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Endemic *Skunk amdoparvovirus* in free-ranging striped skunks (*Mephitis mephitis*) in California

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Abstract

The genus *Amdoparvovirus* includes the newly discovered skunk amdoparvovirus and the well-characterized Aleutian disease virus which causes significant health impacts in farmed mink worldwide. In 2010–2013, an outbreak of fatal amdoparvovirus-associated disease was documented in free-ranging striped skunks (*Mephitis mephitis*) from the San Francisco Bay Area of California. To characterize the geographic distribution, earliest occurrence and abundance of this virus, as well as possible impacts on sympatric mustelids of conservation concern, we tested blood samples from skunks throughout California and fishers (*Pekania pennanti*) from northern California for amdoparvovirus DNA. Amdoparvovirus DNA was detected in 64.8% of sampled skunks (140/216), and test-positive skunks were distributed widely throughout the state, from as far north as Humboldt County and south to San Diego County. The first test-positive skunks were detected from 2004, prior to the 2010–2013 outbreak. No significant spatial or temporal clustering of infection was detected. Although healthy and clinically ill animals tested positive for amdoparvovirus DNA, histopathologic evaluation of a subset from clinically ill skunks indicated that positive PCR results were associated with pneumonia as well as there being more than one inflammatory type lesion. None of 38 fishers were PCR-positive. Given the widespread geographic distribution and lack of a clear epizootic centre, our results suggest the presence of an endemic skunk-associated amdoparvovirus strain or species. However, if the virus is not host-specific, skunks' ubiquitous presence across rural and urban habitats may pose a risk to susceptible domestic and wild species including mustelids of conservation concern such as fishers and Pacific martens (*Martes caurina*).

KEYWORDS
California, emerging infectious disease, Mephitidae, parvovirus

1 | INTRODUCTION

Until recently, the only known member of the little-known *Amdoparvovirus* genus in the family *Parvoviridae* was Aleutian disease virus (ADV, now called *carnivore amdoparvovirus 1*) which has significant negative impacts in farmed mink worldwide. Since 2011, four new amdoparvovirus species have been identified: the grey fox amdoparvovirus (GFAV; Li et al., 2011), raccoon dog and fox amdoparvovirus (RFAV; Shao et al., 2014), red fox amdoparvovirus (RFAV; Bodewes, van der Giessen, Haagmans, Osterhaus, & Smits, 2013) which has not been fully sequenced and classified by the International Committee on Taxonomy of Viruses (ICTV), and skunk

amdoparvovirus (SKAV or *carnivore amdoparvovirus 4*; Canuti, Doyle, Britton, & Lang, 2017). SKAV is the closest related amdoparvovirus to ADV and serological cross-reactivity SKAV, and ADV is likely (Canuti et al., 2017).

Aleutian disease virus, while primarily found in farmed American mink (*Neovison vison*), infects free-ranging and domesticated mustelids, canids, felids, viverrids, procyonids and mephitids, including the striped skunk (*Mephitis mephitis*; Farid, 2013; Fournier-Chambrillon et al., 2004; Ingram & Cho, 1974; Manas et al., 2001). There is an increasing concern that amdoparvoviruses, particularly ADV, may contribute to the decline of wildlife species around the world as ADV is widespread in free-ranging mink (Fournier-Chambrillon et al., 2004; Manas et al., 2001; Guzman et al., 2008). Outbreaks of virulent amdoparvoviruses could significantly impact susceptible wildlife by damaging immune function and reproduction, and causing mortality events (Alexandersen, 1990; Ingram & Cho, 1974).

In mink, ADV infection can be completely cleared by the host, be shed subclinically, or cause reproductive dysfunction, weight loss or the slow progressive fatal wasting Aleutian disease (AD; Farid, Zillig, Finley, & Smith, 2012; Ingram & Cho, 1974). Pathogenicity and clinical progression depend on viral strain and host factors such as species, age and genotype (Hadlow, Race, & Kennedy, 1983; Ingram & Cho, 1974). There is no effective vaccine or treatment. Prolonged environmental persistence, subclinical shedding and multiple transmission routes (feces, urine, saliva, blood and potentially aerosol) have prevented the elimination of ADV despite widespread eradication efforts (Farid et al., 2012; Prieto et al., 2014). Available data on SKAV suggest it may also have environmental persistence, be associated with subclinical shedding and have multiple transmission routes (Canuti et al., 2017; Nituch, Bowman, Wilson, & Schulte-Hostedde, 2015).

Recently, multiple cases of presumed AD were diagnosed in free-ranging striped skunks along the coast of California in the USA (LaDouceur et al., 2015). These cases are the first known to the authors in a free-ranging species anywhere, other than mink, to collectively show high mortality with extensive neurologic signs and lesions associated with an amdoparvovirus. These cases represented 27% of all skunk submissions to the California Animal Health and Food Safety Laboratory (CAHFS) for 2010–2013. Studies have documented amdoparvovirus in free-ranging striped skunks via serology, PCR or immunohistochemistry in British Columbia (Britton et al., 2015) and Ontario (Nituch et al., 2015) Canada, and South Dakota (Oie et al., 1996) and midwestern United States (Giannitti et al., 2017; Woolf & Gremillion-Smith, 1986). Although the association between virus and clinical signs or mortality was not described for many amdoparvovirus-positive individuals, some individuals had AD-like histologic lesions. Clinical signs and mortality attributed to amdoparvovirus in captive striped skunks have also been documented albeit rarely (Allender et al., 2008; Pennick, Latimer, Brown, Hayes, & Sarver, 2007).

Little is known about amdoparvoviruses in California wildlife and whether they could negatively impact the conservation-sensitive fisher (*Pekania pennanti)* and Pacific marten (*Martes caurina*) which are both listed as Species of Special Concern in California (California Department of Fish and Wildlife; Natural Diversity Database, 2017). Accordingly, as a first step to understanding the ecology and potential conservation significance of these viruses for California's wildlife, we aimed to determine the abundance and spatial distribution of amdoparvovirus DNA in free-ranging striped skunks throughout California and a small sample of fishers from northern California. Our null hypothesis was that the distribution is restricted at present to a single epizootic hotspot in the San Francisco Bay Area (SFBA). Alternatively, amdoparvovirus infection in striped skunks may extend broadly in space across California. As limited information is available on the potential for amdoparvoviruses to cause pathology in skunks, samples from a subset of skunks with clinical signs consistent with AD were evaluated by histopathology and presence of histopathologic lesions and demographic risk factors were evaluated as predictors for positive PCR status.

2 | MATERIALS AND METHODS

Using opportunistic sampling, we obtained whole blood or serum samples from 216 free-ranging striped skunks from 21 counties distributed throughout California. We targeted collection of samples from 4 geographic zones based on geographic distance from the initial skunk amdoparvovirus outbreak and overlap with sympatric mustelids of conservation concern. Zone 1 (n = 50 skunks) comprised the 4 counties (Marin, Santa Cruz, San Francisco and Alameda) where the epizootic occurred and adjacent San Mateo County; Zone 2 (n = 63) comprised counties surrounding Zone 1 (Sonoma, Napa, Solano, Yolo, Sacramento, Contra Costa, San Joaquin, Stanislaus, Santa Clara, San Benito, Monterey, Nevada, Placer, El Dorado, Amador, Calaveras, Lake, Merced, Kings, Colusa, Yuba and Sutter); Zone 3 (n = 67) comprised more distant counties where striped skunks are sympatric with fishers and Pacific martens (Humboldt, Del Norte, Shasta, Siskiyou, Mendocino, Trinity, Butte, Lassen, Plumas, Tehama, Madera, Fresno, Glenn, Tuolumne, Mariposa, Tulare, Plumas, Sierra, Alpine, Mono, Inyo and Modoc); and Zone 4 (n = 32) comprised southern California counties (Los Angeles, Orange, Ventura, Kern, Santa Barbara, San Bernardino, Riverside, Imperial, San Luis Obispo and San Diego). Samples were also available from 38 fishers from Fresno and Humboldt Counties.

Archived and prospectively collected samples from collaborators were utilized including 29 archived samples from striped skunks and 38 from fishers live-trapped for unrelated projects between 2004 and 2013. Between January 2015 and September 2016, 187 striped skunk carcasses, blood samples or serum samples were obtained from wildlife rehabilitation centres, zoos, the California Department of Fish and Wildlife (CDFW), and the US Department of Agriculture Wildlife Services (USDA WS) after natural death or humane euthanasia for welfare, depredation or public nuisance reasons. Blood samples were collected from live-trapped animals via venipuncture and euthanized animals via cardiac puncture and submitted as whole blood or serum. Samples were collected under authorization of UC

Davis Animal Care and Use Protocol (#18179) and CDFW Scientific Collecting Permits held by Janet Foley. Cases from the LaDouceur et al. (2015) publication were not included in this analysis.

Necropsies of 131 whole carcasses were performed at CDFW, Wildlife Investigations Lab (EG, DC) and by veterinary pathologists at the California Animal Health and Food Safety Diagnostic laboratory (CAHFS). When present, samples of heart blood or blood in the pericardial sac were collected using a sterile syringe. If blood was not available, serosanguinous fluid was collected from the pleural space. Spleen, liver, kidney, lung, small intestine and mesenteric lymph node samples were collected and fixed in formalin. Additional tissues were sampled and fixed at the discretion of the case coordinator. Tissue samples from a subset of skunks exhibiting any clinical signs of ill health were submitted to CAHFS for histology. Ancillary testing for other pathogens, parasites or toxins for selected individuals was performed based on histology findings.

The sex was determined, and each animal was assigned to an age class (adult ≥12 months, or juvenile <12 months) based on size, body weight and dentition (Verts, 1967). Skunks reported by the submitter to have any clinical signs of poor health when alive were classified as "ill." Indications of poor health on paperwork included abnormal behaviour, neurologic, seizures, circling, hindlimb paraparesis, obtundation, emaciation, severe lethargy, blindness, hypersalivation, twitching and mucoid ocular or nasal discharge. Clinical history was not available for skunks or skunk samples collected for nuisance or welfare reasons such as traumatic injury; however, brief observation in the time prior to euthanasia did not reveal clinical abnormalities. Accordingly, those samples were all classified as healthy, while three that experienced vehicular strike or were found dead were classified as unknown. Of those necropsied, none showed obvious external lesions suggestive of significant disease during a pre-necropsy examination, although lesions were noted on internal organs of some cases once the necropsy was completed.

DNA was extracted from 100 microlitres of each sample of whole blood, serum or serosanguinous fluid using DNeasy Blood and Tissue kits (Qiagen, Valencia, CA) according to manufacturer's directions for extraction from non-nucleated blood. In rare samples, 50 µl was used due to small sample volume. Negative controls were included with each set of extractions including nuclease-free water and a blood sample from a dog (*Canis lupus familiaris*). Fragments of 401 base pairs in length spanning the hypervariable region in the VP2 gene were amplified using published PCR protocols and forward (5′-AGAGCAACCAAACCACCC-3′) and reverse (5′-TCACCCCAAAAGTGACC-3′) primers (Allender et al., 2008; Saifuddin & Fox, 1996). In silico analysis confirms that these primers should amplify ADV and SKAV, with less close matches to RFAV and GFAV. Primers were used in a 25 µl reaction with GoTaq Green Master Mix (Promega). The reaction was cycled as follows: 95°C for 4 min, then 40 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s. Positive controls included DNA from 2 initial samples confirmed positive by PCR and DNA sequencing. Nuclease-free water and the dog DNA were used as negative controls. Electrophoresis on 1.5% agarose gels was used to separate PCR products, and results were viewed with ultraviolet transillumination. A set of randomly chosen positive amplicons was submitted for DNA sequencing to confirm amdoparvovirus. PCR bands were cut from the agarose gel and purified using QIAquick Gel Extraction Kits (Qiagen) according to manufacturer's instructions. Purified PCR product was submitted to the UC Davis College of Biological Sciences DNA Sequencing Facility for DNA sequencing using the forward PCR primer. Ambiguous bases were visually adjusted if possible and trimmed in CLC Main Workbench (Qiagen). Sequences were evaluated against existing amdoparvovirus sequences in GenBank. DNA sequences obtained in this study were submitted to GenBank.

Reports from 55 sick skunks submitted for histopathologic evaluation were reviewed for lymphocytic and/or plasmacytic infiltration of renal, hepatic, nervous, cardiovascular, pulmonary and endocrine organs, and pneumocyte hyperplasia as reported previously for ADV and SKAV (Alexandersen, 1990; Allender et al., 2008; Jensen, Chriel, & Hansen, 2016; Jensen, Hammer, & Chriel, 2014; LaDouceur et al., 2015; Pennick et al., 2007). Lesions were considered significant unless infiltration was classified in the report as minimal or rare.

2.1 | Statistical analysis

We performed exploratory data analysis and calculated descriptive statistics to assess the distribution of variables and identify missing data using Microsoft Excel (Microsoft Office, Redmond, Washington 2013) and "R" version 3.4.1 (R Core Team, 2015; R Core Team, 2017). Statistical associations with a p-value <0.05 were considered significant. The prevalence of PCR-positive samples and associated 95% Clopper–Pearson exact binomial confidence interval (along with stratified sample prevalence and 95% CIs for sex, age class, geographic zone, body condition score (BCS), collector and collection period defined as prior to or after the SFBA epizootic in 2010) were calculated in R using the "prevalence" package (Daniel, 2009; Devleesschauwer et al., 2015). Because most of the 20 collecting organizations submitted small numbers of samples, collectors were condensed into five groups consisting of a single group comprised of all wildlife rehabilitators, and the four other collectors that contributed larger numbers of samples. Trend lines were evaluated in Microsoft Excel using the Data Analysis Toolpak to evaluate regression statistics. Univariate logistic regression was used to evaluate the relationship between a positive PCR result and each variable considered biologically plausible as a predictor (age class, sex, geographic zone, signs of illness, BCS, collector's affiliation and collection period). Pearson's chi-square test for independence, Fisher's exact test or Kruskal–Wallis rank sum test was used to evaluate associations between predictors (McDonald, 2014). Fixed effects and random effects multivariable logistic regression models were fit using age, sex, geographic region and signs of illness as potential predictors for PCR-positivity as well as relevant interaction terms (Daniel, 2009). Akaike's information criterion with a correction for finite sample size (AICc) was used to rank a saturated model representing variables considered from univariate regression (with $p < 0.2$, or deemed essential to explain data set) and relevant interaction terms. Stepwise

backward elimination from the saturated model was used for model selection, removing variables in order of least significance, and using AICc as selection criteria. The lowest AICc value was considered the best model to explain the data, and other models were evaluated based on their difference from this minimum. Odds ratios with 95% confidence intervals were calculated for each variable selected. Likelihood ratio tests were used to assess the overall significance of the selected model in the "epicalc" package in R (Chongsuvivatwong, 2012). A weighted average of the best-fit models with unconditional standard errors was estimated if relevant. Receiver operator curve (ROC) plots were evaluated using the area under the curve (AUC) as a measure of how well the model would be as a "test" to separate positive from negative individuals also using the "epicalc" package in R (Chongsuvivatwong, 2012).

Prevalence of histologic lesions in each organ system was calculated, as well as prevalence of one or more lesions in a single animal. A second multivariable logistic regression model was fit using the primary histologic lesions of interest as well as number of lesions as predictors for amdoparvovirus PCR status, using the same criteria as above to select a model.

All counties and geographic zones were georeferenced, and abundance of amdoparvovirus in each zone was plotted on a geographic information system (ArcGIS, ESRI) using the WGS84 geodetic datum. A space-time cluster analysis using a Bernoulli model was performed with SaTScan software version 9.4.4 (M Kulldorff and Information Management Services Inc, 2016; Kulldorff, 1997). For the time variable, date of death was used for animals obtained after death unless an exact date was not available, then the date that the carcass was obtained by our study was used as a rough approximation of date of death. For live-trapped animals, the date of capture and sample collection was used. Latitude and longitude coordinates of the nearest city were obtained for each animal

TABLE 1 Infection status for amdoparvovirus using PCR on blood and serum samples in striped skunks (*Mephitis mephitis*) in California, USA

	Total sampled	PCR status		Sample prevalence (%)	95% CI
		Negative	Positive		
Sex					
Female	86	29	57	66.28	55.28–76.12
Male	98	33	65	66.33	56.07–75.56
Unknown	32	14	18	56.25	37.66–73.64
Age class					
Juvenile	48	24	24	50.00	35.23–64.77
Adult	152	38	103	73.05	64.93–80.17
Unknown	27	14	13	48.15	26.67–68.05
Zone					
Zone 1	50	18	32	64.0	49.19–77.08
Zone 2	63	24	39	61.90	48.80–73.85
Zone 3	67	18	49	73.13	60.90–83.24
Zone 4	32	12	20	62.50	43.69–78.90
Signs of illness					
Present	84	19	65	77.38	66.71–85.50
Absent	129	57	72	55.81	46.82–64.46
Unknown	3	0	3	100.00	0.31–1.00
Collection period					
Pre-outbreak (2004–2009)	12	7	5	41.67	15.16–72.33
Post-outbreak (2010–2016)	204	69	135	66.17	59.24–72.64
Collector (Grouped)					
Foley	24	16	8	33.33	15.63–55.32
IERC	11	6	5	45.45	16.75–76.62
USDA	85	26	59	69.41	58.47–78.95
Zoos	13	4	9	69.23	38.57–90.91
Wildlife Rehabilitators	74	24	50	67.57	55.68–78.00

Prevalence and 95% Clopper–Pearson exact binomial confidence intervals were calculated for variables of interest including sex, age class, geographic zone, signs of illness, collector organization and collection period (prior to or after an outbreak of presumed ADV in 2010)

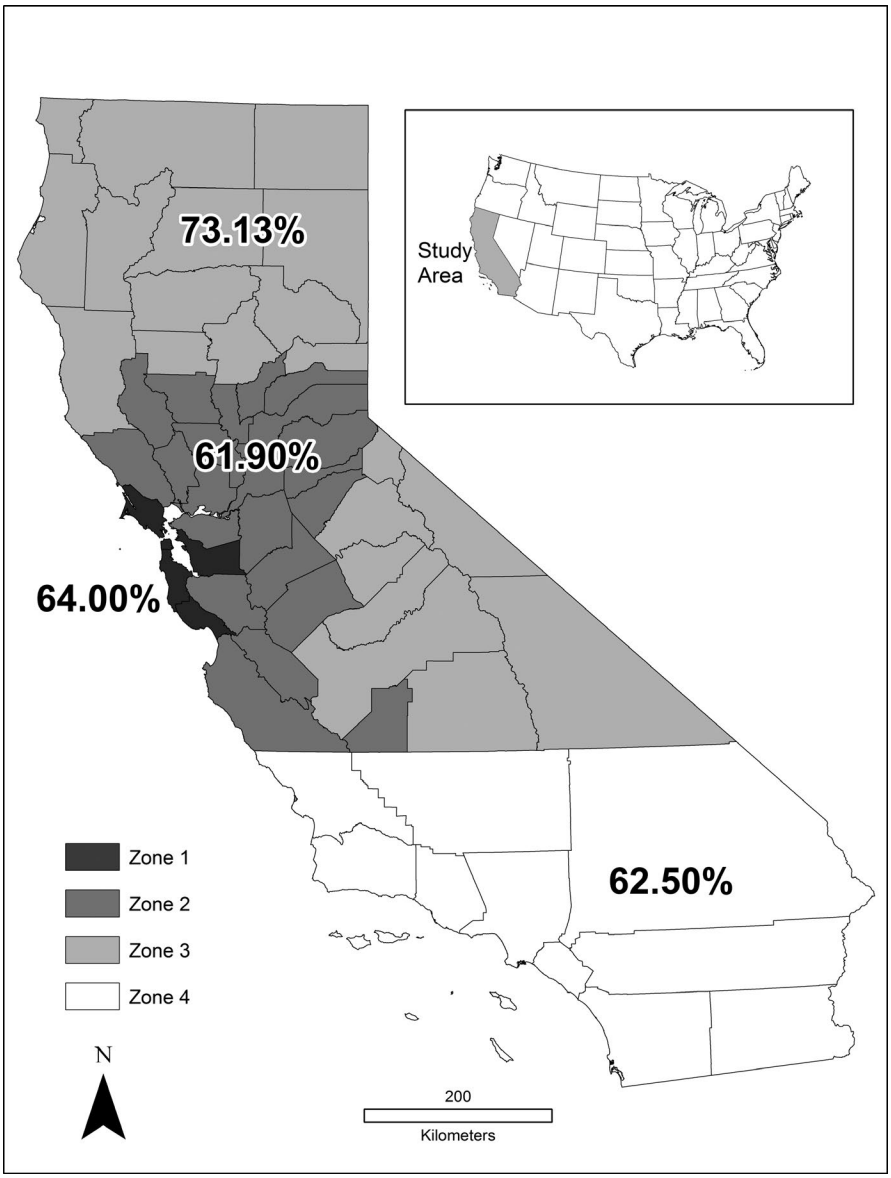

FIGURE 1 California, USA, with 4 geographic zones and their respective sample prevalence of amdoparvovirus infection in striped skunks (*Mephitis mephitis*) as determined by PCR of blood and serum. Zone 1) counties where cases were detected in the original suspected epizootic, Zone 2) counties roughly surrounding Zone 1, Zone 3) counties where striped skunks are sympatric with fishers and Pacific martens and Zone 4) geographically distant southern California counties

using Google Earth version 7.1.8.3036 (Google, 2017). When city-level data were not available the animal was excluded from the space-time analysis. The analysis was run using monthly time increments.

3 | RESULTS

Amdoparvovirus DNA was detected in 140 of 216 skunk blood samples, resulting in a sample prevalence of 64.8% (95% [CI] 58.1%–71.2%). Prevalence for each demographic group and geographic zone is shown in Table 1. Positive skunks were present in all 21 counties sampled. No significant differences were found in numbers across the four geographic zones (Figure 1). The sex ratio of sampled skunks was approximately 1:1, and the majority (65.3%) of sampled skunks were adults (Table 1). Sex and age data were not available for 32 and 27 skunks, respectively. Skunks with clinical signs of illness made up 39.8% of all skunks tested. Of 38 Pacific fisher samples collected from 2 counties, Fresno and Humboldt, none tested positive for amdoparvovirus DNA.

Twelve samples were tested from skunks prior to the epizootic in 2010, including 11 from Humboldt County and one from Santa Cruz County. Of these, 41.7% (5/12) tested positive, all from Humboldt County with collection dates in 2004 (n = 2), 2006 (n = 2) and 2009 (n = 1). Of skunks collected in 2010–2016, 67.0% (126/188) tested positive. There was no statistically significant difference between the proportion of PCR-positive individuals sampled prior to and after 2010. Numbers appeared to increase over the years based on the trendline when graphed, although this increase was not statistically significant. Infections tended to be more common in December–April, but this result was not statistically significant.

Adult skunks were significantly more likely to be PCR-positive than juveniles (OR = 2.7, 95% CI = [1.89, 3.98]), and skunks showing signs of illness were also significantly more likely to test positive

TABLE 2 Optimal multivariate logistic regression model selected to predict amdoparvovirus infection in striped skunks (*Mephitis mephitis*) in California between 2004 and 2016 using PCR of blood and serum samples

	β	*SE*	*p*-value	OR	95% CI
(Intercept)	−0.8304	0.5834	0.1546		
Age class: juvenile[a]	−0.4231	0.6512	0.5158	0.66	0.18–2.35
Signs of illness: present[b]	2.9634	0.6650	<0.0001	19.36	5.26–71.30
Zone 2[c]	0.6669	0.5849	0.2542	1.95	0.62–6.13
Zone 3[c]	2.1319	0.6856	0.0019	8.43	0.56–32.32
Zone 4[c]	0.6874	0.6395	0.2825	1.99	0.57–6.96
Age class*Signs of illness[d]	−1.8657	0.9134	0.0411	0.15	0.03–0.93

[a]Reference category is Adult.
[b]Reference category is absent.
[c]Reference category is Zone 1.
[d]Interaction term between predictor variables age class and signs of illness.

than skunks that appeared healthy (OR = 2.71, 95% CI = [1.44, 5.09]). PCR-positivity was not significantly associated with sex, BCS or geographic zone. The grouped collector variable was a significant predictor of a positive PCR status, but also highly correlated with all other predictors so was dropped from the logistic regression model. Tests for associations between predictors showed that age class and signs of illness had statistically significant associations with all other variables except sex. Interaction terms for age class and zone, and zone and signs of illness, were not statistically significant and did not improve the model fit. The optimal multivariate logistic regression model chosen was "PCR ~ Zone +Age + Illness +Age*Illness" (Table 2). The AUC for this model was 76.10%, considered fair predictive availability. The maximum likelihood ratio test for this model compared to the null model showed a significant difference (χ^2 = 41.20, *df* = 6, *p* = 2.64e-7).

Histologic evaluation of sick skunks for lesions revealed that the most common lesion in PCR-positive skunks was interstitial pneumonia, followed by epi/endo/myocarditis (Table 3). Lesion distributions between PCR-positive and -negative skunks were similar (Table 3). Perivascular lymphocytic and/or plasmacytic cuffing was a frequently noted lesion in multiple organ systems, was present in one or more organs in 41.9% (95% [CI]: 24.5%–60.9%, *n* = 13/31) of skunks with lesions and was evenly distributed between PCR-positive and -negative animals. Splenitis/splenic hyperplasia, thyroiditis, glossitis, laryngitis, sialadenitis, cystitis and gastritis were noted, but were too infrequent for statistical testing of association with PCR status. No intestinal lesions were identified. A single incidence of type II pneumocyte hyperplasia was observed in a very young PCR-negative skunk. This juvenile also tested negative for canine distemper virus (CDV, tested with immunohistochemistry), rabies, anticoagulant rodenticides and bromethalin.

Glomerulonephritis, interstitial nephritis, pyelonephritis, portal hepatitis, meningoencephalitis, encephalitis, epi/endo/myocarditis, vasculitis, interstitial pneumonia, adrenalitis and the presence of one or more lesions were evaluated for association with PCR status using logistic regression. Significant predictors of a positive PCR test included interstitial pneumonia (*p* = 0.032) and the presence of one or more lesions (*p* = 0.0098). The number of organs with lesions was positively associated with a PCR-positive test (*β* = 0.71, *p* = 0.032). While not a significant predictor with *p* > 0.05, renal lesions had the greatest odds ratio of any of the considered lesions (OR: 3.12, 95% CI: 0.31–31.22). Age was included in the univariate models to adjust for potential confounding. Age, nephritis, pneumonia and/or the number of organs with lesions were included in four multivariate logistic regression models best describing the data. In a final model, created by averaging the four best models, age was the only variable of statistical significance (*p* = 0.035) with adults being 4.7 (95% [CI]: 1.12–20.04) times as likely to be PCR-positive as juveniles with similar lesions. Predictive ability of the final model remained fair (AUC: 77.82%).

The space-time analysis did not reveal any statistically significant clusters in space, time or both together. A visually apparent cluster included 21 PCR-positive skunks and centred on Occidental (Sonoma County, Northern California), between 4/1/2015 and 4/30/2016 (*p* = 0.097, relative risk = 1.56).

The subset of skunk samples submitted for histologic evaluation showed evidence of past exposure or current infection with *Leptospira* spp. (5/16, tested using PCR and ELISA), four of which were amdoparvovirus-positive. Four of the *Leptospira* spp.-positive skunks showed histologic lesions in their kidneys, three with interstitial nephritis and one with glomerulonephritis. CDV-positive skunks were also found (*n* = 8/34) using IHC, all of which tested positive for amdoparvovirus. Seven CDV-positive skunks had interstitial pneumonia (*n* = 7/8). Two CDV-positive skunks had been decapitated for rabies testing so could not be evaluated for encephalitis or meningoencephalitis; of those remaining, one had encephalitis (*n* = 1/6) and another had meningoencephalitis (*n* = 1/6). Twenty-four skunks submitted for rabies testing all tested negative. Toxicological analysis revealed anticoagulant rodenticide exposure (22/28, including 8 trace detections), bromethalin exposure (15/26, including 7 trace detections) and vitamin D (cholecalciferol) toxicosis

TABLE 3 Prevalence of histopathologic lesions consistent with SKAV or ADV infection observed in organ systems of amdoparvovirus PCR-positive and -negative striped skunks (*Mephitis mephitis*) in California between 2004 and 2016 with 95% Clopper–Pearson exact binomial confidence intervals

Organ system	PCR negative	PCR positive	Total
Renal/Urinary			
Glomerulonephritis	0/14	2/41	2/55
	0.0% (0.0%–23.2%)	5.0% (12.4%–40.3%)	3.7% (0.4%–12.3%)
Interstitial Nephritis	1/14	10/41	11/55
	7.1% (0.2%–33.9%)	25.0% (12.4%–40.3%)	20.4% (10.4%–33.0%)
Pyelonephritis	0/14	2/41	2/55
	0.0% (0.0%–23.2%)	5.0% (0.6%–16.5%)	3.7% (0.4–12 3%)
Cystitis	0/8	1/30	1/38
	0.0% (0.0%–36.9%)	3.3% (0.1%–17.2%)	2.6% (0.1%–13.8%)
Hepatic			
Portal Hepatitis	0/14	7/41	7/55
	0.0% (0.0%–23.2%)	17.1% (7.2%–32.1%)	12.7% (5.3%–24.5%)
Nervous			
Meningoencephalitis/Encephalitis	2/14	11/36	13/50
	14.3% (1.8%–42.8%)	30.6% (16.3%–48.1%)	26.0% (14.6%–40.3%)
Circulatory			
Epi/Endo/Myocarditis	2/14	10/39	12/53
	14.3% (1.8%–42.8%)	25.6% (13.0%–42.1%)	22.6% (12.3%–36.2%)
Vasculitis	1/14	7/41	8/55
	7.1% (0.2%–33.9%)	17.1% (7.2%–32.1%)	14.5% (6.5%–26.7%)
Pulmonary			
Interstitial Pneumonia	3/14	22/39	25/53
	21.4% (4.7%–50.8%)	56.4% (38.6%–72.2%)	47.2% (33.3%–61.4%)
Endocrine			
Adrenalitis	0/13	4/34	4/47
	0.0% (0.0%–24.7%)	11.8% (3.3%–27.5%)	8.5% (2.4%–20.4%)
Oropharyngeal			
Glossitis	1/12	1/24	2/36
	8.3% (0.2%–38.5%)	4.2% (0.1%–21.1%)	5.5% (0.7%–18.7%)
Laryngitis	0/0	1/8	1/8
	0.0% (0.0%–0.0%)	12.5% (0.3%–52.7%)	12.5% (0.3%–52.7%)
Sialadenitis	0/13	1/40	1/53
	0.0% (0.0%–24.7%)	2.5% (0.1%–13.2%)	1.9% (0.0%–10.1%)
Lymphatic			
Splenitis/Splenic hyperplasia	1/14	1/38	2/52
	7.1% (0.2%–33.9%)	2.6% (0.1%–13.8%)	3.8% (0.5%–13.2%)
Thyroiditis	0/3	1/18	1/21
	0.0% (0.0%–70.8%)	5.6% (0.1%–27.3%)	4.8% (0.1%–23.8%)
Gastroenteric			
Gastritis	0/13	1/40	1/53
	0.0% (0.0%–24.7%)	2.5% (0.1%–13.2%)	1.9% (0.0%–10.1%)
Multiple			
>1 organ system	2/14	22/36	24/50
	14.3% (1.8%–42.8%)	61.1% (43.3–76.9)	48.0% (33.7%–62.6%)

(1/1). Other pathologies noted grossly or on histopathology included gastrointestinal parasitosis, pulmonary nematodiasis, subcutaneous parasitosis, tick/flea/lice infestations, *Skrjabingylus* spp. infestation, intramuscular protozoal cysts, pyometra, upper respiratory infection, brain tumour and skeletal fractures.

Sequenced VP2 PCR products from 14 skunks best matched either mink ADV strains or SKAV strains in NCBI-BLAST (GenBank: MK573213, MK573214, MK573215, MK573216, MK573217, MK573218, MK573219, MK573220, MK573221, MK573222, MK573223, MK573224, MK573225). Sequence identities of the 14 samples were between 88.52% and 100% when evaluated against each other in CLC Main Workbench. Sequences from three SKAV strains (GenBank: NC_034445, KX981925, KX981926) matched our samples with 87.4 to 93.4% identity, and the Pullman, TR, and Utah mink ADV strains (GenBank: U39014, U39013, U39015) matched our samples with 83.9 to 90.2% identity. The sample from the previously documented captive skunk in California matched our sequences with 92.5 to 98% identity (Allender et al., 2008).

4 | DISCUSSION

Amdoparvovirus is far more prevalent and widely distributed in free-ranging striped skunks throughout California than previously known and is well outside the originally identified single epizootic hotspot in the San Francisco Bay region. While no samples prior to 2010 were available near the SFBA, the earliest dated positive samples were in fact from Humboldt County, geographically and ecologically distant from the initially suspected epizootic in the SFBA. All counties tested had amdoparvovirus-positive skunks, without significant evidence of spatial clustering. The abundance of amdoparvovirus also occurs in skunks that are sympatric with fishers and Pacific martens. This spatial distribution suggests that amdoparvovirus is endemic in the California striped skunk population. Most previously detected amdoparvoviruses in skunks have been found in geographically distant areas of North America leading us to speculate our findings may be more broadly applicable than the scope of our study both spatially and temporally.

Amdoparvovirus was present in skunks in California prior to the SFBA 2010–2013 outbreak, with positive samples from 2004, 2006 and 2009 from skunks in Humboldt County. Unfortunately, few samples collected prior to 2010 were available for inclusion in this study and those that were obtained were localized primarily in one county, making it difficult to extrapolate conclusions about the broader epidemiology of amdoparvovirus in California skunks prior to 2010. This also complicates attempts to elucidate changes in the viral epidemiology over time, such as increasing prevalence, or detect mutations in the virus if additional sequencing was an option. There does appear to be a significant increase in cases within our sample over time; however, a temporal cluster analysis did not reveal any significant clustering. The apparent increase in cases may an artefact of selection bias as the majority of skunks collected prior to 2012 were collected from 2 similar sources.

Presence of clinical signs of illness was a significant risk factor for a skunk testing PCR-positive, although in most cases, signs of illness were non-specific. As the skunks in our study are wild, it is likely that any detected clinical signs would be biased towards those that are more severe or obvious. Clinical signs in many skunks were consistent with those reported in captive skunks and the previously identified free-ranging skunks in California, including sudden death, lethargy and other neurologic disease (Allender et al., 2008; LaDouceur et al., 2015; Pennick et al., 2007). These signs are similar to those of mink and ferrets with AD (Alexandersen, 1990; Stevenson, Gates, Murray, & Bloom, 2001).

The majority of PCR-positive skunks had histologic lesions in more than one of the five primarily affected organ systems (i.e. kidneys, heart, brain, liver and lungs), consistent with previous characterization of amdoparvovirus in skunks (LaDouceur et al., 2015) and ADV in mink. Due to the mechanism of disease, with immune complex deposition (Allender et al., 2008; LaDouceur et al., 2015), renal lesions such as glomerulonephritis as well as vascular lesions including arteritis and microangiopathy are reported relatively frequently. In contrast, we found few cases of glomerulonephritis, arteritis and microangiopathy and no perivascular cuffing within the kidneys or encephalomalacia. PCR-positive skunks had more (but not significantly so) cases of vasculitis.

Signs of AD and those in amdoparvovirus PCR-positive skunks overlap with those of multiple other disease processes and toxicities, including several confirmed in skunks in our study, such as canine distemper, leptospirosis, *Skrjabingylus* sp. infestation, anticoagulant rodenticide toxicity and bromethalin toxicity. Other diseases with overlapping clinical signs include influenza, toxoplasmosis, and in California, the second most commonly identified rabid species is the striped skunk (Rabies surveillance in California, Annual Report 2015, 2016). Histologic lesions could also have multiple aetiologies, and coinfections could have altered the histologic patterns we observed, such as with CDV or *Leptospira* spp. particularly in amdoparvovirus-positive skunks CDV and encephalitis or interstitial pneumonia or *Leptospira* spp. infection and nephritis. While no statistical significance was found for coinfections, the data set was small; there was a suggestion that amdoparvovirus PCR-positive animals had a higher prevalence of CDV infection and *Leptospira* spp. infection than amdoparvovirus PCR-negative animals did. Our study could not draw a clear picture of what lesions occur due to the amdoparvovirus per se which may be less pathogenic than some ADV strains in mink, because of coinfections and the opportunistic collection of skunk cases, many of which lacked clinical histories. Additional testing could attempt to confirm the presence of amdoparvovirus antigen or anti-amdoparvovirus antibodies within the lesions, and experimental studies in skunks could help inform understanding of clinical disease progression.

Although skunks of all ages tested positive for amdoparvovirus, juveniles were significantly less likely to be affected than adults. Our results are potentially supportive of both horizontal and vertical transmission if this virus behaves similarly to ADV in mink. Signs in juvenile and adult skunks in this study were similar including

neurologic signs, emaciation and lethargy, similar to mink kits that were infected vertically, or in any juvenile more than a few months old when infected horizontally. Mink kits commonly develop a fatal acute fulminant pneumonia with type II pneumocyte hyperplasia when they are infected horizontally rather than the chronic multiple organ failure classically seen in adults (Alexandersen, 1986; Alexandersen & Bloom, 1987). Of the few skunks in this study thought to be within the 3.5 month age range described in mink kits with acute pneumonia, we found a single juvenile with histologic lesions similar to those previously reported in this form of disease. This juvenile was PCR-negative; however, the blood sample had low volume and was diluted in cavitary fluid. Another juvenile skunk had necrotizing pneumonia and was PCR-positive. Both tested negative for CDV. This leaves open the possibility of a juvenile-specific pneumonia in skunks, similar to mink with ADV.

The regression model that best predicted PCR status had an AUC of 76.1%, which could be considered at best a fair fit for our data suggesting possible undetected confounders or other predictors. As the significance of predictors when evaluated in univariate analysis changed when relatively small numbers of cases were added or subtracted, larger sample size would be useful. Bias from opportunistic sampling likely contributed to associations between predictors that were stronger than biologically expected. Biologically plausible predictors for which we were unable to collect data include urbanicity, exposures at rehabilitation facilities, variation in exposures between different habitat types and degree of carcass degradation. As habitat generalists, skunks' environments can vary greatly, and their behaviours along with it. For example, urban skunks may congregate in large numbers under houses during breeding season providing many opportunities for environmental and direct horizontal transmission, whereas rural skunks can be solitary in self-made dens, perhaps creating a situation where vertical transmission plays a more prominent role. If, as for ADV, this skunk virus has high environmental persistence and multiple modes of transmission (Eklund, Hadlow, Kennedy, Boyle, & Jackson, 1968; Ingram & Cho, 1974; Prieto et al., 2014), small changes in environment and population dynamics could significantly impact disease transmission.

Available genetic information about the virus, the high prevalence in striped skunks and biological behaviour in skunks suggest that this amdoparvovirus is a strain of SKAV. Sequencing performed in our study showed a segment of the VP2 hypervariable region collectively best matched SKAV, closely followed by some ADV strains. Further sequencing of the full genome would be helpful. It has been suggested that all published genetic material from amdoparvoviruses in skunks falls within SKAV regardless of whether it was identified initially as ADV (Canuti et al., 2017). Overall, our data are consistent with SKAV having long been present but undetected in striped skunks in California. Alternatively, there may be a more complex ecology involving multiple closely related viruses that cross-react on PCR but have different clinical implications for skunks. While California itself does not have a recent history of fur farming, nearby states such as Oregon, Utah and Washington (National Agricultural Statistics Service, 2015) have extensive histories, and ADV has been documented in fur farms in Utah as recently as 2015 (Wilson, Baldwin, Whitehouse, & Hullinger, 2015). Farmed American mink occasionally escape from farms, or are let loose by concerned activists, and subsequently can establish the ADV in local free-ranging mink (Nituch, Bowman, Wilson, & Schulte-Hostedde, 2012; Oie et al., 1996). Genetic analysis of SKAV suggested coinfections and recombinant genomes can occur, similar to ADV in mink (Canuti et al., 2017). ADV also displays high genetic diversity, and high pathogenicity is not restricted to particular viral genetic lineages, suggesting that pathogenic strains may arise spontaneously from varied genetic origins (Gottschalck, Alexandersen, Storgaard, Bloom, & Aasted, 1994; Olofsson et al., 1999). A mutant/novel strain of SKAV in the SFBA with higher than average pathogenicity for skunks may explain the high mortality and appearance of emergence seen there, whereas the strains identified in other regions may have been comparatively less pathogenic.

Amdoparvovirus was present in skunks in Butte, Humboldt, Mendocino and Tuolumne Counties in areas of sympatry with mustelid species of conservation concern including fisher and Pacific marten (California Department of Fish and Wildlife; Natural Diversity Database, 2017). Both species occupy only remnants of their historic range in California (Kucera, Zielinski, & Barrett, 1995) and face threats from habitat loss, fragmentation and degradation due to timber harvest, wildfires and roads, predation, disease and toxicants (Hamlin, Roberts, Schmidt, & K., B., & Bosch, R., 2010; Naney et al., 2012). Antibodies to ADV have been documented in other marten species (Fournier-Chambrillon et al., 2004) and in experimentally infected fishers. SKAV was also found in a mink, suggesting it displays some host promiscuity, as does ADV (Canuti et al., 2017). Shifts of only a few amino acids in parvoviruses, including ADV, can shift viral tropism (Tijssen, 1999) in turn allowing transmission to naïve species. While our study found no evidence of amdoparvovirus viral DNA in 2 geographically distinct populations of fisher, their potential susceptibility is concerning.

The striped skunk's ubiquitous distribution in most habitats and high frequency of intra- and interspecies contact make them an ideal reservoir for this amdoparvovirus across the wildland–urban interface and between carnivore species. With both subclinical carriers and animals displaying pathology and mortality, skunks may serve as both a reservoir and true host of the virus. Subclinical carriers could transmit the disease amongst skunks and naïve species, while eluding monitoring and control efforts. This virus' possible deleterious impact on the host immune system could leave animals with increased susceptibility to other infectious diseases. Conversely, other pathogens and conditions such as various rodenticide toxicoses that are prevalent in California skunks can have adverse effects on the immune system and could leave skunks with an increased susceptibility to amdoparvovirus infection (Serieys et al., 2018). These issues may complicate transmission dynamics of amdoparvovirus while also leading to significant changes in transmission dynamics of diseases of known public health and ecologic importance in California, such as rabies and leptospirosis.

Limitations of this study are that the reported overall prevalence is a sample rather than a population prevalence, use of opportunistically collected samples, relatively low sample size in some areas and inability to perform further evaluation of the virus. Our estimate of prevalence is inherently biased due to the use of convenience sampling but this was required to obtain the sample size. Using skunks from wildlife rehabilitation facilities increased chances of obtaining affected skunks and discovering the full spatial distribution of the virus, but also yielded a biased sample that may not be representative of all skunks in California due to a higher than expected proportion of ill animals. Our results indicate animals showing signs of illness are more likely to be infected with amdoparvovirus, so a higher than expected proportion of these animals in our sample could indicate our sample prevalence may be higher than the true population prevalence. On the contrary, previous studies have shown that PCR detection of ADV in mink can wax and wane (Jensen et al., 2014), so we may not have detected all infected animals and our calculated prevalence may be lower than the true prevalence. Across geographic zones, there were different degrees of selection bias with some more likely to be generalizable to the true population. Despite this geographic bias in collection of high risk animals, no significant spatial clustering of positive samples was found anywhere in the state. Zone 3 was collected in what was likely the least biased manner of any zone, as most of the skunks were collected live for nuisance reasons, whereas much larger proportions of all other zones were collected due to poor health. Despite this, Zone 3 had the highest proportion of PCR-positive individuals, suggesting that these data may come close to approximating the true prevalence of amdoparvovirus in skunks in California. Another limitation was missing data for some predictors such as age class and sex. A more complete data set would likely improve model discovery and thus our model's explanatory power.

4.1 | Conclusions and recommendations

We still have much to learn about the true impacts of the virus we are detecting in California skunks. ADV and other parvoviruses such as canine parvovirus have established a precedent for the potential devastation highly adaptive parvoviruses can cause in domestic and wildlife populations. These viruses often feature rapid mutations and cross-species transmission, characteristics which raise concern for skunks hosting an amdoparvovirus that may potentially be transmitted across a variety of species. The results of our study suggest that SKAV or a closely related virus is endemic in California striped skunks, with potential recent emergence of a strain with increased virulence. Traditional test and cull methods of outbreak control are unlikely to be successful if an SKAV or ADV-like virus is introduced into a naïve species or if a virulent strain circulates. With limited options for control, continued monitoring and additional research are needed in both skunks and other mesocarnivores such as fishers, martens, spotted skunks (*Spilogale gracilis*), raccoons (*Procyon lotor*) and foxes (*Vulpes* spp. and *Urocyon cinereoargenteus)* to better understand the threat to vulnerable wildlife. Future studies should focus on additional genetic testing to confirm species identity, increasing sample size and spatial distribution of tested skunks, testing of additional species—fishers and martens in particular, environmental testing in facilities that house ill skunks as well as skunk habitat in the wild, investigation of molecular epidemiology and possibly experimental trials to investigate the progression of SKAV in skunks.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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