Circulating neurohormone imbalances in canine sudden acquired retinal degeneration syndrome and canine pituitary-dependent hypercortisolism

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Abstract
Background: Sudden acquired retinal degeneration syndrome (SARDS) has clinical similarity to pituitary-dependent hypercortisolism (PDH) in dogs. Some studies have identified a greater frequency of SARDS in seasons with reduced daylight hours. Neurohormone imbalances contribute to retinal lesions in other species, warranting further study in dogs with SARDS.

Hypothesis: Dysregulation of circulating melatonin concentration is present in dogs with SARDS but not in dogs with PDH.

Animals: Fifteen client-owned dogs with spontaneous SARDS (median time of vision loss 18 days), 14 normal dogs, and 13 dogs with confirmed PDH.

Procedures: Prospective case-control study. ELISA on samples (obtained in the morning) for measurement of plasma melatonin and dopamine, serum serotonin, urine 6-sulfatoxymelatonin (MT6s), and creatinine. Statistical analysis was performed using 1-way ANOVA, Spearman correlation and receiver operator characteristic area under the curve analysis.

Results: There were no significant differences in circulating melatonin, serotonin or dopamine concentrations between the 3 groups, although the study was underpowered for detection of significant differences in serum serotonin. Urine MT6s:creatinine ratio was significantly higher in dogs with PDH (4.08 ± 2.15 urine [MT6s] ng/mL per mg of urine creatinine) compared with dogs with SARDS (2.37 ± .51, P < .01), but not compared with normal dogs.

Conclusions and Clinical Relevance: We have identified neurohormone differences between dogs with SARDS and PDH.

Keywords
dog, dopamine, melatonin, PDH, retina, SARDS, serotonin

1 | INTRODUCTION

Sudden acquired retinal degeneration syndrome (SARDS) is a leading cause of acute incurable vision loss in dogs. The pathogenesis of SARDS is poorly understood and there are currently no proven safe
and effective treatments. There are existing hypotheses for the etiology of SARDS including autoimmunity and intoxication, however, a recent clinical trial failed to show that oral immunosuppressive medication was effective in restoring vision. Thus, exploration of novel hypotheses is warranted to facilitate more effective approaches to treatment. Advancement of our understanding of the disease pathogenesis might also allow identification of useful diagnostic biomarkers for SARDS, allowing earlier therapeutic intervention.

The central hypothesis of this study was that an imbalance of neurohormones is associated with SARDS. There are primary observations underlying this hypothesis, as there is an increased incidence of SARDS cases in seasons with reduced daylight hours. Melatonin influences, and fluctuates with, circadian rhythm and seasonal biology and therefore might explain the seasonal incidence of SARDS. Furthermore, the retina contains receptors for melatonin. Although the pineal gland is the primary source of melatonin, it is also produced by other organs. In the retina, melatonin is almost exclusively produced by photoreceptor cells, with melatonin dysregulation proposed to contribute to photoreceptor apoptosis observed in SARDS. Urine 6-sulfatoxymelatonin (MT6s) concentrations represent accumulated amounts of systemic melatonin over several hours and are therefore less affected by fluctuations in circulating concentrations. Additionally, urine MT6s accounts for more than 70% of the melatonin secreted, and its concentration in urine is 2-3 orders of magnitude higher than that of urine melatonin. Related monoamines, serotonin and dopamine, might also be associated with fluctuations in melatonin as serotonin is a precursor to melatonin and dopamine is involved in a cellular feedback loop with melatonin in the retina.

The purpose of this study was to provide baseline measurements of selected systemic neurohormone concentrations to determine whether this hypothesis merits future mechanistic investigation and whether neurohormone assays might aid in the early diagnosis of SARDS. We measured plasma melatonin, serum serotonin, plasma dopamine, urine MT6s, creatinine in dogs with SARDS, normal dogs, and dogs with pituitary-dependent hypercortisolism (PDH). We included dogs with PDH as this condition shares some clinical signs in common with SARDS, and comparison with this group could potentially provide insight into the pathogenesis of some of the systemic clinical signs, such as polyphagia and polyuria-polydipsia, in dogs with SARDS.

**2 | MATERIALS AND METHODS**

**2.1 | Study design**

A prospective case-control study was designed to compare concentrations of morning-sampled plasma melatonin, serum serotonin, plasma dopamine, urine MT6s, and urine creatinine between client-owned dogs with confirmed SARDS, normal dogs, and dogs with PDH. A prospective power analysis was performed on available data to calculate sample size to detect a specific change in plasma melatonin concentration. Normal dogs have a serum melatonin mean level of 2.39 ± 0.18 pg/mL (SEM) using data obtained from a group of 19 dogs living in New York State. Therefore, to detect a proportional difference in melatonin levels comparable to those seen in postmenopausal women with obesity, at a power of 0.8 and alpha of 0.05, we would need to observe a level of 1.74 pg/mL in our SARDS population compared with normal dogs resulting in a minimum sample size of 12 animals per group. This study was conducted with full institutional approval (Institutional Animal Care and Use Committee approval) and client written informed consent. Full details of recruitment and inclusion/exclusion criteria for the dogs used in this study are described in a previously published study.

**2.2 | Clinical evaluation**

All dogs underwent full physical and ophthalmic examination, electroretinography (ERG), spectral-domain optical coherence tomography (OCT), fasted CBC, serum biochemistry, urinalysis, and ACTH (cosyntropin) stimulation testing as previously described.

**2.3 | Sampling and analysis**

Voided first morning urine was collected by the owner (approximately 5-8 am) and samples were stored at −80°C until analysis of MT6s. Fasted blood samples were drawn (between 8 and 9 am) by venipuncture from the jugular vein into 8 mL glass Becton Dickinson Vacutainer Mononuclear Cell Preparation Tubes (CPT, Becton Dickinson Vacutainer, Fischer Scientific, Pittsburg, Pennsylvania) and 4 mL plastic serum blood collection tubes. The CPT tubes were centrifuged at 1500g for 20 minutes in a refrigerated centrifuge maintained at 20°C according to manufacturer’s recommendation. The serum blood collection tube samples were allowed to clot at room temperature for 30 minutes, with shielding from light, and were then centrifuged at 1500g for 10 minutes in a refrigerated centrifuge maintained at 20°C. Serum was collected, and stored at −80°C. Samples were not subject to freeze-thaw cycles before analysis.

Melatonin was extracted from plasma using manufacturer-supplied extraction columns (Sep-pak C18 Vac cartridges with a hydrophobic, reverse-phase, silica-based bonded phase: KEME761, IBL International, Hamburg, Germany) and an evaporator centrifuge, according to manufacturer’s instructions. Validated and commercially available human ELISA kits were used for all neurohormone assays (Table 1). Although these kits were not previously validated for use with canine serum or plasma, human and canine melatonin are predicted to be identical, and it was anticipated that assays developed for human samples would also be suitable for canine samples, as previously described for a melatonin radioimmunoassay. In accordance with recent recommendations for reporting of neurohormone assays in publications, we calculated inter and intra-assay coefficients of variation for canine samples, and present these data in comparison with manufacturer’s published data in Table 1. In addition, any values obtained that were less than the lowest standard were reported as that value and used in statistical analysis.

Urine creatinine (mg/mL) was quantified using a kinetic colorimetric assay (Jaffe method; Roche Cobas c501 Chemistry analyzer, Indianapolis, Indiana) validated for use in canine clinical samples.

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### 2.4 Statistical analysis

One-way ANOVA with Bonferroni posttest (to correct for type I error) was used to compare dogs with SARDS, normal dogs, and dogs with PDH for the following data sets: daylight duration at time of sampling, post-ACTH serum cortisol concentration, plasma melatonin concentration, serum serotonin concentration, plasma dopamine concentration, urine specific gravity (USG), creatinine, and MT6s:creatinine ratio. Because of the non-normal distribution of data (assessed using the D’Agostino and Pearson omnibus normality test), Spearman correlation analysis was completed to model the relationship between serum/plasma neurohormones and post-ACTH cortisol. Analysis was performed with computerized statistical software (GraphPad Prism version 5.0 for Mac, GraphPad Software, La Jolla, California). Data are presented as mean ± SD and significance was set at $P < .05$.

Receiver operator characteristic (ROC) analysis determines the sensitivity and specificity at different cutoff values for the evaluation of medical diagnostic tests. One index available from an ROC analysis, the area under the curve (AUC) measures the ability of a diagnostic test to discriminate between the “diseased” and “non-diseased.” The AUCs were used to analyze the diagnostic ability of plasma melatonin, serum serotonin, plasma dopamine, and urine MT6s:creatinine ratio between groups: SARDS versus normal dogs, SARDS versus PDH, and PDH versus normal dogs. The AUC is classified as follows: .9 to 1.0 = excellent, .80 to .89 = good, .70 to .79 = fair, .60 to .69 = poor, and .50 to .59 = worthless. The best cutoff values were established as the ones for which sensitivity and specificity were maximal (Youden-Index). The cutoff maximizing the Youden index minimizes the total number of misclassified patients (false positive and false negatives). ROC statistical analyses were performed using MedCalc version 14.12.0 (MedCalc Software bvba, Mariakerke, Belgium). The significance was set at $P < .05$.

### 3 RESULTS

#### 3.1 Clinical demographics and examination findings

Clinical demographics, examination, and laboratory findings for the 3 groups were previously described in detail. All enrolled dogs were included in the analysis. Briefly, 15 dogs with SARDS (8.04 ± 1.7 years) were enrolled and included in data analysis. The median number of days of vision loss at the time of examination was 18 (interquartile range 9 days). No retinal function was identified in any dog in the SARDS group at any electroretinographic (ERG) light intensity (dark- and light-adapted conditions), confirming the diagnosis of SARDS. The control group consisted of 14 normal dogs (8.91 ± 2.68 years; normal retinal function on ERG). The PDH group consisted of 13 dogs (12.0 ± 3.5 years; normal retinal function on ERG). Diagnosis of PDH was based on ACTH stimulation test results, abdominal ultrasound examination that demonstrated bilaterally symmetrically enlarged adrenal glands, endogenous ACTH concentrations and/or results of low-dose dexamethasone suppression testing. There was a significant difference in post-ACTH serum cortisol concentrations between groups (1-way ANOVA $P < .0001$). Bonferroni posttest showed that dogs with PDH had a significantly higher concentration of serum cortisol post-ACTH (30.9 ± 8.9 μg/dL) compared with both normal dogs (15.2 ± 2.6 μg/dL $P < .0001$) and dogs with SARDS (17.4 ± 4.1 μg/dL, $P < .0001$). There were no differences in post-ACTH serum cortisol concentrations between normal dogs and dogs with SARDS.

The season of recruitment was not controlled for during the enrollment for the study. Dogs with SARDS were recruited over a 1 year period (4/15 spring March-May, 2/15 summer June-August, 6/15 fall September-November, 3/15 winter; December-February). Dogs with PDH were recruited over a 1 year and 2 month period (7/13 spring, 3/13 summer, 2/13 fall, and 1/13 winter). Normal dogs were all recruited in either spring (9/14) or fall (5/14). There was a significant variation in daylight duration (source aa.usno.navy.mil) on the date of enrollment between groups using 1-way ANOVA ($P = .04$). Post hoc Bonferroni test identified that dogs with PDH were experiencing a significantly longer daylight duration (779 ± 80 minutes) compared with dogs with SARDS (696 ± 91 minutes, $P < .05$). There was no significant difference between normal dogs (741 ± 85 minutes) and dogs with either SARDS or PDH.

#### 3.2 Plasma melatonin and urine 6-sulfatoxymelatonin:creatinine ratio

When 1-way ANOVA was performed on data from all dogs, there were no significant differences in plasma melatonin concentration...
between dogs with SARDS (7.49 ± 4.88 pg/mL), normal dogs (8.04 ± 4.20 pg/mL), and dogs with PDH (16.4 ± 23.32 pg/mL, overall P = .17, Figure 1A).

Urine creatinine was significantly different between groups using 1-way ANOVA (P < .0001). Bonferroni posttest identified dogs with PDH had significantly lower urine creatinine (61.4 ± 43.7 mg/dl, P < .01) and normal dogs (220.5 ± 76.6 mg/dl, P < .001, Figure 1B), reflecting less concentrated urine in this group. Urine specific gravity was also significantly different between groups using 1-way ANOVA (P < .0001).

Bonferroni posttest identified that dogs with PDH had significantly lower USG (1.012 ± 0.007) compared to dogs with SARDS (1.031 ± 0.013; P < .001), and USG was also significantly lower in dogs with PDH compared to normal dogs (1.039 ± .008; P < .001). There was no significant difference in USG between dogs with SARDS and normal dogs.

Urine MT6s:creatinine ratio was significantly different between the 3 groups using 1-way ANOVA (P = .009). Bonferroni posttest identified that urine MT6s:creatinine ratio was significantly higher in dogs with PDH (4.08 ± 2.15 urine [MT6s] ng/mL per mg of urine creatinine) compared to dogs with SARDS (2.37 ± .51 urine [MT6s] ng/mL per mg of urine creatinine, P < .01, Figure 1C). There was no difference in the MT6s:creatinine ratio between dogs with either SARDS or PDH and normal dogs (3.11 ± 1.14 urine [MT6s] ng/mL per mg of urine creatinine, P > .05, Figure 1C). Based on AUC values, the urine MT6s:creatinine ratio had "good" diagnostic ability to discriminate between dogs with SARDS and dogs with PDH (AUC: .856, at >3.047 ng/mL per mg of urine creatinine sensitivity: 84.6%, specificity: 93.3%), but only had "fair" diagnostic ability to differentiate dogs with SARDS from normal dogs (AUC: .714, at <2.363 ng/mL per mg of urine creatinine sensitivity: 60.0%, specificity: 78.6%), and "poor" diagnostic ability to discriminate between normal dogs and dogs with PDH (AUC: .681, at >3.288 ng/mL per mg of urine creatinine sensitivity: 76.9%, specificity: 71.4%; Table 2).

Spearman correlation analysis determined that when including all groups together, post-ACTH serum cortisol and plasma melatonin were not significantly correlated (Spearman r = .19, P = .23). However, when groups were considered separately, post-ACTH serum cortisol and plasma melatonin were significantly positively correlated in dogs with PDH (r = .56, P = .05) but not in normal dogs (r = .02, P = .94) or dogs with SARDS or PDH.

**FIGURE 1** Vertical scatter plots showing plasma melatonin, urine creatinine, and urine 6-sulfatoxymelatonin concentrations. A. There were no significant differences in plasma melatonin concentrations between groups. B. Dogs with PDH had significantly lower urine creatinine concentrations than both of the other groups, reflecting a reduction in urine specific gravity. C. Dogs with PDH had significantly higher urine MT6s:creatinine ratios compared to dogs with SARDS. Each column in graphs represents mean value (horizontal line in the middle) ± SD. Lines connecting columns represent significantly different comparisons, 1-way ANOVA with Bonferroni posttest **P < .01, ***P < .001. MT6s, 6-sulfatoxymelatonin; PDH, pituitary-dependent hypercortisolism; SARDS, sudden acquired retinal degeneration syndrome

**TABLE 2** Area under the receiver operating characteristic curves (AUCs) and 95% confidence intervals (CIs) for each neurohormone parameter to discriminate between dogs with sudden acquired retinal degeneration syndrome (SARDS), normal dogs, and dogs with pituitary-dependent hypercortisolism (PDH)

| Variables | Units | AUC (95% CI) |
|-----------|-------|-------------|
| **Comparison 1: normal dogs versus SARDS** | | |
| Melatonin | pg/mL | .556 (.342-.772) |
| 6-sulfatoxymelatonin:creatinine ratio | ng/mL/mg | .714 (.520-.909) |
| Serotonin | ng/mL | .724 (.530-.918) |
| Dopamine | pg/mL | .538 (.325-.751) |
| **Comparison 2: SARDS versus PDH** | | |
| Melatonin | pg/mL | .656 (.450-.862) |
| 6-sulfatoxymelatonin:creatinine ratio | ng/mL/mg | .856 (.682-1.030) |
| Serotonin | ng/mL | .697 (.494-.901) |
| Dopamine | pg/mL | .513 (.288-.739) |
| **Comparison 3: normal dogs versus PDH** | | |
| Melatonin | pg/mL | .599 (.380-.818) |
| 6-sulfatoxymelatonin:creatinine ratio | ng/mL/mg | .681 (.463-.900) |
| Serotonin | ng/mL | .522 (.296-.748) |
| Dopamine | pg/mL | .529 (.309-.748) |
with SARDS ($R = -0.07, P = .80$). Spearman correlation analysis determined that when including all groups together, post-ACTH serum cortisol and urine MT6s:creatinine ratio were positively correlated (Spearman $r = .39, P = .0098$; Figure 2B). When groups were considered separately, post-ACTH serum cortisol and urine MT6s:creatinine ratio were not significantly correlated in dogs with PDH ($r = .50, P = .08$), in normal dogs ($r = .13, P = .65$) and dogs with SARDS ($r = .03, P = .92$).

### 3.3 Serum serotonin

There were no significant differences in the concentration of serum serotonin between dogs with SARDS (768.4 ± 242.9 ng/mL), normal dogs (525.2 ± 295.3 ng/mL), and dogs with PDH (581.8 ± 322.5 ng/mL, overall $P = .08$, Figure 3A). The original study sample size calculation was made based on canine plasma melatonin, therefore we performed a post hoc power analysis using our generated serotonin ELISA data from dogs with SARDS and controls, to form the basis for future studies. Based on the observed variation and effect size, an alpha of .05 and 80% power, a sample size of 16 animals per group would be needed to detect a significant difference in serum serotonin, indicating that the serotonin assay was underpowered to detect a significant difference. Serum serotonin values did not correlate significantly with post-ACTH cortisol in either dogs with SARDS or dogs with PDH (data not shown).

**FIGURE 2** X-Y scatter plot showing correlation between melatonin assays and post-ACTH cortisol concentrations. A, Linear regression analysis showed significant correlation between plasma melatonin and post-ACTH serum cortisol concentration. B, Linear regression analysis showed significant correlation between urine MT6s:creatinine ratio and post-ACTH serum cortisol concentration. MT6s, 6-sulfatoxymelatonin; PDH, pituitary-dependent hypercortisolism; SARDS, sudden acquired retinal degeneration syndrome.

**FIGURE 3** Vertical scatter plots showing serum serotonin concentrations. Each column in graphs represents mean value (horizontal line in the middle) ± SD. PDH, pituitary-dependent hypercortisolism; SARDS, sudden acquired retinal degeneration syndrome

**FIGURE 4** Vertical scatter plots showing plasma dopamine concentrations. There were no significant differences in the concentrations of dopamine in plasma between normal dogs, dogs with SARDS, and dogs with PDH. Each column in graphs represents mean value (horizontal line in the middle) ± SD. PDH, pituitary-dependent hypercortisolism; SARDS, sudden acquired retinal degeneration syndrome.
3.4 | Plasma dopamine

Using 1-way ANOVA, there were no significant differences in the concentration of dopamine in plasma between dogs with SARDS (5.27 ± 1.32 pg/mL), normal dogs (5.54 ± 1.82 pg/mL), and dogs with PDH (5.54 ± 1.56 pg/mL, P = .86, Figure 4). Plasma dopamine values did not correlate significantly with post-ACTH cortisol in either dogs with SARDS or dogs with PDH (data not shown).

4 | DISCUSSION

This study evaluated differences in melatonin, serotonin, and dopamine between dogs with SARDS, normal dogs, and dogs with PDH. Urine MT6s:creatinine ratio was higher in dogs with PDH compared to dogs with SARDS. Serum serotonin concentrations were higher in dogs with SARDS, although differences were not significant between the groups. No differences were noted in plasma dopamine concentrations among the 3 groups. The differences in neurohormone concentrations between groups found in this study suggest that future research is warranted in this area, and might also propose new directions for therapeutic strategies.

Our observation that plasma melatonin correlates with post-ACTH cortisol in dogs with PDH raises some interesting questions. The initial motivation was to study SARDS and utilize PDH as a comparison group because these 2 diseases have shared clinical features.1-3,35,45,46 It is likely that our results were affected by the season of recruitment, as daylight duration was significantly longer in dogs with PDH compared to dogs with SARDS. However, considering that melatonin in dogs peaks in winter (a season with shorter daylight duration),33 our results most likely represent a relative underestimate of the melatonin levels in dogs with PDH. Melatonin has been proposed as a therapeutic agent for atypical hypercortisolism in dogs. A study performed in dispersed cells and explants from primate adrenal glands showed that melatonin inhibited corticotropin (ACTH)-stimulated cortisol production, possibly through a melatonin receptor, MT1.47 Others showed in cell cultures of human and porcine, and murine models, exogenous serotonin induced vasoconstriction at the level of the caudal ocular segment.61-60 Furthermore, in pri-mates with atherosclerotic lesions, infusion of serotonin resulted in vasospasm of the central retinal artery and/or caudal ciliary artery61 and profoundly decreased retinal ERG responses.62 The potential for serotonin, platelet aggregation and ischemia as key players in the pathogenesis of SARDS warrants further investigation.

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CONFlict OF INTEREST DECLARATION

Authors declare no 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

Approval by the North Carolina State University IACUC, protocol number 15-132-O.
HUMAN ETHICS APPROVAL DECLARATION

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

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