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**Hernández, and Thomas B. Waltzek. PSEUDORABIES (AUJESZKY'S DISEASE) IS AN UNDERDIAGNOSED CAUSE OF DEATH IN THE FLORIDA PANTHER (*PUMA CONCOLOR CORYI*).**

## **Materials and Methods**

### Capture and handling

We fitted captured panthers ( $\geq 6$  mo of age) with VHF or VHF/GPS radio-collars (Land et al. 2008; Onorato et al. 2010; Criffield et al. 2018). Panther locations were determined by detecting VHF radio-collar signals on a receiver in a fixed-wing aircraft flying the area three times a week. We programmed the GPS collars to transmit its position 1-24 times per day. Depending on research and management objectives, radio-collars were 1) replaced at recapture to maintain battery life and remained on the panther for life, 2) were removed either remotely or at recapture, or 3) failed and remained non-functioning on the panther unless the animal was opportunistically recaptured. Radio-collars emitted a mortality signal when still for longer than 2-8 hr. Carcasses were recovered and stored on ice or frozen until necropsy. Panthers dying of unknown causes were necropsied within 1-2 d; those dying of known trauma (e.g. vehicular collision) were occasionally frozen up to 90 d before necropsy. We categorized as collared those

Florida panthers with functioning radio-collars at the time-of-death and all others (not radio-collared or whose radio-collar was not functioning) as uncollared.

### Diagnostics

*Virus Isolation* – Briefly, tissue samples (~ 0.5 cm<sup>3</sup>) were homogenized in 650 µl of virus isolation media (1X minimum essential media, 2.2 g/L NaHCO<sub>3</sub>, 3% fetal bovine serum, 400 units/ml penicillin, 400 µg/ml streptomycin, 1 µg/ml amphotericin B) and centrifuged at 6700 x g for 10 min. Duplicate 100µl aliquots of supernatant were inoculated into Vero MAURU cells incubated at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and passaged at 10-d intervals. At the end of each passage (1<sup>st</sup> or 2<sup>nd</sup>) DNA was extracted from culture supernatant using QIAamp® DNA Mini Kit (Qiagen, Germantown, Maryland USA).

*Real-time Polymerase Chain Reaction (real-time PCR)* – DNA was extracted using the DNeasy Blood and Tissue Kit (Qiagen) and the QIAamp® DNA FFPE Tissue Kit (Qiagen), respectively. A PRV-specific real-time PCR assay that targets a region of the glycoprotein D (gD) gene was performed as described by Cramer et al. (2011) with slight modifications as follows. Forward primer (US6-1190F) 5'-ACACCGACGAGCTAAAAGCG-3' and reverse primer (US6-1252R) 5' ATCATCATCGACCGGTA-3' were added to the reaction at a final concentration of 0.5 µM, and the minor groove binder probe (US6-1211T) 5'FAM-AGCCCGGTCCGTAGC-3' at 0.2 µM in a 25µL total reaction volume. Extracted DNA (8µL) was used as a template for each reaction. The assay was run in the Smartcycler II (Cepheid Inc., Sunnyvale, California USA) with the following cycling parameters: a denaturation step of 95 C for 10 min followed by 40 cycles of 95 C for 15 s and 60 C for 1 min. Tissues tested, if available, were brainstem, brain, adrenal gland, bladder, ovary, kidney, liver, spleen, lung, salivary gland, and lymph node (Supplementary Table 3). For testing of fixed tissues, we cut 5µm scrolls from archived

formalin-fixed paraffin-embedded tissue blocks (Supplementary Table 4). For negative controls, we performed PCR on archived adrenal gland and/or brainstems collected from 10 randomly selected panthers that had been killed by vehicles in Central and South Florida (2010-2015). We also tested tissues (brain, brainstem, adrenal, and/or salivary gland) from virus-isolation positive panthers as positive controls (Table 1).

*Genetic characterization and phylogenetic analysis of PRV strains* –To genetically characterize PRV-positive tissues, the partial PRV glycoprotein C (gC) sequences were obtained using primers PRV F: 5'-TTTTTAAAACCGCGATGGG-3' (Dufour and De Boisséson 2003) and PRV-R1: 5'-ACGTGTAGACGCCCTCGGTCT-3' (Hahn et al. 2010). Each PCR reaction included 25µL of 2× Platinum® Taq DNA Polymerase High Fidelity Reaction Mix (ThermoFisher, Carlsbad, California USA), 1µL of forward and reverse primers at 10µM, 1µL of enzyme, 1.7µL of detection enhancer (AgPath-ID; ThermoFisher), and 2.25µL of viral DNA. Thermocycler conditions were as follows: denaturation at 94 C for 2 min followed by 40 cycles of 94 C for 15 s, 57°C for 30 s, and 68 C for 1 min with a final extension of 68 C for 5 min. PCR products were gel purified on a 1% agarose gel visualized with ethidium bromide. Appropriately sized amplicons (708bp) extracted using a QIAquick Gel Extraction Kit (Qiagen) and sent to Macrogen USA (Rockville, Maryland, USA) for Sanger sequencing.

We performed PRV genome sequencing on the PRV DNA-positive Florida feral swine. A DNA library from this sample was prepared using a NEBNext® Ultra™ II DNA Library Prep Kit for Illumina® (Ipswich, Massachusetts USA) and sequenced using a 600-cycle V3 Kit on an Illumina® MiSeq sequencer (Illumina, San Diego, California USA). The paired-end reads were quality trimmed and assembled *de novo* in CLC Genomic Workbench V7 (Qiagen) using default settings. Basic Local Alignment Search Tool analysis of the assembled contigs was conducted

against the National Center for Biotechnology Information (NCBI) GenBank non-redundant protein sequence database to determine the contig which contained the complete gC sequence.

The partial gC sequences generated from Florida panther isolates (FP117, FP156, and FP173), the complete gC sequence from the Florida feral swine, and the PRV gC sequences retrieved from the NCBI GenBank Nucleotide database were aligned using the Multiple Alignment tool on the Fast Fourier Transform server (<https://mafft.cbrc.jp/alignment/software/>) (Kato et al. 2019). Missing characters in the alignment for the PRV strains from panthers and a dog (dog/Italy/15608/2016; GenBank accession # MF040159) were coded as question marks. The final alignment consisted of 85 PRV gC sequences with 1,486 nucleotide characters (including gaps and question marks). A maximum-likelihood-analysis (MLA) was performed in IQ-TREE with the Bayesian information criterion to determine the best model fit and 1000 non-parametric bootstraps to test the robustness of the clades (Nguyen et al. 2015). A sequence difference count matrix was calculated using the same sequence alignment in BioEdit Sequence Alignment Editor 7.2.5 (<https://bioedit.software.informer.com/7.2/>) after columns containing gaps and missing characters were removed. The final dataset used to determine the sequence difference consisted of 85 PRV gC sequences and 541 nucleotide characters.

*Immunohistochemistry (IHC)* – IHC was performed at the Michigan State University Veterinary Diagnostic Laboratory as described by Quiroga et al. (1998) on sections of brain, brainstem, and adrenal gland from PRV-positive panthers for which suitable tissues were available (Table 1, Supplementary Table 6). IHC was also performed on spinal cord and cranial nerves 9 and 10 from FP118.

*Serology* – Archived serum samples collected 1988-2012 from 46 live-captured adult panthers that occupied habitat with a known feral swine population were tested by virus neutralization for

antibodies against PRV at the National Veterinary Services Laboratories (Ames, Iowa USA) (Beach 2018). Unpublished data from sera tested for PRV antibodies by latex agglutination (Animal Health Diagnostic Center, Cornell University, Ithaca, New York USA) from 35 panthers captured 2005-2008 throughout their range also were included (FWC, unpubl. data).

*Other Diagnostics* – To screen for additional viruses in panthers with PRV or undiagnosed mortalities (Supplementary Table 7), total RNA was extracted from homogenized tissues using the QIAamp Viral RNA Mini Kit (Qiagen) according to the manufacturer's instructions. Screening for eastern equine encephalitis virus RNA was performed using the Florida Department of Health Bureau of Laboratories protocol (Florida Department of Health 2011). Specimens were screened for the presence of flavivirus vRNA using consensus primers in a nested reaction as described by Sánchez-Seco et al. (2005) followed by gel electrophoresis and visualization of products using GelStar nucleic acid stain (Lonza, Basel, Switzerland). When postmortem condition allowed, all necropsied panthers were tested for rabies virus by direct fluorescent antibody (DFA) at the Bureau of Public Health Laboratory (Jacksonville, Florida USA). Serological testing for feline leukemia virus and feline immunodeficiency virus (FIV) were completed for necropsied panthers with suitable tissues (see Supplementary Table 1) as described by Cunningham et al. (2008).

### Statistics

*Co-variates* – We tested the effects of the covariates (listed below) on PRV with logistic regression. We used as positive cases ( $n=9$ ) collared panthers with a Confirmed or Probable PRV diagnosis and as negative cases ( $n=134$ ) panthers having either a cause of death other than PRV or an unlikely PRV diagnosis. We excluded Suspect and Possible cases and all kittens ( $n=2$ ) because of small sample size and lack of PRV diagnosed in the kitten age class.

Co-variates used included sex, geographic region (Fig. 1, north or south of Interstate [I]-75, approximate latitude 26° 10.017'), age class, and individual heterozygosity. Greater predation on feral swine by panthers/puma occurred north of I-75 (Caudill et al. 2019). Age classes were delineated as kitten (0 to <1 yr), subadult (1 to <2.5 yr for females, and 1 to <3.5 yr for males), adult (2.5 to <10 yr for females, 3.5 to <10 yr for males), and older adult ( $\geq 10$  yrs) (Hostetler et al. 2013). Panther ages were either known (i.e. they had been handled as neonates) or estimated from tooth wear (Anderson and Lindzey 2000) and gum line recession (Laundre et al. 2000). Individual heterozygosity values for panthers were determined using genotype data from 16 microsatellites as described by van de Kerk et al. (2019).

Supplementary Table 1. Free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested or evaluated for pseudorabies virus infection at necropsy 1981-2018.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Prior COD <sup>c</sup>	New COD	Radio-collar	COD type	Age (years)	Rabies DFA <sup>d</sup>	FeLV <sup>e</sup>
FP029	5/27/1992	Confirmed	PRV	PRV	Yes	Acute	3.9	Unknown	Unknown
FP077	7/12/2003	Confirmed	Unknown	PRV	No	Acute	6.1	Unknown, vaccinated	Unknown
FP108	11/16/2002	Confirmed	Unknown-ISA	PRV	Yes	Acute	1.8	Unknown	Unknown
FP117	7/28/2004	Confirmed	PRV	PRV	Yes	Acute	2.3	Not tested	Not tested
FP118	4/4/2003	Confirmed	Unknown	PRV	Yes	Acute	1.1	Unknown, vaccinated	Unknown
FP156	7/29/2011	Confirmed	Unknown-HBC	PRV	Yes	Acute	6.1	Unknown, vaccinated	Negative
FP173	12/22/2010	Confirmed	N/A	PRV	Yes	Acute	4.9	Negative	Negative
FP242	8/29/2016	Confirmed	N/A	PRV	Yes	Acute	10	Unsuitable	Unknown
FP245	4/11/2018	Confirmed	N/A	PRV	Yes	Acute	7	Not tested	Not tested
UCFP057	6/16/2003	Confirmed	Unknown	PRV	No	Acute	5	Unknown	Unknown
FP073	6/28/2003	Probable	Unknown	PRV	Yes	Acute	7.8	Unknown	Unknown
UCFP055	6/13/2003	Probable	Unknown	PRV	No	Acute	1.5	Unknown	Unknown
UCFP056	6/14/2003	Probable	Unknown	PRV	No	Acute	1.5	Unknown	Unknown
FP084	4/20/2000	Suspect	Unknown	Unknown	Yes	Acute	1.2	Negative	Unknown
FP128	9/27/2007	Suspect	Unknown	Unknown	Yes	Acute	7.3	Unknown	Negative
FP141	1/5/2011	Suspect	Unknown	Unknown	Yes	Acute	8.6	Unknown, vaccinated	Negative
FP197	7/18/2012	Suspect	Unknown	Unknown	Yes	Acute	4.5	Not tested	Negative
FP244	8/26/2017	Suspect	N/A	Unknown	Yes	Acute	7	Unknown	Not tested
TX101	3/29/2000	Suspect	Unknown	Unknown	Yes	Acute	9.1	Negative	Unknown
TX103	8/19/1999	Suspect	Unknown	Unknown	Yes	Unknown	8.4	Unknown, vaccinated	Unknown
UCFP163	9/3/2011	Suspect	Unknown	Unknown	No	Unknown	3	Nd	Unknown
FP005	11/18/1983	Possible	Unknown	Unknown	Yes	Unknown	9	Unknown	Unknown
FP017	7/23/1990	Possible	Unknown	Unknown	Yes	Acute	12.5	Not tested	Unknown
FP023	12/1/2000	Possible	Unknown	Unknown	Yes	Acute	14	Not tested	Not tested
FP024	8/22/1988	Possible	Unknown	Unknown	Yes	Acute	4	Not tested	Unknown
FP027	7/23/1989	Possible	Unknown	Unknown	Yes	Acute	3.8	Negative	Unknown
FP036	10/10/1998	Possible	Unknown	Unknown	Yes	Unknown	12.7	Unknown	Unknown
FP042	6/22/1995	Possible	Unknown	Unknown	Yes	Acute	6	Unknown, vaccinated	Unknown
FP048	10/23/2006	Possible	Unknown	Unknown	Yes	Acute	15	Unknown	Negative
FP093	11/28/2015	Possible	N/A	Unknown	Yes	Acute	17	Unknown	Negative
FP100	1/25/2007	Possible	Unknown	Unknown	Yes	Acute	10	Unknown, vaccinated	Negative

FP105	1/16/2002	Possible	Unknown	Unknown	Yes	Acute	6.7	Unknown, vaccinated	Negative
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Supplementary Table 1. Continued

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Prior COD <sup>c</sup>	New COD	Radio-collar	COD type	Age (years)	Rabies DFA <sup>d</sup>	FeLV <sup>e</sup>
FP107	8/6/2008	Possible	Unknown	Unknown	Yes	Unknown	8.3	Unknown, vaccinated	Negative
FP138	5/12/2008	Possible	Unknown	Unknown	Yes	Acute	7.3	Unknown, vaccinated	Negative
FP155	9/15/2008	Possible	Unknown	Unknown	Yes	Acute	3.3	Not tested	Not tested
FP164	6/30/2008	Possible	Unknown	Unknown	Yes	Acute	2.3	Not tested	Not tested
FP188	9/14/2011	Possible	Unknown	Unknown	Yes	Acute	3.5	Unknown, vaccinated	Not tested
UCFP017	12/19/1987	Possible	Unknown	Unknown	No	Unknown	10	Unknown	Unknown
UCFP068	9/30/2004	Possible	Unknown	Unknown	No	Unknown	4.5	Unknown	Negative
UCFP082	2/27/2006	Possible	Unknown	Unknown	No	Unknown	>0.5	Not tested	Not tested
UCFP109	9/15/2008	Possible	Unknown	Unknown	No	Unknown	8	Not tested	Not tested
UCFP127	10/9/2009	Possible	Unknown	Unknown	No	Unknown	3.5	Unknown	Negative
UCFP138	1/29/2010	Possible	Unknown	Unknown	No	Unknown	7	Not tested	Not tested
UCFP157	3/15/2011	Possible	Unknown	Unknown	No	Unknown	3	Not tested	Not tested
UCFP234	1/1/2014	Possible	N/A	Unknown	No	Unknown	>0.5	Not tested	Not tested
UCFP235	2/4/2015	Possible	N/A	Unknown	No	Unknown	0.83	Not tested	Negative
UCFP260	1/3/2016	Possible	N/A	Unknown	No	Unknown	0.5	Not tested	Negative
FP006	4/16/1982	Unlikely	Unknown	Trauma-Unknown	Yes	Unknown	7	Not tested	Not tested
FP014	6/20/1991	Unlikely	Unknown	Unknown-suspect renal failure	Yes	Unknown	9	Negative	Unknown
FP016	1/3/2000	Unlikely	Unknown	Unknown	Yes	Unknown	14.5	Not tested	Unknown
FP022	7/20/1991	Unlikely	Unknown	Unknown	Yes	Unknown	5	Unknown	Unknown
FP032	9/12/2002	Unlikely	Other-degenerative	Other-degenerative	Yes	Acute	16	Unknown, vaccinated	Unknown
FP068	3/1/2000	Unlikely	Unknown	ISA-probable	Yes	Unknown	6.6	Unknown, vaccinated	Unknown
FP085	3/1/2004	Unlikely	Unknown	Unknown-toxin suspected	Yes	Acute	6.5	Unknown, vaccinated	Negative
FP092	9/1/2001	Unlikely	Unknown	ISA <sup>f</sup>	Yes	Unknown	2.2	Not tested	Not tested
FP104	3/9/2006	Unlikely	Unknown	Unknown	Yes	Chronic	5.4	Unknown, vaccinated	Negative
FP122	2/13/2004	Unlikely	FeLV	FeLV	Yes	Acute	2.2	Negative	Positive
FP131	4/16/2008	Unlikely	Endogenous lipid pneumonia	Endogenous lipid pneumonia	Yes	Acute	9.1	Unknown, vaccinated	Negative



FP159	4/25/2015	Unlikely	N/A	Unknown-susp. ISA	Yes	Acute	13	Unsatisfactory	Negative
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Supplementary Table 1. Continued

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Prior COD <sup>c</sup>	New COD	Radio-collar	COD type	Age (years)	Rabies DFA <sup>d</sup>	FeLV <sup>e</sup>
FP175	1/23/2014	Unlikely	N/A	Trauma- Unknown	Yes	Acute	6.5	Unsatisfactory	Negative
FP191	2/13/2015	Unlikely	N/A	Trauma- Unknown	Yes	Acute	16	Not tested	Not tested
FP223	1/6/2014	Unlikely	N/A	Bartonella	Yes	Acute	2.7	Negative	Negative
FP229	11/14/2014	Unlikely	N/A	Trauma-ISA probable	Yes	Acute	4	Not tested	Not tested
FP231	8/4/2014	Unlikely	N/A	Unknown – suspect FeLV	Yes	Acute	2.4	Not tested	Positive
TX107	1/18/2001	Unlikely	Unknown	Unknown	Yes	Chronic	9	Negative	Unknown

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>COD = cause of death

<sup>d</sup>DFA = direct fluorescent antibody test

<sup>e</sup>FeLV = feline leukemia virus

<sup>f</sup>ISA = Intraspecific aggression

Supplementary Table 2. Results for free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested for pseudorabies virus infection by virus isolation (VI) at necropsy 1981-2018. Only individuals that had tissues available for VI testing are described. ‘—’ indicates that the tissue was not tested.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland
FP029	5/27/1992	Confirmed	—	Positive	—
FP077	7/12/2003	Confirmed	—	Negative	—
FP108	11/16/2002	Confirmed	—	Negative	Negative
FP117	7/28/2004	Confirmed	—	Positive	—
FP118	4/4/2003	Confirmed	—	Negative	—
FP156	7/29/2011	Confirmed	—	Negative	Negative
FP173	12/22/2010	Confirmed	—	Negative	Positive
FP141	1/5/2011	Suspect	—	Negative	—
FP197	7/18/2012	Suspect	—	Negative	—
TX103	8/19/1999	Suspect	—	Negative	—
FP105	1/16/2002	Possible	—	Negative	—
FP085	3/1/2004	Unlikely	Negative	Negative	—
FP092	9/1/2001	Unlikely	—	—	—
FP104	3/9/2006	Unlikely	—	Negative	—
FP122	2/13/2004	Unlikely	Negative	—	—
TX107	1/18/2001	Unlikely	—	Negative	—

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

Supplementary Table 3. Results for free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested for pseudorabies virus infection by real-time polymerase chain reaction (PCR) on fresh tissues performed at Michigan State University. Only individuals that had fresh tissues available for PCR testing are described. N indicates the tissue was negative and P indicates the tissue as positive. ‘—’ indicates that the tissue was not tested.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland	Salivary gland	Bladder, Uterus	Kidney	Pancreas	Heart	Lung	Spleen	Lymph node	Liver	Other
FP029	5/27/1992	Confirmed	—	N	—	—	—	—	—	N	N	—	—	—	—
FP077	7/12/2003	Confirmed	—	N	—	P	—	—	—	N	N	—	N	—	—
FP108	11/16/2002	Confirmed	—	P	P	N	—	N	—	—	N	—	N	—	—
FP117	7/28/2004	Confirmed	—	—	—	—	—	N	—	N	N	—	N	—	—
FP118	4/4/2003	Confirmed	—	P	N	P	—	N	—	N	N	—	—	—	—
FP156	7/29/2011	Confirmed	—	N	P	—	—	P	—	—	N	—	—	—	—
FP173	12/22/2010	Confirmed	—	—	—	—	N	N	—	—	N	—	P	P	—
FP245	4/11/2018	Confirmed	P	—	—	N	—	—	—	—	—	—	—	—	—
UCFP057	6/16/2003	Confirmed	—	P	—	—	—	N	—	N	N	—	—	—	—
UCFP055	6/13/2003	Probable	—	—	—	—	—	—	—	N	N	—	—	—	—
UCFP056	6/14/2003	Probable	—	—	—	—	—	—	—	N	N	—	—	—	—
FP141	1/5/2011	Suspect	—	—	N?	—	—	—	—	N	N	N	N	—	—

Supplementary Table 3. Continued

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland	Salivary gland	Bladder, Uterus	Kidney	Pancreas	Heart	Lung	Spleen	Lymph node	Liver	Other
FP197	7/18/2012	Suspect	—	—	—	—	N	N	—	N	N	N	—	N	N <sup>c</sup>
TX103	8/19/1999	Suspect	—	N	—	—	—	N	—	—	—	—	—	—	—
UCFP163	9/3/2011	Suspect	—	—	—	—	—	—	—	N	N	—	—	—	—
FP036	10/10/1998	Possible	—	—	—	—	—	—	—	—	—	—	—	N	—
FP042	6/22/1995	Possible	—	—	—	—	—	N	—	—	—	—	—	N	—
FP100	1/25/2007	Possible	—	N	—	—	—	—	—	—	—	—	—	—	—
FP105	1/16/2002	Possible	—	N	—	—	N	N	—	—	—	—	—	—	N <sup>d</sup>
FP107	8/6/2008	Possible	—	N	N	N	—	—	—	N	—	—	—	—	N <sup>c</sup>
FP138	5/12/2008	Possible	N	N	—	—	—	—	—	N	N	—	—	—	—
UCFP235	2/4/2015	Possible	N	—	—	—	—	—	—	—	—	—	—	—	—
FP014	6/20/1991	Unlikely	—	N	—	—	—	N	—	—	—	—	—	N	—
FP022	7/20/1991	Unlikely	—	—	—	—	—	N	—	—	N	—	—	—	—
FP032	9/12/2002	Unlikely	—	—	N	—	—	—	—	—	—	—	—	—	—
FP085	3/1/2004	Unlikely	—	N	N	—	N	N	—	—	—	—	—	—	—

Supplementary Table 3. Continued

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland	Salivary gland	Bladder, Uterus	Kidney	Pancreas	Heart	Lung	Spleen	Lymph node	Liver	Other
FP104	3/9/2006	Unlikely	—	—	N	—	N	N	—	—	—	—	—	—	—
FP122	2/13/2004	Unlikely	N	—	—	—	—	—	—	—	—	—	—	—	—
FP159	4/25/2015	Unlikely	N	N	N	—	—	—	—	—	—	—	—	—	N <sup>f</sup>
FP175	1/23/2014	Unlikely	—	N	N	—	—	—	—	—	—	—	—	—	—
FP191	2/13/2015	Unlikely	N	N	—	—	—	—	—	—	—	—	—	—	—
FP223	1/6/2014	Unlikely	—	—	—	—	—	—	—	—	N	—	—	—	—
FP231	8/4/2014	Unlikely	—	—	—	—	—	N	N	—	N	—	—	—	—
TX107	1/18/2001	Unlikely	—	N	—	—	—	N	—	N	N	—	—	—	—

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>Tissue = pancreas

<sup>d</sup>Tissue = ovaries

<sup>e</sup>Tissue = spine

<sup>f</sup>Tissue = tonsil

Supplementary Table 4. Results for free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested for pseudorabies virus infection by real-time polymerase chain reaction (PCR) on fixed tissues performed at Michigan State University. Only individuals that had fixed tissues available for PCR are described. ‘—’ indicates that the tissue was not tested.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland	Ovary	Kidney	Other
FP118	4/4/2003	Confirmed	—	Positive	Negative	—	—	—
UCFP057	6/16/2003	Confirmed	—	—	—	—	Negative	—
FP084	4/20/2000	Suspect	Negative	Negative	Negative	—	—	—
FP128	9/27/2007	Suspect	—	—	—	—	—	—
FP141	1/5/2011	Suspect	Negative	Negative	Negative	—	—	Negative <sup>c</sup>
TX101	3/29/2000	Suspect	—	Negative	—	—	Negative	—
FP105	1/16/2002	Possible	Negative	Negative	Negative	—	—	—
FP107	8/6/2008	Possible	—	—	Negative	Negative	Negative	—
TX107	1/18/2001	Unlikely	—	Negative	Negative	—	Negative	—

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>Tissue = spine

Supplementary Table 5. Results for free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested for pseudorabies virus infection by real-time polymerase chain reaction (PCR) on frozen tissues at the University of Florida. Only individuals that had frozen tissues available for PCR are described. ‘—’ indicates that the tissue was not tested.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brainstem	Cerebrum	Adrenal gland	Salivary gland	Other
FP077	7/12/2003	Confirmed	—	Positive	—	—	—
FP108	11/16/2002	Confirmed	—	Positive	Positive	—	—
FP117	7/28/2004	Confirmed	—	Negative	—	—	—
FP118	4/4/2003	Confirmed	—	Negative	Negative	Positive	—
FP156	7/29/2011	Confirmed	—	Negative	Positive	—	—
FP242	8/29/2016	Confirmed	—	Negative	Negative	Positive	—
FP245	4/11/2018	Confirmed	—	—	—	Negative	—
UCFP057	6/16/2003	Confirmed	—	Positive	—	—	—
UCFP055	6/13/2003	Probable	—	Negative	—	—	Negative <sup>c</sup>
UCFP056	6/14/2003	Probable	—	Negative	—	—	Negative <sup>c</sup>
FP141	1/5/2011	Suspect	—	—	—	—	Negative <sup>d</sup>
FP197	7/18/2012	Suspect	—	—	—	—	Negative <sup>e</sup>
TX103	8/19/1999	Suspect	—	Negative	—	—	Negative <sup>f</sup>
UCFP163	9/3/2011	Suspect	—	Negative	—	—	Negative <sup>c</sup>
FP036	10/10/1998	Possible	—	Negative	—	—	Negative <sup>g</sup>
FP042	6/22/1995	Possible	—	Negative	—	—	Negative <sup>g</sup>



FP100	1/25/2007	Possible	—	Negative	—	—	—
FP105	1/16/2002	Possible	—	Negative	—	—	—
FP138	5/12/2008	Possible	Negative	Negative	—	—	—
FP085	3/1/2004	Unlikely	—	—	Negative	—	—
TX107	1/18/2001	Unlikely	—	Negative	Negative	—	—

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<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>Tissue= lung

<sup>d</sup>Tissue = lung and kidney

<sup>e</sup>Tissue = spleen

<sup>f</sup>Tissue = kidney

<sup>g</sup>Tissue = liver and kidney

Supplementary Table 6. Results for pseudorabies virus infection in free-ranging Florida panthers (*Puma concolor coryi*) in Florida, USA by immunohistochemistry (IHC). Only individuals that had tissues available for IHC testing are described. ‘—’ indicates that the tissue was not tested.

<b>Panther ID<sup>a</sup></b>	<b>Mortality Date</b>	<b>PRV<sup>b</sup> category</b>	<b>Brainstem</b>	<b>Cerebrum</b>	<b>Adrenal gland</b>	<b>Ovary</b>	<b>Lung</b>	<b>Other</b>
<b>FP029</b>	5/27/1992	Confirmed	Negative	Negative	—	Negative	—	—
<b>FP117</b>	7/28/2004	Confirmed	Negative	Negative	Negative	—	—	—
<b>FP118</b>	4/4/2003	Confirmed	Positive	Negative	Negative	—	Negative	Positive <sup>c</sup>
<b>FP156</b>	7/29/2011	Confirmed	Negative	Negative	Negative	—	—	—
<b>FP173</b>	12/22/2010	Confirmed	—	—	Positive	—	—	—
<b>FP084</b>	4/20/2000	Suspect	—	Negative	Negative	—	—	—

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>Tissue = spine and cranial nerve

Supplementary Table 7. Results for free-ranging Florida panthers (*Puma concolor coryi*), and Texas pumas (*P. concolor stanleyana*) in Florida, USA tested for eastern equine encephalitis virus by real-time polymerase chain reaction (PCR) and for flavivirus RNA by conventional PCR at the University of Florida. Only cats that had tissues available for PCR are described. ‘—’ indicates that the tissue was not tested.

Panther ID <sup>a</sup>	Mortality Date	PRV <sup>b</sup> category	Brain	Other
FP117	7/28/2004	Confirmed	Negative	—
FP242	8/29/2016	Confirmed	Negative	Negative <sup>c</sup>
UCFP055	6/13/2003	Probable	—	Negative <sup>c</sup>
UCFP056	6/14/2003	Probable	—	Negative <sup>c</sup>
FP141	1/5/2011	Suspect	—	Negative <sup>d</sup>
FP197	7/18/2012	Suspect	—	Negative <sup>e</sup>
TX103	8/19/1999	Suspect	—	Negative <sup>f</sup>
UCFP163	9/3/2011	Suspect	—	Negative <sup>c</sup>
FP036	10/10/1998	Possible	—	Negative <sup>g</sup>
FP042	6/22/1995	Possible	—	Negative <sup>g</sup>

<sup>a</sup>ID = identification

<sup>b</sup>PRV = pseudorabies virus

<sup>c</sup>Tissue = lung

<sup>d</sup>Tissue = lung and kidney

<sup>e</sup>Tissue = spleen

<sup>f</sup>Tissue = kidney

<sup>g</sup>Tissue = liver and kidney

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