., 2015). The combined shedding of CWD positive material in conjunction with the evidence shown here of plant contamination with PrP^{RES} illustrates a new source and potential reservoir that may be contributing to continued spread of CWD. Further research needs to be conducted in order to determine the extent of plant contamination within the park as well as the length of time plants remain contaminated. Additionally, there's only one study thus far showing that CWD transmission can occur in animals that are orally inoculated with *in-vitro* contaminated plants (Pritzkow et al., 2015). In order to ascertain whether plants could transmit disease, oral inoculations with environmentally contaminated material would need to be performed. These experiments are currently ongoing. Mice were intra-cranially inoculated with the surface rinses of the two positive plants. Mice are showing evidence of clinical signs including hyperactivity, akinesia, and lack of grooming in some animals (313 days' post-inoculation). The knowledge of this environmental reservoir would allow researchers to implement strategic management plans in order to curtail spread and implement possible environmental decontamination plans.

CHAPTER 3 – CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The specific aims of the thesis project were to optimize detection of PrP^{RES} in the presence of plant material using the prion amplification assay PMCA to ensure this assay could be used without any inhibition or false positives from the plant material. We also wanted to know the limitations of the assay in the presence of plant material by determining the lower limit of detection or sensitivity. The second aim was to sample plants from Rocky Mountain National Park and look at both the surface and interior tissues for PrP^{RES} using PMCA if the assay was appropriate after optimization. We found that amplification of PrP^{RES} was possible in the presence of plant material however there was a slight inhibition of amplification rate indicating some inhibition. Nevertheless, after optimizing PMCA we were able to detect 1 LD_{50} unit/g of plant tissue in PrP^{RES} spiked dilution series in the presence of plant material. We never saw any amplification of PrP^{RES} or false positives in plant only amplified samples. We then chose to analyze plants collected from three separate locations, with known history of CWD positivity, in Rocky Mountain National Park. Once plants were collected they were rinsed before sectioning and homogenizing to look at interior tissue contamination. The surface rinses of all of the plants were analyzed by sPMCA and we detected surface contamination on two of the plants collected from the first location. This is the first evidence of surface plant contamination collected in an environmentally relevant setting, an endemic area of CWD contamination, and it supports experimental evidence showing plant contamination can occur (Pritzkow et al., 2015). We chose to intra-cranially inoculate mice that over-express PrP^{C} (Tg(cerPrP)5037 mice) with the two positive plant rinses in order to determine if the amount of PrP^{RES} found on the surface of the plants is relevant in transmitting disease. These experiments are currently ongoing and mice are showing clinical signs of infection. Detection of PrP^{RES} in these mice would be the first evidence of environmental plant transmission

causing disease, solidifying plants as a culpable environmental reservoir. When looking at the interior tissues of the surface positive plants we did not find any evidence of PrP^{RES} contamination. There could be numerous reasons for this with the most obvious being there was no internalization and contamination in the roots, stems, or leaves of these plants even though they were contaminated on their exterior. Another plausible reason for lack of PrP^{RES} internalization could be due to the tight binding of PrP^{RES} to soil and other soil components which would inhibit uptake through the roots of the plants which is not to say it couldn't occur in other scenarios (C. J. Johnson et al., 2006; Wyckoff et al., 2013). PrP^{RES} could've been internalized however degradation of the protein could've occurred during uptake truncating PrP^{RES} or altering the abnormal protein form in such a way that it could no longer template conversion of PrP^{C} in PMCA. Lastly, it might be impossible in an environmental setting where the levels of PrP^{RES} are already very low, for this to occur or even be detectable even though uptake was shown in an experimental setting (Pritzkow et al., 2015). All in all, elucidation of a route of transmission provides management with more knowledge about transmission in order to better implement plans to curtail spread of CWD.

Future Directions

Experimental Directions

As mentioned in the previous section bioassays are ongoing to determine if the level of PrP^{RES} on the surface of the plants is sufficient to cause infection. Currently those experiments are 313 days post inoculation and the mice are showing evidence of clinical signs. Disease will be allowed to progress naturally after which relevant tissues will be analyzed by WB and IHC for signs of PrP^{RES}. If necessary sPMCA will be run to try and amplify and low level PrP^{RES} present. To better understand uptake of PrP^{RES} into plants an uptake experiment using osmosis and dye as an indicator was performed with carnations (*Dianthus carophyllus*). White carnations were placed in 15mL conical tubes containing water alone, dye

alone, water and dye, and water and dye with PrP^{RES} spiked in. At two, four, and twenty-four hours after the experiment was set up both petals and leaves from each carnation plant were collected and homogenized. We plan on analyzing these collected tissues and looking for presence of PrP^{RES} at the varying time-points to see if uptake of PrP^{RES} mimics the dye front or if it occurs at all. To continue to understand uptake of prion proteins inside of the plants we plan to grow grass plants in soil that's spiked with fluorescent prion rods. At different time points plants would be harvested and visualized using confocal microscopy to show uptake can occur. In order to determine the mechanisms of uptake plants would again be grown in PrP^{RES} spiked soil but plants with mutations in nitrogen synthesis or other root synthesis inhibitions would be used in order to determine whether uptake of PrP^{RES} could be halted.

Management Strategies

While the knowledge now exists of plants playing a role in environmental indirect transmission of Chronic Wasting Disease it is still very challenging to try and reduce environmental contamination. Free ranging animals can continue to shed PrP^{RES} in the environment where it will bio-accumulate and may persist for long periods of time wherein naïve animals may get exposed. Due to the size of the host-range it would also be near impossible to mount an environmental decontamination strategy especially when positive animals aren't cordoned or quarantined and the rate of re-contamination would most likely be very high. Ultimately it seems as though animal extinction in this area will be the end result unless a strategy can be reached. More work would need to be taken to understand the percentage of environmental contamination that exists in different areas. Both composting and use of enzyme treatment may help to degrade PrP^{RES} environmentally (Quiquampoix et al., 2002; Saunders et al., 2008; Xu et al., 2013).

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