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A case-control survey study of environmental risk factors for primary hypoadrenocorticism in dogs

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Abstract

Background: Primary hypoadrenocorticism in dogs is thought to be multifactorial with roles for both genetic and environmental factors. The contributions of environmental factors remain unexplored.

Objective: Identify environmental and lifestyle exposures associated with primary hypoadrenocorticism in 2 dog breeds with high risk of developing the disease.

Animals: Animals were not used in this study. Owners of Standard Poodles (STPDs) and Portuguese water dogs (POWDs) participated in a survey.

Methods: Retrospective case-control study. Dog owners were invited to participate in an online survey through convenience sampling. Questions regarded the demographics, health histories, and indoor/outdoor environments in which their dogs live and play. Responses for dogs with primary hypoadrenocorticism were compared to those without the disease using univariate and multivariate logistic regression models.

Results: Five thousand forty-seven responses (358 cases, 4689 controls) met initial inclusion criteria. Significant associations with modest effect size were found for community type, ingestion of canned food, and use of lawn fertilizer in some analysis models. Reproductive (spay/neuter) status exhibited the strongest association with high effect size across all models with adjusted odds ratio (OR) 2.5 (95% confidence interval [CI], 1.4-4.5; $P = .003$) for spayed females and 6.0 (95% CI, 2.6-13.9; $P < .001$) for neutered males.

Conclusions and Clinical Importance: The large effect size for reproductive status reflects its high potential clinical relevance, whereas modest effect sizes for other environmental variables suggest lower potential clinical relevance. These findings are associations and do not necessarily imply causation. Before any actionable recommendations are warranted, additional evidence regarding biological mechanisms is needed.

Abbreviations: AKC, American Kennel Club; AQI, air quality index; CDC, Centers for Disease Control and Prevention; CI, confidence interval; CTLA4, cytotoxic T-lymphocyte associated protein 4; DOB, date of birth; Dx, diagnosis; EPA, Environmental Protection Agency; GIS, geographic information system; IQR, interquartile range; IRB, Institutional Review Board; km, kilometers; MHC, major histocompatibility complex; MLR, multivariate logistic regression; NA, not applicable; NO_2, nitrogen dioxide; O_3, ozone; OR, odds ratio; PM_{10}, particulate matter <10 μm in diameter; POWD, Portuguese Water Dogs; REF, reference; STPD, Standard Poodles; UK, United Kingdom; ULR, univariate logistic regression; UMN, University of Minnesota.

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KEYWORDS
Addison's disease, autoimmune disease, dogs, environmental exposures, primary hypoadrenocorticism, survey

1 | INTRODUCTION

Primary hypoadrenocorticism is an immune-mediated endocrinopathy in which the adrenal cortex is destroyed, resulting in dangerously low concentrations of glucocorticoids and mineralocorticoids.[1,2] Although prevalence in the general dog population is low (ie, 0.06%-0.31%)[3] the disease is concentrated in certain breeds.[4-10] Standard Poodles (STPDs) and Portuguese water dogs (POWDs) have among the highest prevalence, with some studies estimating 8% to 10% being affected.[6,7] Breed predisposition suggests a hereditary component, and genetic contributions have been studied.[5,8,11-17] Major histocompatibility class (MHC) class II alleles,[11,13,18] cytotoxic T-lymphocyte protein 4 (CTLA4),[12,18] and other immune system-related genes[5,19,20] have shown modest associations with the disease (henceforth referred to as hypoadrenocorticism) in dogs. Heritability estimates of 0.49 for POWD[6] and 0.75 for STPD[7] suggest genetic variation explains a large portion of the disease phenotype. However, a complex etiology with roles for both genetic and nongenetic factors is likely.[21]

Environmental risk factors for hypoadrenocorticism in dogs remain poorly understood. Few studies have investigated roles for nongenetic factors. In addition to breed,[4,22] associations with age,[22] sex,[4,22] body weight,[22] insured status,[22] and spay/neuter status[22-24] have been identified. However, some of these variables (eg, breed, body weight)[25] are not independent of genetic influence and are not exclusively exogenous environmental exposures. To the best of our knowledge, no published study has comprehensively evaluated environmental exposures for association with hypoadrenocorticism in dogs.

Our objective was to conduct a survey of STPD and POWD owners to identify environmental risk factors associated with hypoadrenocorticism in these breeds. We hypothesized that environmental exposures associated with autoimmune disease in humans (eg, pollution, diet, pesticides, tobacco)[26-35] would be present at a higher frequency in dogs with hypoadrenocorticism compared to dogs without the disease. Identifying environmental factors associated with hypoadrenocorticism in dogs may benefit the health of dogs through improvements in clinical management using targeted interventions of associated exposures, and by identifying novel therapeutic targets by research investigating the molecular consequences of these exposures.

2 | METHODS

2.1 | Study population

The target population consisted of STPDs and POWDs with and without hypoadrenocorticism. Owners of STPDs and POWDs meeting the following eligibility requirements could participate: (1) survey respondents must be ≥18 years old, (2) live in the United States (US) or Canada, and (3) currently live with ≥1 STPD or POWD. A convenience sampling approach was used. Invitations to participate were advertised through breed club announcements, via social media targeting owners of pet STPDs and POWDs, and through emails, newsletters, and word-of-mouth and included a link to the survey generated by Qualtrics[XM].[36] This study was deemed exempt by the Institutional Review Board (IRB) of University of Minnesota (UMN) because the survey topic focused on animal health.

2.2 | Survey

The survey was administered through UMN's secure online survey software platform Qualtrics[XM].[36] Questions were written in English and designed in consultation with an epidemiologist (DMC) and a survey methodologist (KJY). Three veterinarians and 3 dog owners piloted the survey. Revisions were made based on their feedback.

Informed consent was collected before participants could answer the survey. Participants were instructed to answer for only 1 dog per household. Households with dogs both with and without hypoadrenocorticism were asked to answer for a dog with hypoadrenocorticism. Respondents with multiple eligible dogs were advised to respond for the dog with the name that came first alphabetically to minimize selection bias. Owners responding for dogs affected with hypoadrenocorticism were asked to answer with regard to the time before their dog's diagnosis.

The total number of questions administered varied from 37 to 61, because of branching logic and skip patterns (eg, respondents with hypoadrenocorticism dogs received additional questions that controls did not, such as age at hypoadrenocorticism diagnosis). Questions about the dog's demographic characteristics and health history included: name, breed, date of birth (DOB), age when acquired by owner, sex, spay/neuter status, affected/unaffected with hypoadrenocorticism, other chronic health conditions besides hypoadrenocorticism, number of dogs in the household, diet, and flea and tick product use. Questions regarding household variables included community type (eg, urban, suburban, rural); size of residence; bath frequency; use of tobacco products, incense, scented candles, wood burning stoves, insecticides, pesticides, carpet cleaner, and paint strippers; and whether cats lived in the household. Questions on the environment outside the home concerned attendance at daycare, dog parks, or professional groomers; outdoor insecticide or pesticide use; outdoor swimming or bonfires; and tobacco use in cars. Respondents had the option to enter their contact information in case of need for follow-up. A copy of the survey exported from Qualtrics[XM][36] is available in Supplemental Document 1.

2.3 | Data analysis

2.3.1 | Survey data

Responses were collected from February 15, 2021, to March 2, 2022. Incomplete surveys (<20% completed), ineligible responses (those selecting "no" to the consent/eligibility question), and duplicates (≥1 completed survey per household) were excluded. The first entry for each duplicate was retained.

2.3.2 | Geographic information system data

Qualtrics[XM][36] adds geotags, for the latitude and longitude of the IP address of the device used to access the survey, to each response. As an exploratory subgroup analysis, geotags were used to join survey responses for eligible United States participants to data from publicly available geospatial databases on air, water, and soil pollution, and human intoxication mortality from illicit drugs, alcohol, or both. This approach uses IP address location as a proxy for the respondent's household. Analysis of geographic information system (GIS) data was restricted to US participants because Canadian data were not available from the identical sources.

Soil data consisted of locations of brownfields as determined by the US Environmental Protection Agency (EPA), defined as a property the use of which may be complicated by the presence of a hazardous substance, pollutant, or contaminant. Data from June 2022 were downloaded at http://www.epa.gov/enviro.[37] This database records brownfield sites as geographic point locations of longitude and latitude. Distance between each household and the closest brownfield was calculated in kilometers (km). Water data consisted of the US EPA 303 geodatabase of impaired waters.[38] This database consists of point, linear, and area events of contaminated water sources. Distance between each household and the closest contaminated water source was calculated in kilometers. Annual air quality data by county from 2020 were obtained from the US EPA.[39] Each household was assigned air quality data for its county of residence. Drug overdose data per county were obtained from the Centers for Disease Control and Prevention (CDC) WONDER database of underlying cause of death using averages spanning 1999 to 2020.[40] Data were downloaded on deaths per population caused by intoxication, drug overdose, or both, which was used as a proxy for potential exposures to these substances in dogs.[41] These data were joined to participant data by location of the county; participants were assigned the overdose death rate for their county of residence. All spatial joins were performed in ArcGIS version 10.6.1 (ESRI, Redlands, California).[42]

2.4 | Statistical analyses

2.4.1 | Frequency tables

Response counts for survey and GIS variables were tabulated and compiled in frequency tables. Distributions of these variables were compared between cases and controls using Chi-squared tests for binary and categorical variables and Kruskal-Wallis tests for continuous variables. Although the study's original focus was on environmental variables, spay/neuter status emerged with an unexpectedly large effect (high odds ratio [OR]) relative to all other test variables and was highly significant (small P value). To better understand this relationship, subsequent univariate and multivariable analyses were restricted to the dataset of dogs for which spay/neuter status was known. Statistical analyses were performed in Stata version 15.1 (College Station, Texas),[43] with $P < .05$ considered significant.

2.4.2 | Univariate logistic regression

Univariate logistic regression models compared response distributions between cases and controls. We calculated ORs for survey variables reaching significance in the frequency tables. To control for potential confounding effects of age on the distribution of spay/neuter status in cases vs controls, the analysis was restricted to dogs old enough to have been eligible for spay/neuter (conservatively estimated at ≥25 months old at the time of the survey). Variables reaching significance in univariate models were chosen for effect size and a priori knowledge and were subsequently investigated in a stratified analysis of spayed or neutered subgroups.

2.4.3 | Multivariable logistic regression

Multivariable logistic regression models compared cases and controls using spay/neuter status as the primary exposure. Models were stratified by sex, and were adjusted for potential confounding effects from certain variables (eg, breed, age, medical condition, canned food consumption, community type, and exposure to campfires, lawn fertilizer, and tobacco) chosen from variables with significant differences in distribution in the univariate models based on effect size and a priori knowledge.[26-30] All confounders were retained in the final model in an effort to isolate the independent association between spay/neuter status and hypoadrenocorticism. Missing data for answers to questions related to all variables included in MLR models were compared between cases vs controls using a Chi-squared test.

2.4.4 | Subgroup analysis of neutered and spayed dogs

Additional information not included in the original survey (eg, dates of spay/neuter and date of hypoadrenocorticism diagnosis) was needed to investigate the temporal relationship between spay/neuter status and hypoadrenocorticism diagnosis. To collect this information, we conducted a follow-up survey of a subset of respondents. Analysis cohorts were selected by filtering the initial pool of all qualifying responses for owners of spayed or neutered dogs who had provided an email address. From these responses, all resulting cases (n = 276;

156 females, 120 males) were sent a follow-up email asking for the dog's DOB, date of hypoadrenocorticism diagnosis, and date of spay/neuter. Concurrently, a randomly selected subgroup of owners of controls (n = 831; 411 females, 420 males) was sent a follow-up email requesting the dog's DOB and date of spay/neuter. Responses qualified for analysis if the follow-up questions were completely answered. Distribution of months between spay/neuter and hypoadrenocorticism diagnosis was calculated for cases, and distributions of age at the time of the spay or neuter procedure were compared between cases and controls using a Kruskal-Wallis test.

2.4.5 | Comparative geographic mapping of the sampled vs target population (US data only)

We evaluated whether our convenience sampling of STPD and POWD was geographically representative of the distribution of breed-registered dogs in the United States. An anonymized list containing the zip codes of all registered STPD and POWD in the United States (n = 33 077) was provided by the American Kennel Club (AKC). Distribution of the sampled population was compared to the AKC-registered dog population using a Cross K-function analysis.

3 | RESULTS

A total of 6286 survey responses were attempted. Duplicates (n = 6), ineligible dogs (owners selecting "no" to the consent/eligibility question; n = 7), and incomplete surveys (<20% of key questions answered; n = 1226) were excluded, leaving 5047 responses for initial analysis (Figure 1).

3.1 | Basic population characteristics

Among all participants, STPD was a more common breed (n = 4856; 96.2%) than POWD (n = 191, 3.8%). A hypoadrenocorticism diagnosis was reported by 358 (7.1%) participants, including 9 dogs that were diagnosed before living with their current owners and thus were excluded from subsequent analysis. Of respondents providing country information, US residents outnumbered Canadians, 4705 vs 226, respectively. Significant differences between cases and controls were observed for breed ($P < .001$), median age at the time of the survey ($P < .001$), sex ($P = .03$), spay/neuter status ($P < .001$), and comorbidity with other chronic health conditions ($P < .001$). Fifteen percent of cases were POWDs, whereas only 3% of control dogs were POWDs. Conversely, STPDs made up a higher proportion of controls than cases, (97% vs 85%, respectively). The control population was younger at the time of the survey: median age and interquartile range (IQR) in years for control dogs was 3 (1-6) vs 7 (4-10) for dogs with hypoadrenocorticism. Cases were more commonly female (55%), whereas control dogs were evenly divided (51% males, 49% females). Dogs with hypoadrenocorticism were more likely to have been spayed (91.3%) or neutered (93.1%) compared to control dogs (63.9% spayed, 58.2% neutered). More dogs with hypoadrenocorticism, (18%; 95% confidence interval [CI], 12-20) had other chronic medical conditions compared to controls (7%; 95% CI, 6-8).

Variables in the home environment associated with hypoadrenocorticism included living in households with >1 dog ($P = .001$), eating primarily canned food ($P < .001$) or commercial raw food ($P = .03$), seasonal vs year-round use of flea/tick medications ($P < .001$), use of "spot on" flea/tick medications ($P < .001$), and community type ($P = .001$). Variables outside of the home environment associated

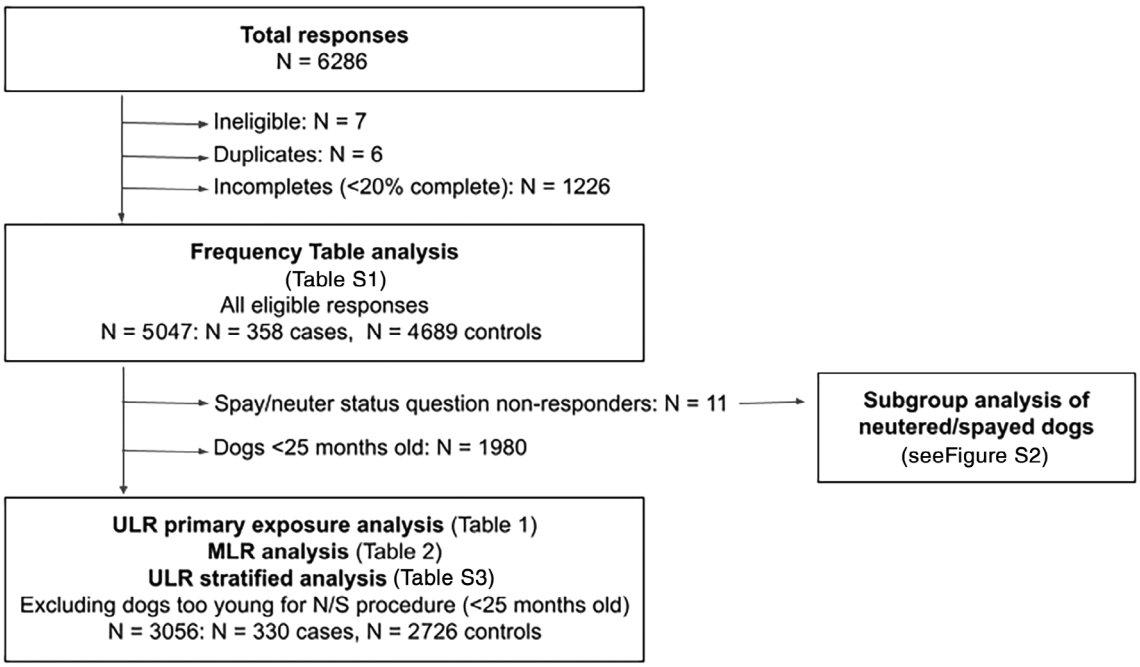

FIGURE 1 Analysis workflow. MLR, multivariate logistic regression; ULR, univariate logistic regression.

TABLE 1 Univariate logistic regression analysis.

Variable	Cases (n = 330) n (%)	Controls (n = 2726) n (%)	Odds ratio (95% CI)	*P* value
Breed				<.001
Portuguese Water Dog	48 (14.6%)	117 (4.3%)	3.8 [2.7-5.4]	
Standard Poodle	282 (85.5%)	2609 (95.7%)	REF	
Neutered				<.001
No	8 (5.5%)	377 (27.8%)	REF	
Yes	138 (94.5%)	981 (72.2%)	6.6 [3.2-13.7]	
Spayed				<.001
No	14 (7.7%)	318 (23.3%)	REF	
Yes	167 (92.3%)	1045 (76.7%)	3.6 [2.1-6.4]	
Other medical conditions				<.001
No	254 (80.6%)	2441 (89.7%)	REF	
Yes	57 (18.1%)	260 (9.6%)	2.1 [1.5-2.9]	
Do not know	4 (1.3%)	20 (0.7%)	OMIT	
Community				<.001
Farm	4 (1.3%)	196 (7.3%)	0.2 [0.1-0.5]	<.001
Rural	71 (23.1%)	687 (2.4%)	0.8 [0.6-1.2]	.27
Suburban	176 (57.3%)	1325 (49.6%)	1 [0.8-1.5]	.82
Urban/city	51 (16.6%)	399 (14.9%)	REF	
Other	5 (1.6%)	65 (2.4%)	OMIT	
Food				
Canned	52 (15.8%)	317 (11.6%)	1.4 [1-2]	.03
Campfires				.02
Never	234 (77.2%)	1779 (67.2%)	REF	
A few times a year	55 (18.2%)	676 (25.5%)	0.6 [0.5-0.8]	.002
A few times a month	8 (2.6%)	98 (3.7%)	0.6 [0.3-1.3]	.2
Monthly	3 (1.0%)	58 (2.2%)	0.4 [0.1-1.3]	.12
Weekly	3 (1.0%)	34 (1.3%)	0.7 [0.2-2.2]	.5
Lawn fertilizer				.05
No	152 (53.7%)	1499 (59.7%)	REF	
Yes	131 (46.3%)	1011 (40.3%)	1.3 [1-1.6]	

Note: Qualified participants were restricted to those with known spay/neuter status ≥25 months old at the time of survey (n = 330 dogs with hypoadrenocorticism, n = 2726 controls).
Abbreviations: CI, confidence interval; OMIT, the variable was omitted from evaluation due to low frequency; REF, the variable choice which was set as the reference to which other choices for the same variable were compared.

with hypoadrenocorticism included use of lawn fertilizer ($P = .02$). In the GIS analysis, residing in a county with higher airborne particulate matter$_{10}$ (PM_{10}; $P = .02$) was associated with hypoadrenocorticism (Table S2A). Variables outside of the home environment associated with the control population included frequent use of a professional groomer ($P = .002$) and frequent presence at campfires ($P < .001$). Complete details regarding all survey characteristics and variables evaluated are presented in Table S1. The GIS variable analyses are presented in Table S2A.

Preliminary calculations of OR from the frequency table identified spay/neuter status as having a very high OR (9.7; 95% CI, 5.2-18.0) for neutered dogs and for spayed dogs (5.9; 95% CI, 3.6-9.8). Consequently, subsequent analyses focused on testing the robustness of the association between hypoadrenocorticism and spay/neuter status.

3.2 | Univariate logistic regression

Survey variables with significant differences in distributions in the initial frequency table and GIS analysis were evaluated in ULR models (Table 1 and Table S2B, respectively). The study population was

restricted to dogs eligible for spay/neuter, conservatively estimated at ≥25 months old at the time of the survey, and to those with known spay/neuter status (330 cases and 2726 controls). Variables associated with hypoadrenocorticism included breed ($P < .001$), spayed status ($P < .001$), neutered status ($P < .001$), concurrent medical conditions ($P < .001$), community type ($P < .001$), eating canned food ($P = .03$), and exposure to lawn fertilizer ($P = .02$). Neutered dogs had the highest odds of having hypoadrenocorticism (OR, 6.6; 95% CI, 3.2-13.7), followed by breed (OR, 3.8; 95% CI, 2.7-5.4), and spay status (OR, 3.6; 95% CI, 2.1-6.4; Table 1).

Significant variables in ULR models were further evaluated by spayed or neutered status separately (stratified analysis). Spayed- and neutered-specific associations were found, consistent with a role for spay/neuter status as an effect modifier for other significant variables (Table S3). Neutered-specific associations included attending campfires a few times a year vs never ($P = .02$; OR, 0.5; 95% CI, 0.3-0.9). Spayed-specific associations included chronic medical conditions ($P < .001$; OR 2.4; 95% CI, 1.6-3.7) and community type, specifically farm vs urban ($P = .03$; OR, 0.1; 95% CI, 0.01-0.8; Table S3).

3.3 | Multivariable logistic regression

Multivariable logistic regression analysis tested the strength of association between spay/neuter status and hypoadrenocorticism while adjusting for contributions of other potential primary variables (eg, significant variables in ULR analysis and tobacco). Analysis cohorts matched those described for ULR models. Adjusted ORs for spayed and neutered status are shown in Table 2 ($P = .003$; OR, 2.5; 95% CI, 1.4-4.5 and $P < .001$; OR, 6.0; 95% CI, 2.6-13.9), respectively. Among survey respondents with dogs ≥25 months at the time of the survey, 83% had complete data for all variables (cases, 85%; controls, 83%). Among explanatory variables in the MLR analysis, age had the largest percentage of missing data, 6.6% of the dataset. All other variables had <5% of values missing. No significant differences were found in missing data between cases and controls for age or any other MLR variable.

3.4 | Subgroup analysis of spay/neutered temporal data

Response rates for the follow-up survey, which sought dates of spay/neuter and hypoadrenocorticism diagnosis, were 54% (148/276) for cases and 35% (294/831) for controls. Responses qualified if they provided (1) a DOB consistent with the original survey response, (2) a known or estimated date of spay/neuter, and (3) a known or estimated date of hypoadrenocorticism diagnosis (cases only), and included 70% (104/148) of cases and 88% (258/294) of controls (Figure S1). Distribution of age at the time of spay/neuter did not differ between cases and controls ($P = .97$) for all qualifying responses or for the subset of dogs ≥25 months at the time of the survey ($P = .99$; 91 dogs with hypoadrenocorticism; 174 dogs without hypoadrenocorticism; Figure S2). Most affected dogs, 79%, (82/104) were spayed or neutered before hypoadrenocorticism diagnosis (median, 17.9 months; IQR, 5.1-40.5; Figure S3A). A higher proportion of males (88%; 38/43) was altered before hypoadrenocorticism diagnosis than females (72%; 44/61; Figure S3B,C, respectively). Females were spayed a median of 14.9 months before diagnosis (IQR, 5.5-30.2). Males were neutered a median of 21.1 months before diagnosis (IQR, 7.3-46.6).

3.5 | Comparative geographic mapping of the sampled vs target population (US data only)

Geographic distributions of the sampled US population and the target US population were compared to determine whether the sampled population represented the target population. Figure S4 shows that the study population (red dots) resembles that of the AKC-registered STPD and POWD populations (blue dots), as well as the general distribution of population density in the United States (based on 2020 census data).[44] Cross K-function analysis confirmed that the distributions of the sampled and AKC-registered populations were not significantly different.

4 | DISCUSSION

We evaluated common environmental exposures for association with hypoadrenocorticism in 2 dog breeds with a high prevalence of the disease. Significant associations were found for certain variables across multiple analysis models, including community type (eg, urban, rural, farm), ingestion of canned food, and exposure to lawn fertilizer (Tables 1 and 2, Table S1). Although biologically plausible, the modest effect sizes of these associations suggest low practical relevance.[45] Modest effect sizes may reflect indirect effects (eg, as a proxy for some other variable), insufficient power, or the influence of sample population characteristics (eg, overrepresentation of breeder-owned dogs in the control group).[45,46] Nevertheless, our study provides the first identification of environmental risk factors associated with hypoadrenocorticism in dogs. Follow-up studies that target these variables using probabilistic sampling in a larger number of cases will determine the reproducibility and clinical relevance of these findings.

The strongest association found was for spay/neuter status, a factor previously reported to be associated with hypoadrenocorticism in dogs.[22-24] Despite being a secondary aim, the potential high clinical relevance suggested by the large effect size compelled us to further investigate this variable. The OR here was >2 times higher than previously reported for neutered males in our study (6.0; 95% CI, 2.6-13.9) vs (2.1 ± 0.5)[23] and (2.5; 95% CI, 1.4-3.5) in a study which evaluated males and females together.[22] The OR for spayed females resembled those of prior studies: (2.5, 95% CI [1.4-4.5]) in our study vs (1.5 ± 0.3)[23] and (2.5; 95% CI, 1.4-3.5) in previous studies.[22] Also consistent with a prior study[23] was the higher OR for neutered males compared to spayed females. Although our CIs were wider than those in

TABLE 2 Multivariable logistic regression analysis.

Variable	Unadjusted OR (95% CI)	Adjusted OR (95% CI)	Adjusted *P* value
Neutered			
Yes	6.6 (3.2-13.7)	6.0 (2.6-13.9)	$P < .001$
Spayed			
Yes	3.6 (2.1-6.4)	2.5 (1.4-4.5)	$P = .003$

Note: Evaluation of the independent association between spay/neuter status and hypoadrenocorticism using a multivariable logistic regression model adjusted for contributions from age, breed, medical condition, canned food, community type, bonfires, lawn fertilizer, and tobacco use. Study population was restricted to participants with known spay/neuter status ≥25 months old of age at time of survey (n = 330 cases, n = 2726 controls).
Abbreviations: CI, confidence interval; OR = odds ratio.

prior studies, the differences likely result from the smaller number cases vs controls.[45,46] Furthermore, they do not overlap with effect size estimates reflecting significant differences.

The breed-restricted approach of our study may explain the higher ORs for spay/neuter status and hypoadrenocorticism compared to those of previous studies. Breed has been shown to influence the likelihood of developing hypoadrenocorticism in dogs.[4] Prior studies evaluating spay/neuter status for association with hypoadrenocorticism were open to dogs from any breed.[22,23] Additionally, each study evaluated geographically distinct populations: 1 study evaluated United Kingdom (UK) dogs,[22] another study included dogs seen at a university clinic in California,[23] whereas our study was open to dogs across the United States and Canada. That 3 independent studies in different validation populations found the same association, with the same pattern of higher ORs for neutered male vs spayed female dogs emphasizes the robustness of this association.

We initially were surprised by the high odds of developing hypoadrenocorticism in spayed/neutered dogs. We therefore took several steps, including a small follow-up survey, to increase our confidence in these findings. For example, we observed that, in our initial study population, the median age of control dogs was significantly lower than the median age of cases (3 years [IQR 1-6] for controls and 7 years [IQR 4-10] for cases; $P < .001$). We hypothesized that dogs in the younger control population may not have been eligible for a spay or neuter procedure. We therefore restricted our analysis to include only dogs with owners who had answered the spay/neuter status question, and dogs that were old enough to have been eligible for a spay or neuter procedure (conservatively estimated at ≥25 months at the time of the survey). Age distributions of cases and controls were not significantly different after this adjustment ($P = .53$; Table 1). Although the values of the initially observed OR contracted, they remained high (6.6; 95% CI, 3.2-13.7) for neutered males and (3.6; 95% CI, 2.1-6.4) for spayed females (Table 1).

In our final MLR analysis, these ORs decreased slightly for males (6.0; 95% CI, 2.6-13.9) and compared to females (2.5; 95% CI, 1.4-4.5). Therefore, we concluded that these associations were significant and not driven by factors we could otherwise control for in our study population. We acknowledge the many approaches to MLR analysis. Given our case-control study design, we approached our analysis with a focus on identifying independent predictors of hypoadrenocorticism. In our exploratory analysis and ULR models, we found spay/neuter status had the largest effect size of all potential exposures. This observation led to our decision to explore spay/neuter status as the primary exposure and consider other potential confounding factors in our analysis. Because we had a large sample size and many potential confounders, we applied both a stringent cutoff (*P* value of .05) and a priori knowledge to move variables into the MLR model. Because we intended to identify the independent association between spay/neuter status and hypoadrenocorticism, all other variables in the MLR model were considered only as confounders. This analytic approach was most appropriate for our objectives, because other approaches may have allowed for only consideration of the statistical significance, and spurious results might have been identified.

Given the strong association observed between hypoadrenocorticism and spay/neuter status, we followed this finding up in a subgroup of study participants to explore the timing between spay/neuter and hypoadrenocorticism diagnosis because our initial survey did not ask questions designed to interrogate this relationship. The majority of dogs (79%) were found to have been spayed or neutered before the hypoadrenocorticism diagnosis. More males (88%) were neutered before the hypoadrenocorticism diagnosis than females (72%). Therefore, we concluded that the higher proportion of spayed/neutered dogs among cases was not entirely explained by owners seeking this procedure for their dogs in response to a hypoadrenocorticism diagnosis to prevent further breeding. Furthermore, the median months for a hypoadrenocorticism diagnosis after the spay/neuter procedure was >1 year later (17.9 months for males/females combined' 14.9 months for females and 21.1 months for males). This finding suggests that, in most cases, the spay/neuter procedure did not trigger an adrenal crisis nor did it uncover pre-existing disease. Although these data are consistent with the hypothesis that being spayed or neutered contributes to the development of hypoadrenocorticism, they are only associations.[47]

Additionally, our findings are supported by other evidence. For example, in a large epidemiological study in a Swedish population of dogs,[4] POWD and STPD were found to be breeds commonly diagnosed with hypoadrenocorticism.[6,7] However, the prevalence in these breeds (1.2% for both POWD and STPD)[4] was much lower than those found in another study of North American populations (8.6% for

STPD[7] and 7.0% for POWD,[48] respectively). Lower rates of hypoadrenocorticism might be expected in populations for which spay and neuter procedures are less common if spay/neuter status contributes to the pathogenesis of the disease. In Sweden, only 1.1% of dogs are estimated to be spayed or neutered,[49] compared to an estimated 64% of dogs in the United States.[23,50] Our subgroup analysis was limited by containing only approximately a third of the case population, 29% (104/358) of all eligible cases or 32% (104/330) of age-restricted cases. It is possible that the association between spay/neuter status is a proxy for some other yet to be identified causative variable or a latent confounding variable.[47,51,52]

Other novel insights include evidence of effect modification by spay/neuter status. A univariate stratification analysis showed different environmental variables were associated with spayed vs neutered dogs (Table S3). This finding suggests sex hormone profiles may influence both overall risk as well as susceptibility to other environmental risk factors for developing hypoadrenocorticism in dogs. Future etiological studies should account for the influence of spay/neuter status in study design. Other research is needed to determine if spay/neuter status plays a contributing role to the development of hypoadrenocorticism that can be explained by biological mechanisms or if spay/neuter status is a proxy for other variables such as better access to veterinary care.

Although we did not evaluate the biological mechanisms for the role of spay/neuter status in the development of hypoadrenocorticism, the literature supports several potential explanations. For example, sex hormones have immune-modulating effects in mammals. Gonadal androgens have broad immunosuppressive effects in both the central and peripheral immune system,[53] including suppressing antibody production, T-cell proliferation, natural killer cell activity, and cytokine production.[54,55] Loss of these hormones through neutering and their corresponding immunosuppressive functions may contribute to an immune-mediated pathogenesis in hypoadrenocorticism. Additionally, both androgen and estrogen receptors can be found on immune[56,57] and adrenocortical cells, and sex hormones influence the physiology of these cells.[58,59] Sex hormones also play roles in cellular repair, turnover, and proliferation of the adrenal glands, as well the recruitment of adrenal stem cells.[58,60] Loss of these hormones may contribute to dysfunction of these cells resulting in adrenocortical atrophy that is observed in these dogs.[61]

We acknowledge several limitations inherent to the methodology of our study. Nonprobabilistic convenience sampling is associated with several issues: (1) sampling error cannot be calculated, making results not generalizable beyond the study population, and (2) potential for participation/nonresponse bias.[62] That is, participants self-select into the study and those with a higher motivation to participate may be overrepresented (eg, the higher proportion of POWDs relative to STPDs in the cases may reflect higher motivation to participate among owners of POWDs with hypoadrenocorticism). Also, advertising the survey through breed clubs could have led to sampling bias such that breeder-owned dogs are overrepresented, possibly explaining the overrepresentation of certain breeder-associated behaviors in the control group (eg, use of professional grooming and not spaying or neutering). Furthermore, a nonprobabilistically sampled population may not represent a target population because of poor sampling coverage or selection effects.[62] Comparing our nonprobabilistically derived US sample to a well-defined estimate of the US sample frame using spatial mapping suggested that our sampled US population was geographically representative of the target US population (Figure S4). Despite these known limitations, we used non-probabilistic sampling for certain advantages to performing exploratory research (eg, low cost, expedience, ability to collect a large sample).[63]

Self-reported survey data are vulnerable to several types of bias (eg, misclassification, recall, response, item, and unit nonresponse bias).[64-66] Among these concerns are potential effects from missing data. We calculated missing data for each explanatory variable in the multivariable analysis and compared the differences between cases and controls using a Chi-squared test; statistically significant differences in missing data were not detected for these variables. Self-reported data are also susceptible to misclassification bias. For example, some respondents may have incorrectly categorized their dogs as not having hypoadrenocorticism because of misdiagnosis or misunderstanding of the term. However, misclassification of hypoadrenocorticism status is unlikely in our target population for the following reasons: (1) hypoadrenocorticism is common in these breeds and most owners are aware of its importance; (2) hypoadrenocorticism is fatal if not treated and treatment is highly specific; (3) there were several opportunities to self-correct classification (including several hypoadrenocorticism-specific questions); and (4) pilot testing of the survey tested comprehension of terms.

Spatial data have other limitations. For example, the scale of GIS data influences whether or not an association might be detected.[67,68] Differences in how data from diverse municipalities are collected, managed, and stored may introduce standardization errors. Other limitations stem from the data analysis (eg, type II error). To illustrate, although tobacco exposure was evaluated, its use was low for both case and control cohorts (approximately only 3% of households sampled; Table S1). Our study was likely underpowered to detect a real effect for this variable. Therefore, the lack of association for a particular variable in our study should not be regarded as evidence of a lack of risk. Future studies with sufficient power are required to detect an effect for variables with low minimum effect sizes.

Likewise, significant results are not inherently meaningful. For example, several air quality variables had statistically significant associations with hypoadrenocorticism (eg, PM_{10}, NO_2, O_3, yearly total and median number of days above the unhealthy air quality index [MD > UAQI]; Table S2A,B). However, medians compared between cases and controls for many of these variables were often near zero (eg, PM_{10}, NO_2, and MD > UAQI), small enough to question if these differences are meaningful even if a statistically significant differene exists. Different study design methods may be required in future studies to more effectively make use of GIS databases in understanding environmental exposures associated with hypoadrenocorticism in dogs.

Although the main objective of our study was to identify environmental exposures associated with hypoadrenocorticism in dogs, the most important finding in our study population was the association of

spay/neuter status. This finding confirms prior work[22,23] and lays the groundwork for future studies to test the reproducibility of our work and to investigate underlying biological mechanisms that might contribute to the disease. Future epidemiologic studies might use probabilistic sampling (eg, prospective studies) with attention to the temporality between spay/neuter status and hypoadrenocorticism diagnosis. Other studies in cell-based model systems such as canine adrenal organoids or adrenocortical cell culture could generate insights into how different hormone profiles affect these tissues. These studies would represent a promising new approach to understanding underlying triggers of this common disease that carries a high degree of morbidity in certain dog breeds.

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CONFLICT OF INTEREST DECLARATION

Steven G. Friedenberg serves as Associate Editor for the Journal of Veterinary Internal Medicine. He was not involved in review of this manuscript. No other authors declare a conflict of interest.

OFF-LABEL ANTIMICROBIAL DECLARATION

Authors declare no off-label use of antimicrobials.

INSTITUTIONAL ANIMAL CARE AND USE COMMITTEE (IACUC) OR OTHER APPROVAL DECLARATION

Authors declare no IACUC or other approval was needed.

HUMAN ETHICS APPROVAL DECLARATION

Authors declare human ethics approval was not needed for this study.

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