Supplementary materials for Journal of Wildlife Diseases DOI: 10.7589/JWD-D-21-00029: Lisa L. Wolfe, Karen A. Fox, Karen A. Griffin, Michael W. Miller. MOUNTAIN LIONS RESIST LONG-TERM DIETARY EXPOSURE TO CHRONIC WASTING DISEASE.

Supplemental Materials and Methods

Clinical assessments & operant conditioning

Our experiences observing early signs of chronic wasting disease (CWD) in cervids and published accounts of spongiform encephalopathy in nondomestic felids including mountain lions (*Puma concolor*; Willoughby et al. 1992; Kirkwood and Cunningham 1994, 1999) underscored the clinical value of having tractable and readily observed subject animals to facilitate detecting the subtle earliest signs of prion infection. Conventional veterinary neurological examinations are designed to assess mentation, posture and gait, cranial nerve function, and postural reactions that may reveal deficits in coordination and strength. However, performing many of the manipulations necessary for proper assessment (e.g., "hopping", "wheel barrowing", "hemiwalking" to assess postural reactions) is neither feasible nor advisable in captive mountain lions and other nondomestic animals. Consequently, we devised a way to conduct a running clinical assessment of subject animals that would provide a sensitive indicator of infection, important from a scientific as well as an ethical standpoint.

To facilitate tractability, veterinary care, enrichment, and to provide a baseline for evaluating behavioral and other neurological changes over time, we trained all three mountain lions via positive reward-based operant conditioning (Skinner 1938; Pryor 1999; Westlund 2014). Mark-reward operant conditioning has been used in a variety of nondomestic animals to ease handling and reduce injuries during veterinary procedures (Pryor 1999; Westlund 2014). For example, Phillips et al. (1998) demonstrated that bongo (*Tragelaphus eurycerus*) could be conditioned to stand in a handling crate for successful blood sampling. Their work showed that cortisol levels were lower in conditioned animals than in restrained animals, indicating less stress in the former. Training and conditioning also offer broader welfare benefits to animals held in captivity (Westlund 2014).

We selected trained behaviors that served as proxies for the elements of a conventional neurological examination to test mentation, balance, coordination, strength, and tractability, essentially capturing and reinforcing natural behaviors for use as on-demand assessment tools. We used a whistle, clicker or voice command as the bridge with chunks of deer or wapiti meat given as the food reward. Meat reward amounts varied depending on duration and intensity of work and served as a supplemental source of prion exposure. Behaviors were shaped as approximations until the final behavior was established as a response to both a verbal and hand signal. The specific trained behaviors and their relationship to neurological exam components are described in detail in Table S1.

We initially did training with each animal at least three times per week until behaviors were well established. We used these behaviors regularly (at least weekly) to assess behavior, coordination and muscle tone, all affected by spongiform encephalopathy in felids (Willoughby 1992). The training also allowed us to routinely weigh animals to monitor body condition. Beyond the immediate health monitoring and animal welfare benefits, the extent of their training and willingness to work also allowed all three animals to be further trained and used in other complementary studies over time (see Supplemental Video).

Histopathology and immunohistochemistry

Multiple sections of brain, spinal cord, tonsil, multiple lymph nodes, and other tissues (Table S2; appended reports) were examined by light microscopy after hematoxylin and eosin (H&E) staining for lesions and after IHC staining for evidence of disease-associated prion protein (PrP^d) accumulation. We used published reports on distribution of spongiform lesions and PrP^d accumulation in mule deer (Sigurdson et al. 1999, 2001; Fox et al. 2006) and felids (Ryder et al. 2001; Seelig et al. 2015) to guide sampling and microscopic examinationss.

The entire set of tissues for IHC was stained using the CSUVDL protocol optimized for detecting PrP^d in cervids (Spraker et al. 2002a) using monoclonal antibody (mAb) F99/97.6.1 (O'Rourke et al. 2000) targeting the PrP epitope at mule deer amino acids (aa) 220–225. For immunohistochemistry (IHC), sections were mounted on positively charged glass slides, dewaxed, and hydrated. Pretreatments included 3% hydrogen peroxide to quench endogenous peroxidase, antigen retrieval with 98% formic acid for 5 min and 20 min heat and pressure (decloaking) in citrate buffer (DAKO Retrieval), followed by incubation with proteinase K (DAKO Ready-to-use Proteinase K reagent) for 1 min at room temperature. Sections were immunolabeled with 10 min incubations at room temperature with primary antibody (monoclonal antibody F99/97.6.1; Spraker et al. 2002a), biotinylated anti-mouse antibody, streptavidin-horseradish peroxidase conjugate, and 500 µl diaminobenzidine (DAB) added as enzyme substrate with intervening wash buffer rinses, carried out in an automated slide stainer. Sections were counterstained with hematoxylin.

In addition, we examined a subset of tissue sections using a second IHC protocol described by Seelig et al. (2015; Prion Research Center, CSU, Fort Collins, Colorado USA) optimized for detecting PrP^d in cats experimentally infected with CWD (Mathisaon et al. 2013). This subset included caudal brainstem (dorsal motor nucleus of the vagus nerve and solitary tract nucleus), basal nuclei, hippocampus, cerebral cortex, ileum, cecum, trigeminal ganglion, tonsil, and one or more cranial lymph nodes, thereby covering the sites most consistently reported as showing PrP^d accumulations in prion-infected felids and cervids (Spraker et al. 2002b; Hilbe et al. 2009; Seelig et al. 2105). The Seelig et al. (2015) protocol employed mAb L42 targeting PrP aa 145–163 (Vorberg et al. 1999). Caudal brainstem sections from experimentally infected and uninfected cats (Mathiason et al. 2013) were used as L42-IHC controls.

We referenced established criteria (e.g., Fraser and Dickinson 1968; Wells and Wells 1989; Wyatt et al. 1991; Willoughby et al. 1992; Williams and Young 1993; Gould et al. 2003; Wohlsein et al. 2012) in interpreting spongiform changes consistent with prion disease.

Similarly, we referenced negative and positive control slides, as well as multiple publications in establishing criteria for interpreting IHC staining patterns as consistent with prion disease (Bell et al. 1997; Ryder et al. 2001; Spraker et al. 2002b; Vidal et al. 2006; Jeffrey et al. 2011, 2012; Gill et al. 2013, 2020; Seelig et al. 2015; Williams et al. 2018). Our criteria, modeled after the clear descriptions by Gill et al. (2020), are summarized as follows: To be considered IHC-positive, a section needed to show characteristic immunostaining (e.g., staining within or along the periphery of the neuronal perikaryon, staining of a follicular dendritic cell network within a germinal center of a secondary lymphoid follicle); that characteristic staining also needed to be present either in consecutive sections, or nearby in a deeper section. We did not regard sections with staining identified as non-specific, or diffuse reactivity, or poor definition of the positively labelled cells, or very weak immunoreactivity (including repeats) as indicative of prion infection. Sections showing no immunostaining or reactivity at all, or showing only non-specific including staining of atypical structures (e.g., macrophages within lymphoid follicles, non-specific background staining inside follicles, staining of parasitic sarcocysts with mAb F99/97.6.1, and inconsistent staining of vessel walls with mAb L42), or staining of structures including nerve fibers, macrophages, mucosa epithelium and myofibroblasts, and occasionally small parasites were regarded as negative (Gill et al. 2013, 2020).

PRNP gene sequencing

We extracted DNA from lymph node or whole blood buffy coat tissue using the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland, USA) according to manufacturer's instructions. Polymerase chain reaction (PCR) amplification was performed with the forward primer 5'-GGTTCCAACATGAACGTAAGATG-3' and reverse primer 5'-TAAAGGGCTGTAGGTAGACAC-3' used by Stewart et al. (2012; primer sequences kindly provided by A. Robinson, The Roslin Institute, University of Edinburgh, Midlothoan, Scotland, UK) with the following conditions: Illustra PuReTaq Ready-to-Go PCR beads (Cytiva, Marlborough, Massachusetts, USA) were combined with 0.4 µM of each primer, 100 ng of template DNA, and PCR grade water to a final volume of 25 µL. PCR conditions were: 3 min at 95 C followed by 40 cycles of 30 sec at 95 C, 30 sec at 62 C, and 1 min at 72 C, with a final extension of 10 min at 72 C (Stewart et al. 2012). PCR products were visualized on an agarose gel, and

products with a single band of the expected size (841 bp) were sequenced using forward primer 5'-CGTAAGATGCTGACGCCCTTC-3' or reverse primer 5'-GACCCGTAAAAGATGAAGAAG-3' (Stewart et al. 2012) by a commercial lab (Eurofins, Lancaster, Pennsylvania, USA). We aligned the resulting sequences with the published *Puma concolor PRNP* sequence (Stewart et al. 2012; JX218980.1) using MUSCLE alignment software.

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Table S1. Core set of behaviors established via operant conditioning as a basis for conducting neurological evaluations in captive mountain lions (*Puma concolor*) receiving long-term dietary exposure to chronic wasting disease (CWD)-infected cervid carcasses. We selected trained behaviors that would serve as proxies for the manipulations used to evaluate mentation, posture and gait, cranial nerve function, and postural reactions in domestic animals to detect clinical signs reported in association with spongiform encephalopathy in felids. Those signs, which have included "unusual" behaviors (aggression or timidity), tactile and auditory hyperesthesia, altered grooming habits, ataxia, polyphagia, anorexia, polydipsia, hypermetria and falling, weight loss, head and tail tremors, patterned motor behaviors, stilted gait, and ataxia, would have been readily observed in captive mountain lions using this approach.

Trained behavior	**Description**	**Neurological examination components covered**
target	touch nose to target	mentation, vision, proprioception
point follow	follow trainer's pointed index finger to a location at varied speeds	mentation, vision, proprioception, locomotion, gait, postural reactions
sit	sit facing trainer	proprioception, postural reactions
down	lie down	proprioception, postural reactions
platform	step or jump up onto an elevated platform (included standing on scale)	proprioception, balance, locomotion, gait, postural reactions
shift	move to a different cage (or to a different location within large enclosure)	mentation, proprioception, locomotion, gait, postural reactions
up	stand on hind with front legs braced against fence	proprioception, balance, postural reactions (especially hind limb strength & muscle tone)
side or parallel station	moving sideways into a source of pressure (focal or broad)	mentation, proprioception, tactile response, postural reactions
paw present	extend forepaw under gate for handling by trainer	proprioception, tactile response, postural reactions (especially forelimb strength & muscle tone)
over	roll over (side-to-side)	proprioception, postural reactions, righting reflex
lick	approach & lick target or hand	tactile response, prehension, licking/chewing (also used to deliver oral medications)
reward feeding in conjunction with training	pieces of fresh meat offered to animal as a reward for completing behavior/task	mentation, tactile response, coordination, appetite, prehension, chewing/swallowing (also used to deliver oral medications)

Table S2. Multiple sections of brain, spinal cord, tonsil, multiple lymph nodes, and other tissues were collected and examined by light microscopy after hematoxylin and eosin staining for lesions and after immunohistochemistry staining for evidence of disease-associated prion protein (PrP^d) accumulation. We used published reports on distribution of spongiform lesions and PrP^d accumulation in mule deer and felids to guide sampling and microscopic exams. See text for references.

Tissues processed for histology

Brain:
- Caudal medulla oblongata near C1
- Medulla oblongata at obex
- Rostral medulla oblongata (pons/cerebellar peduncles)
- Cerebellum – vermis
- Cerebellum – hemisphere with dorsal roof nuclei
- Midbrain – inferior colliculi
- Midbrain – superior colliculi
- Hippocampus
- Caudal thalamus with pineal gland
- Rostral thalamus
- Basal ganglia
- Occipital cortex
- Temporo-parietal cortex
- Frontal cortex

Spinal cord:
- Cervical sections C3, C4
- Thoracic sections T9, T11
- Lumbar sections L3, L6

Lymphoid system:
- Spleen
- Mesenteric LN
- Submandibular LN
- Medial retropharyngeal LN
- Palatine tonsil

Endocrine:
- Pancreas
- Adrenal gland
- Pituitary

Viscera:
- Liver
- Kidney
- Small intestine, to include distal ileum
- Lung
- Right ventricle
- Left ventricle

Skeletal muscles:
- *Longissiums lumborum*
- *Semitendinosis*
- Triceps

Other organs:
- Eye

Supplemental Video

Video S1. To facilitate tractability, veterinary care, enrichment, and to provide a baseline for evaluating behavioral and neurological changes over time, we used operant conditioning to capture natural behaviors lending to ongoing assessments. We selected trained behaviors that tested mentation, balance, coordination, strength, and tractability, often capturing and reinforcing natural behaviors for use as assessment tools. We used a whistle, clicker or voice command as the bridge with chunks of cervid meat given as the food reward. We used these and other behaviors regularly (at least weekly) to assess behavior, coordination and muscle tone, all affected by spongiform encephalopathy in felids. Beyond the immediate health monitoring benefits, the extent of their training and willingness to work also allowed all three animals to be further trained and used in other complementary studies over time.