Chronobiology of the renin-angiotensin-aldosterone system in dogs: relation to blood pressure and renal physiology

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The renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in the regulation of blood pressure and volume homeostasis. Its contribution to the development of cardiovascular diseases has long been recognized. Extensive literature has shown that peptides of the RAAS oscillate with a circadian periodicity in humans, under strong influence of posture, sleep, and age. Although observations of time-variant changes in the renin cascade are available in dogs, no detailed chronobiological investigation has been conducted so far. The present studies were designed to explore the circadian variations of plasma renin activity (RA) and urinary aldosterone-to-creatinine ratio (U A:C) in relation to blood pressure (BP), sodium (U Na, U Na,fe), and potassium (U K, U K,fe) renal handling. Data derived from intensive blood and urine sampling, as well as continuous BP monitoring, were collected throughout a 24-h time period, and analyzed by means of nonlinear mixed-effects models. Differences between the geometric means of day and night observations were compared by parametric statistics. Our results show that variables of the renin cascade, BP, and urinary electrolytes oscillate with significant day-night differences in dogs. An approximately 2-fold (1.6–3.2-fold) change between the average day and night measurements was found for RA (p = 0.001), U A:C (p = 0.01), U K,fe (p = 0.01), and U Na (p = 0.007). Circadian variations in BP, albeit small (less than 10 mm Hg), were statistically significant (p < 0.01) and supported by the model-based analysis. For all variables but U Na and U Na,fe, the levels were higher at night than during the day. The data also indicate that blood pressure oscillates in parallel to the RAAS, such that, as opposed to healthy humans, BP does not drop at night in dogs. The postprandial decrease in RA is assumed to be related to body fluid volume expansion secondary to water and sodium intake, whereas the reduction of U A:C reflects aldosterone-stimulated secretion by the renin-angiotensin II pathway. U Na and U Na,fe peaked in the afternoon, about 7–8 h after food intake, which is consistent with the “impulse-response pattern” of sodium excretion described in previous publications. Finally, U K and U K,fe mirrored aldosterone-mediated potassium secretion in the kidney tubules. To describe the circadian variations of the various variables, two different mathematical representations were applied. A cosine model with a fixed 24-h period was found to fit the periodic variations of RA, U A:C, U K, U K,fe, and BP well, whereas changes in U Na and U Na,fe were best characterized by a surge model. The use of nonlinear mixed effects allowed estimation of population characteristics that can influence the periodicity of the RAAS. Specifically, sodium intake was found to interact with the tonic and the phasic secretion of renin, suggesting that varying feeding time could also impact the chronobiology of the renin cascade. (Author correspondence: jonathan.mochel@novartis.com)

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INTRODUCTION

Within the mammalian kidney, the juxtaglomerular apparatus (JGA) consists of tubular and vascular structures whose role is critical in the regulation of renal blood flow and glomerular filtration rate (Thurau, 1966). As part of the JGA, the macula densa (MD) is an area of specialized cells that respond to changes in sodium chloride concentration in the tubular fluid by mediating...
renin release from the granular cells of the JGA. Renin release triggers a cascade of biochemical events, with the subsequent formation of angiotensin I, angiotensin II, and aldosterone. The so-called renin-angiotensin-aldosterone system (RAAS) plays a pivotal role in the control of blood pressure (BP) and volume homeostasis by regulating sodium and potassium exchanges in the kidney tubules. Its contribution to the development of hypertension and chronic heart failure has long been recognized (Kunita et al., 1976; Nicholls et al., 1993; Pedersen et al., 1995; Roche et al., 2002). Further, it has been shown that inhibition of the renin-angiotensin pathway is an effective approach to treat these disorders (Allikmets, 2007; Turnbull, 2003).

It has been established that RAAS peptides oscillate with a circadian periodicity in rodents and humans (Cugini et al., 1981, 1984, 1985, 1986, 1987; Kawasaki et al., 1990). Several physiological influences have been shown to influence day-night variations of the renin-angiotensin cascade, including alterations in posture (Muller et al., 1958), sleep cycles (Brandenberger et al., 1985, 1994), and age (Cugini et al., 1985).

Parallel to day-night changes in RAAS peptides, data from telemetry monitoring in humans have consistently demonstrated that BP has a reproducible circadian rhythm, with highest levels measured in the morning, and lowest values around midnight (Smolenski & Haus, 1994; Staessen et al., 1992).

Although observations of time-variant changes in RAAS peptides in dogs have been published (Corea et al., 1996; Gordon & Lavie, 1985; Reinhart et al., 1996), no detailed characterization of the chronobiology of these variables is currently available in the literature. In addition, the question of whether BP oscillates over the 24-h span in dogs is still a matter of debate (Miyazaki et al., 2002; Piccione et al., 2005, Soloviev et al., 2006).

Benefits of acquiring knowledge on the chronobiology of the RAAS and BP in dogs are twofold. First, it provides a scientific rationale to support further research on administration time-dependent efficacy of treatment in the management of RAAS-related diseases, such as chronic canine valvular heart disease. This condition is characterized by the thickening and shortening of the atrioventricular valves, and affects about 75% of dogs over the age of 16 (Guglielmini, 2003). Second, human and canine heart failure share common pathophysiological features. Specifically, activation of the renin cascade is one of the key neurohumoral responses to the reduced cardiac output observed in both species (Sayer et al., 2009; Watkins et al., 1976). Therefore, knowledge on the circadian periodicity of renin, in relation to BP and renal physiology in dogs, also constitutes valuable information to the understanding of RAAS biology in humans.

This investigation offers a comprehensive description of the chronobiology of the renin cascade in dogs, in relation to BP and renal sodium-potassium handling, using a nonlinear mixed-effects modeling approach (also referred to as population modeling).

**MATERIALS AND METHODS**

Sample collection for measurement of plasma and urinary variables and telemetry recordings were performed in two separate studies and distinct groups of animals, in order to preclude manipulation-related disturbances (e.g., venipuncture) on blood pressure (Baumgart, 1991).

**Animals**

The studies were performed in compliance with a registered Swiss permit covering animal experiments for cardiovascular research in dogs as approved by the Cantonal Animal Welfare Committee and the Veterinary Services. The study protocols were designed to use the fewest number of animals possible while being consistent with the scientific needs of the study, and conformed to international ethical standards (Portaluppi et al., 2010).

In the first experiment (Study a; Figure 1), blood and urinary samples for measurement of RA, urinary aldosterone, and renal sodium-potassium exchanges were taken from 18 adult (9 males and 9 females) healthy, non-neutered beagle dogs weighing between 12.0 and 18.0 kg (Marshall Europe, Green Hill, Montichiari, Italy).

In the second experiment (Study b; Figure 1), systolic (SBP) and diastolic (DBP) arterial blood pressures were continuously recorded from 6 adult healthy, non-neutered telemetered male beagle dogs weighing from 10.4 to 15.2 kg (Marshall Europe, Green Hill).

Suitability for inclusion was evaluated by a physical examination and confirmed by measuring selected hematological (red and white blood cells counts, hemoglobin [Hb], hematocrit [Hct]) and clinical chemistry (albumin, total protein, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine) parameters in blood. Telemetry signal quality and BP waveform analysis were used as additional criterion for telemetry dogs.

**Housing Conditions**

Prior to the experiments, the dogs were acclimatized to the experimental facility for a week. Animals were housed in pens (about 2 m²/animal) containing granulate bedding material and an additional elevated platform for resting. On the sampling days, dogs were housed in metabolism cages. Over many sessions, the dogs were trained to rest in metabolism cages for up to 12 h. Dogs from the telemetry study were group housed and pair housed on days of BP recording. The study rooms had natural daylight and additional artificial light of similar intensity (400 lux) from 07:00 to 19:00 h. Room temperature and relative humidity were within the target ranges of 17–23°C and 35–75%, respectively.
Drinking water quality was compliant with the Swiss Federal Regulation on Foodstuffs, and was offered ad libitum. One week before and throughout the studies, water and food intake were recorded on a daily basis. The dogs were offered a normal-sodium diet (Biomill Adult Medium, 0.5% sodium; Biomill, Herzogenbuchsee, Switzerland) once daily at 07:00 h. Depending on the size of the ration, the individual daily sodium intake ranged from 34 to 80 mEq. The amount of food given per dog was kept constant throughout the experiments.

**Experimental Procedure**

**Study a: Periodicity of the RAAS and Urinary Electrolytes in Dogs**

Blood specimens were collected every 2 h (starting from 07:00 h) into 1.2- or 2.7-mL S-Monovette tubes (Sarstedt, Newton, NC, USA). Due to the known sensitivity of the renin-angiotensin cascade to posture and external stimuli (Muller et al., 1958), specific precautions were taken: dogs were kept and maintained in the same position (up and standing) during blood collection, sampling was performed in a sound-protected room, and low-intensity lighting was used for night sampling.

Blood samples were cooled on ice immediately after withdrawal and centrifuged under refrigeration (2 ± 1 °C, 15 min) within 30 min of sampling. Plasma was then transferred into cooled propylene aliquots, snap-frozen, and stored at −80 °C before determination of RA. Urine samples were collected from the metabolism cage every 4 h (starting from 07:00 h) into cooled Erlenmeyer flasks and transferred into two distinct plain tubes, for determination of urinary (i) aldosterone (UA) (stored at −80 °C), (ii) sodium (UNa) (stored at 4 °C), and (iii) potassium (UK) (stored at 4 °C) concentrations. Urinary aldosterone-to-creatinine ratios (UA:Cr) were derived by measuring plasma and urinary creatinine, as proposed by Gardner et al. (2007). The glomerular filtration rate (GFR), as well as sodium and potassium fractional excretions (UNa,fe and UK,fe respectively), was determined using the renal clearance of creatinine\(^1\) as described by Lefebvre et al. (2008).

**Study b: Periodicity of Blood Pressure in Dogs**

The telemetry system consisted of an implantable transmitter (chronic use TL11M2-D70-PCT implant; Data Sciences International [DSI], St. Paul, MN, USA), cage receivers (RMC-1 general purpose receiver for metal cages; DSI), ambient pressure monitor (APR-1 ambient pressure reference; DSI), data exchange matrix (Dataquest ART data exchange matrix; DSI), and an electronic data acquisition system (DQ ART 2.1 Dataquest acquisition and analysis system; DSI). The transmitter contained a pressure sensor for BP measurement, an electric potential sensor for electrocardiogram (ECG) recording, and a thermometer for measuring core body temperature. The pressure sensor was connected to a fluid-filled urethane catheter inserted in the arteria femoralis with the tip placed in the arteria inguinalis. ECG leads were implanted intramuscularly in an Einthoven lead II configuration. The transmitted signal was captured by the cage receivers and transformed by the data exchange matrix. After surgery, the dogs were allowed to recover for 1 month.

The procedures were in compliance with the recommendations of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement regarding telemetry (Morton et al., 2003). Beginning around 07:00 h and ending about 24 h later, the electrocardiogram and arterial BP and core body temperature were recorded continuously. Data from every third quarter of an hour were analyzed and averaged to derive an hourly value.

\(^1\)Because of limited tubular secretion and reabsorption (Guignard & Drukker, 1999; O’Connell et al., 1962), creatinine clearance can be used to provide estimates of the glomerular filtration rate (GFR) in dogs.
**Analytical Methods**

Renin activity was determined by measuring the rate of angiotensin I (AI) formation after incubation of endogenous renin and angiotensinogen in plasma (2 h, 37 °C, pH 7.2). AI concentrations were measured after liquid-solid extraction using a validated enzyme immunoassay (EIA) (S-1188 Angiotensin I-EIA kit; host: rabbit high-sensitivity European Conformity (CE)-marked; Bachem, Bubendorf, Switzerland). Analyses were performed in duplicates; values with a coefficient of variance (CV%) below 25% were retained for statistical evaluation. Urinary aldosterone concentrations were determined with a liquid chromatography–tandem mass spectrometry method using an isotope dilution technique. Urine samples were not subjected to acid hydrolysis before extraction, that is, only nonconjugated aldosterone concentrations were measured. Calibration standards ranging from 0.05 to 10.0 ng/mL were used for quantification. Sodium and potassium concentrations were quantified using an ion-selective electrode measurement method (Olympus AU 400; Beckmann Coulter International, Nyon, Switzerland). A colorimetric Jaffé reaction was used for quantification of plasma and urinary creatinine concentrations.

**Data Analysis**

**Quantification of Day-Night Differences**

*Comparison of area under the curves (AUCs).* For rich data (i.e., RA, SBP, and DBP), the individual area under the curve of day (AUCs[07:00–19:00]) vs. night (AUCs[19:00–07:00]) observations were derived using the linear trapezoidal rule, and compared by paired t tests using R version 2.15 (The R Foundation for Statistical Computing, Vienna, Austria). The homogeneity of variances between day and night AUCs was assessed using the Bartlett’s test. Covariate search was performed using the stepwise covariate model building tool of Perl-speaks-NONMEM (Mats Karlsson, Niclas Jonsson, and Andrew Hooker, 2008) with forward inclusion based on p = 0.05 and afterwards backward exclusion based on p = 0.01. Analyzed covariates were sex, dietary sodium, and body weight.

**Chronobiological Analysis**

*Chi-square statistics for testing the zero-amplitude hypothesis.* For rhythm detection, a p value was derived from the difference in objective function value (OFV) between the fit of a straight line approximation of the mean (1 model parameter) and that of a cosine function (3 model parameters). In nonlinear mixed-effects modeling, the OFV is approximated to minus 2 times the logarithm of the likelihood of the data given the model, with a lower value indicating a better model (Sheiner & Ludden, 1992). It is used to draw statistical inference about the goodness-of-fit of the mathematical models. A negative OFV would indicate that the likelihood is greater than 1.

The difference in OFV between two models follows an asymptotic chi-square distribution, with degrees of freedom equal to the difference in the number of parameters between contending models. A periodic rhythm was considered as statistically significant for a drop in OFV ≥ 5.9 for a risk level α = 0.05.

**Nonlinear mixed-effects (population) modeling.** Nychtemeral variations in RA, BP, and urinary variables were characterized by means of a nonlinear mixed-effects modeling approach, using NONMEM version 7.2 (Icon Development Solutions, Ellicott City, MD, USA). Individual model parameters were obtained post hoc as empirical Bayes estimates.

Nonlinear mixed-effects models were written using the following format (Equation 1):

\[
y_{ij} = f(\phi_i, t_{ij}) + g(\phi_i, t_{ij}, \beta) \cdot \varepsilon_{ij}, \quad j = 1, \ldots, n_i
\]

\[
\phi_i = \mu \cdot \exp^{\alpha}, \quad i = 1, \ldots, N
\]

where \(y_{ij}\) is the observed variable (e.g., RA, U_{x,C}) measured on the \(i\)th individual at time \(t_{ij}\), \(\phi_i\) is the vector of individual parameters, \(f(\phi_i, t_{ij})\) is the value of that observed variable at time \(t_{ij}\) for an individual with parameters \(\phi_i\), and \(\varepsilon_{ij}\) is an independent random variable. The function \(g(\phi_i, t_{ij}, \beta)\) is the standard deviation of the error of a given measurement at time \(t_{ij}\), \(\mu\) is the population average of a model parameter. The sources of variation between the individual parameters \(\phi_i\) can be explained by population characteristics (i.e., covariates) that can be included additionally or proportionally to \(\mu\).

The independent random variables \(\eta_i\) represent the unexplained difference between the value of the individual parameters \(\phi_i\) and their average \(\mu\). Random variables \(\varepsilon_{ij} \sim \eta_{ij}\) were assumed to be log-normally distributed.

The first-order conditional estimation method with interaction was used for all analyses. Covariate search was performed using the stepwise covariate model building tool of Perl-speaks-NONMEM (Mats Karlsson, Niclas Jonsson, and Andrew Hooker, 2008) with forward inclusion based on p = 0.05 and afterwards backward exclusion based on p = 0.01. Analyzed covariates were sex, dietary sodium, and body weight.

Standard goodness-of-fit diagnostics, including population and individual predictions vs. observations, and the distributions of weighted residuals over time, were performed to assess the adequacy of selected
models. Graphical assessment was performed using the R-based software Xpose version 4.1 (Jonsson & Karlsson, 1999) in R version 2.15.

Model selection was based on statistical significance between competing models using the objective function value obtained from NONMEM, graphical evaluation, and validity of parameter estimates. Residual error estimates from the population models were used as supportive information for evaluation of lack of fit. The normality and independence of residuals were evaluated using the Shapiro-Wilk and Durbin-Watson tests, respectively.

Correlations Between signals
Phase relationships between observed variables were investigated using a circular version of the Pearson’s product moment correlation in R version 2.15 (Agostinelli & Lund, 2011; R package “circular”: Circular Statistics).

Differences between the average phases of blood pressure, plasma, and urinary variables were characterized by introducing a phase shift in the population models. Chi-square statistics were used to decide on the statistical significance of the drop in OFV when including this additional model parameter.

p values were adjusted for multiple comparisons using the Sidak procedure, and were reported for $\alpha = 0.05$.

RESULTS
Study a: Periodicity of the RAAS and Urinary Electrolytes in Dogs
Quantification of Day-Night Differences
For RA, $U_{A:C}$, $U_K$, $U_{K,fe}$, $U_{Na}$, and $U_{Na,fe}$, the p values derived from the Bartlett’s test were greater than the chosen alpha level of 0.05. Therefore, the null hypothesis that the variances of day and night measurements were identical could not be rejected.

As presented in Figures 2 and 3, in dogs fed early in the morning RA, $U_{A:C}$, $U_K$, and $U_{K,fe}$ oscillated with circadian changes over the 24-h span. Values were low in the morning, rose through the afternoon, and peaked in the evening (around 21:00, 19:00 and 19:00 h for RA, respectively).

![Figure 2](image-url)

**FIGURE 2.** Circadian changes in renin activity and urinary aldosterone-to-creatinine ratio in dogs. (Top) Geometric mean plasma renin activity (pg/mL/h) (left panel) and urinary aldosterone-to-creatinine ratio (pg/mg) (right panel) vs. time profile in dogs fed a normal-sodium diet (0.5% sodium) at 07:00 h ($n = 18$). Vertical bars indicate 1 standard error of the geometric mean. The data show that RA and $U_{A:C}$ oscillate with circadian changes over the 24-h span. Measurements were low in the morning, rose through the afternoon, and peaked in the evening (around 21:00, 19:00 and 19:00 h for RA, respectively), with an apparently shorter time to return to baseline for $U_{A:C}$ levels compared with RA. (Bottom) Area under the curve of day (AUC$_{07:00-19:00}$) vs. night (AUC$_{19:00-07:00}$) renin activity (left panel) and urinary aldosterone-to-creatinine ratio (right panel) in dogs fed a normal-sodium diet (0.5% sodium) at 07:00 h ($n = 18$). Vertical bars indicate 1 standard error of the geometric mean. Significant differences are indicated by double asterisks (**). Night measurements were on average 2.0 and 3.2 times significantly greater than day measurements for RA ($p<0.001$) and $U_{A:C}$ ($p = 0.01$), respectively.

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For rhythm detection, a p value was derived from the difference in OFV between the fit of a straight line approximation of the mean and that of a cosine function. A periodic rhythm was considered as statistically significant for a drop in OFV of 5.9 (for a risk level of \( \alpha = 0.05 \)). Model estimates of the amplitudes can be found in Table 2.

\( U_{AC} \) and \( U_{K,fe} \), respectively, with an apparently shorter time to return to baseline for \( U_{AC} \) and \( U_{K,fe} \) levels compared with RA. Night measurements were on average 2.0 and 3.2 times significantly greater than day measurements for RA (\( p < 0.001 \)) and \( U_{AC} \) (\( p = 0.01 \)), respectively (Figure 2).

\( U_K \) and \( U_{K,fe} \) oscillated in parallel, showing similar dynamics to \( U_{AC} \) and a peak around 19:00 h (Figure 3). Day \( U_K \) and \( U_{K,fe} \) values were on average 1.2 (\( p > 0.05 \)) and 1.6 (\( p = 0.01 \)) times smaller than night measurements. In contrast, \( U_{Na} \) and \( U_{Na,fe} \) levels were high from morning to the middle of the afternoon (peak around 15:00 h), and decreased from the latter half of the afternoon to the early morning (Figure 3). \( U_{Na} \) and \( U_{Na,fe} \) values were respectively 1.9 (\( p = 0.007 \)) and 1.3 (\( p = 0.05 \)) times higher during daytime. Unlike sodium, day \( U_{K,fe} \) and \( U_K \) values were on average 1.6 (\( p = 0.001 \)) and 1.2 (\( p > 0.05 \)) times smaller than night measurements.

**Chi-Square Statistics for Testing the Zero-Amplitude Hypothesis**

The cosinor fit of the data was statistically significant (\( p < 0.001 \)) for RA, \( U_{AC} \), \( U_K \), \( U_{K,fe} \), \( U_{Na} \), and \( U_{Na,fe} \), supporting the hypothesis of time-varying dynamics with a 24-h period (Table 1). In contrast, no circadian
Nonlinear Mixed-Effects Modeling

A cosine model (Figure 4) was found to fit the periodic nature of plasma renin activity, aldosterone, and potassium urinary elimination well, as shown by the standard goodness-of-fit diagnostics, and the individual predictions in Figures 5 and 6.

The selected structural model was written as follows (Equation 2):

$$f(t_{ij}) = M_i \cdot \left(1 + A_i \cdot \cos \left(t_{ij} - \psi_i \cdot \frac{2\pi}{\tau_i}\right)\right)$$

where $f(t_{ij})$ is the predicted RA, $U_{AC}$, $U_K$, or $U_{K,fe}$ value at time $t_{ij}$, $M_i$ is the mesor (daily average of rhythm) for the $i$th individual, $A_i$ is the amplitude of the cosine, $\psi_i$ is the acrophase (or time of peak), and $\tau_i$ is the fixed 24-h period of the cosine for that individual.

The residual error model combined an additive and a proportional error term. Estimates of residual errors (CV%) from the population models were 30%, 80%, 18%, and 30% for RA, $U_{AC}$, $U_K$, and $U_{K,fe}$, respectively. The $p$ values derived from the Shapiro-Wilk and Durbin-Watson tests were greater than the chosen alpha level of 0.05. Hence, the null hypothesis that the residuals were independent and came from a normally distributed population could not be rejected.

Changes in $U_{Na}$ and $U_{Na,fe}$ were best characterized by means of a surge function, reflecting an afternoon peak sodium excretion followed by a monotonous decay, rather than periodic oscillations around the clock.

A proportional error model was used to account for the residual noise in the measurement of $U_{Na}$ and $U_{Na,fe}$. Estimates of residual errors (CV%) from the population models were 20% and 30% for $U_{Na}$ and $U_{Na,fe}$, respectively. The $p$ values derived from the Shapiro-Wilk and Durbin-Watson tests were greater than the chosen alpha level of 0.05. Therefore, the null hypothesis that the residuals were independent and came from a normally distributed population could not be rejected.
Standard goodness-of-fit diagnostics and individual predictions can be found in Figures 5 and 6.

Population parameter estimates, relative standard errors (RSEs), and 90% confidence intervals (CIs) are listed in Table 2. The precision of the final model parameters was considered satisfactory (RSE<30%). Data from the various variables were fitted simultaneously, leveraging the richness of the plasma data (RA) to derive the acrophase of the (more sparse) urinary variables. The model estimated a 3-h delay between the acrophase of RA and that of UA:C (respectively 26%, 24%, and 21% of the mesor/baseline value).

Correlations

Statistics confirmed the positive correlation between the phase of UA:C and that of UK,fe (r=0.3, p=0.009). The correlation between plasma renin activity and the renal elimination of aldosterone was also supported by the Pearson’s product moment correlation test (r=0.2, p=0.03). Finally, a positive correlation was found between the phase of UK,fe and that of UNa,fe (r=0.4, p<0.001). Results are summarized in Table 3.

Influence of Population Characteristics on the Periodicity of RAAS-Related Variables

Dietary sodium intake and sex were found to be significant covariates to explain part of the between-subject variability in RA, UA:C, and urinary electrolytes (Table 4).

Results from the covariate analysis indicate that sodium intake (which was proportional to the size of the ration) had a significant effect on the mesor of UA:C (p<0.001), RA (p<0.001), UK,fe (p<0.001), the baseline of UNa,fe (p<0.001), and the amplitude of RA (p<0.001) and UNa,fe (p<0.001). The effect of sodium intake on the periodicity of RA is further illustrated in Figure 7, using predictions from the population model. Specifically, the mesor RA of a typical 13-kg healthy female dog fed around 190 g of dry food per day (i.e., 34 mEq Na/24 h) would be 140 pg/mL/h, with an amplitude of 27 pg/mL/h. Increasing the portion size from 190 to 440 g (i.e., 80 mEq Na/24 h) per day would result in a decrease of both
the mesor (from 140 to 100 pg/mL/h) and the amplitude (from 27 to 12.8 pg/mL/h) of RA oscillations.

Sex had a significant impact on the amplitude of RA ($p < 0.001$), $U_{K,fe}$ ($p < 0.01$), and $U_{Na,fe}$ ($p < 0.001$). An almost 5-fold difference in the amplitude of RA was observed between male and female dogs (Figure 7). Finally, body weight had limited influence on the periodicity of the RAAS, with a statistically significant effect on the amplitude of RA only ($p = 0.01$).

Study b: Periodicity of Blood Pressure in Dogs

DBP and SBP oscillated parallel to RA throughout the observation span (Figure 8). Accordingly, DBP and SBP were found to be highly correlated, as indicated by the significance of the Pearson’s product moment correlation test ($r = 0.8$, $p < 0.001$) (Table 3).

$AUC_{[07:00–19:00]}$ were on average 6.5% and 7% smaller than $AUC_{[19:00–07:00]}$ for DBP ($p = 0.007$) and SBP ($p < 0.001$), respectively. This corresponds to an average nocturnal increase of 5 mm Hg for DBP and 9 mm Hg for SBP. The $p$ values derived from the Bartlett’s test were greater than the chosen alpha level of 0.05, indicating similar variances between day and night $AUC$s for SBP and DBP.

Concurrently, the model fit of blood pressure data led to the rejection of the hypothesis that SBP and DBP measurements ($p = 0.01$) were constant over time (Table 1). Similar to RA, a cosine model with a fixed 24-h period was found to describe the periodic nature of DBP and SBP well, as shown by the quality of the population and individual fits (Figures 5 and 6). The selected structural model was the same as indicated in Equation 2, but with $f(t_{ij})$ being the predicted DBP or SBP value at time $t_{ij}$.

Based on the drop in OFV between competing models, the average phase of blood pressure measurements was found to be statistically different from that of plasma and urinary variables ($p < 0.04$, $p = 0.002$, and $p < 0.001$ for RA, $U_{A,C}$ and $U_{K,fe}$, and $U_{Na,fe}$, respectively).

Noise in DBP and SBP measurements was modeled using a proportional error term. Estimates of residual errors (CV%) from the population models were 7% and 10% for DBP and SBP, respectively. Based on the results of the Shapiro-Wilk and Durbin-Watson tests, the null hypothesis that the residuals were independent and came from a normally distributed population could not be rejected.

Body weight and dietary sodium were not found to have an effect on BP model parameters. Population parameter estimates, RSEs, and 90% CIs can be found in Table 2. The precision of the final model parameters appeared highly satisfactory (RSE < 20%).
DISCUSSION

Various authors have amply reviewed the time-varying changes of the renin-angiotensin cascade in humans. In contrast, little is known about the chronobiology of RAAS peptides in dogs. Gordon & Lavie (1985) described nocturnal increases in renin activity with concomitant peaks in urine osmolality and potassium excretion in four adult, female mongrel dogs fed a normal-sodium diet. More work on the circadian variations of BP in dogs has been published. Most of these investigations failed to demonstrate daily rhythmicity in BP (Miyazaki et al., 2002; Soloviev et al., 2006), although Piccione et al. (2005) disclosed strong evidence that BP and heart rate oscillated around the clock.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Point estimate (unit)</th>
<th>RSE</th>
<th>90% CI</th>
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<tbody>
<tr>
<td>Renin activity (RA)</td>
<td></td>
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<tr>
<td>Mesor</td>
<td>96 (pg/mL/h)</td>
<td>12%</td>
<td>(76–116)</td>
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<tr>
<td>Amplitude</td>
<td>25 (pg/mL/h)</td>
<td>20%</td>
<td>(17–34)</td>
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<tr>
<td>Acrophase</td>
<td>22:40 (h)</td>
<td>9%</td>
<td>(20:40–23:50)</td>
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<td>Urinary aldosterone-to-creatinine ratio (U_{A:C})</td>
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<tr>
<td>Mesor</td>
<td>20 (pg/mg)</td>
<td>19%</td>
<td>(14–26)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>11.5 (pg/mg)</td>
<td>28%</td>
<td>(6.0–17.0)</td>
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<tr>
<td>Acrophase</td>
<td>19:40 (h)</td>
<td>9%</td>
<td>(18:10–20:20)</td>
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<tr>
<td>Potassium fractional excretion (U_{K,fe})</td>
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<td></td>
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<tr>
<td>Mesor</td>
<td>12.8 (%)</td>
<td>4%</td>
<td>(11.9–13.7)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>2.7 (%)</td>
<td>17%</td>
<td>(1.9–3.5)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>19:40 (h)</td>
<td>9%</td>
<td>(18:10–20:20)</td>
</tr>
<tr>
<td>Urinary potassium concentration (U_{K})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>88 (mmol/L)</td>
<td>11%</td>
<td>(72–104)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>21 (mmol/L)</td>
<td>26%</td>
<td>(12–30)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>19:40 (h)</td>
<td>9%</td>
<td>(18:10–20:20)</td>
</tr>
<tr>
<td>Sodium fractional excretion (U_{Na,fe})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.20 (%)</td>
<td>9%</td>
<td>(0.17–0.23)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.27 (%)</td>
<td>27%</td>
<td>(0.15–0.39)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>14:30 (h)</td>
<td>4%</td>
<td>(13:30–15:30)</td>
</tr>
<tr>
<td>Width</td>
<td>4:00 (h)</td>
<td>16%</td>
<td>(2:54–5:04)</td>
</tr>
<tr>
<td>Urinary sodium concentration (U_{Na})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>49 (mmol/L)</td>
<td>14%</td>
<td>(37–61)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>88 (mmol/L)</td>
<td>15%</td>
<td>(66–110)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>14:30 (h)</td>
<td>4%</td>
<td>(13:30–15:30)</td>
</tr>
<tr>
<td>Width</td>
<td>4:00 (h)</td>
<td>16%</td>
<td>(2:54–5:04)</td>
</tr>
<tr>
<td>Systolic BP (SBP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>141 (mm Hg)</td>
<td>6%</td>
<td>(127–155)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>7.0 (mm Hg)</td>
<td>8%</td>
<td>(6.0–8.0)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>23:30 (h)</td>
<td>4%</td>
<td>(22:43–00:17)</td>
</tr>
<tr>
<td>Width</td>
<td>4:00 (h)</td>
<td>16%</td>
<td>(2:54–5:04)</td>
</tr>
<tr>
<td>Diastolic BP (DBP)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>82 (mm Hg)</td>
<td>3%</td>
<td>(78–86)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>5.0 (mm Hg)</td>
<td>16%</td>
<td>(3.6–6.4)</td>
</tr>
<tr>
<td>Acrophase</td>
<td>23:30 (h)</td>
<td>4%</td>
<td>(22:43–00:17)</td>
</tr>
</tbody>
</table>

<table>
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<tr>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>$U_{A:C}$</td>
<td>$U_{K,fe}$</td>
</tr>
<tr>
<td>RA</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>$U_{A:C}$</td>
<td>$r = 0.2$ ($p = 0.03$)</td>
<td>1</td>
</tr>
<tr>
<td>$U_{K,fe}$</td>
<td>NS</td>
<td>$r = 0.3$ ($p = 0.009$)</td>
</tr>
<tr>
<td>$U_{Na,fe}$</td>
<td>NS</td>
<td>NS</td>
</tr>
<tr>
<td>SBP</td>
<td>DBP</td>
<td></td>
</tr>
<tr>
<td>DBP</td>
<td>$r = 0.8$ ($p &lt; 0.001$)</td>
<td>1</td>
</tr>
</tbody>
</table>

NS = nonsignificant $p$ value; 1 = diagonal elements of the correlation table.

| TABLE 4. Summary information on the effect of dietary sodium and sex on the mesor/baseline and the amplitude of RA, $U_{A:C}$, as well as potassium and sodium renal excretion ($U_{K,fe}$ and $U_{Na,fe}$). |
|--------------------------|----------------|----------------|
| (A) Effect of dietary Na on model parameters | (B) Effect of sex on model parameters |
| Low to high Na intake ratio | Male-to-female ratio |
| Mesor or baseline | 1.4** | 3.8** | 0.8** | 1.9** |
| Amplitude | 2.1** | 1 | 1 | 0.25** |
| (A) Model parameter (M, B, A) ratio of low (34 mEq Na) to high (80 mEq Na) daily sodium intake. (B) Model parameter (M, B, A) ratio of male to female dogs. Note that body weight had limited influence on the periodicity of the RAAS. The covariate search was performed using the stepwise covariate model building tool of Perl-speaks-NONMEM. ** $p < 0.01$. |

has been published. Most of these investigations failed to demonstrate daily rhythmicity in BP (Miyazaki et al., 2002; Soloviev et al., 2006), although Piccione et al. (2005) disclosed strong evidence that BP and heart rate oscillated around the clock.
The present studies offer a comprehensive characterization of the chronobiology of the renin cascade in dogs in relation to BP and renal sodium-potassium exchanges, using a nonlinear mixed-effects modeling approach. The core value of population modeling lies in its ability to separate the (between- and within-subject) variability from the measurement error (noise), in order to determine population characteristics (i.e., covariates) that are able to explain the sources of variation between individuals.

Telemetry recordings were performed in a distinct cohort of animals to preclude manipulation-related disturbances, such as venipuncture, on BP. Extensive literature has demonstrated the influence of environmental factors (e.g., noise, stress) on BP in humans (Attarchi et al., 2012; Baumgart, 1991; Lindquist et al., 1997; Rocha et al., 2002). The relation of stress to elevated heart rate and BP in dogs is well known, as most recently confirmed by Höglund et al. (2012). In this observational study, both SBP and DBP increased when owners left dogs in the examination room while recordings were made by the veterinarian alone.

The RAAS and Urinary Electrolytes Exhibit a Clear Circadian Periodicity in Dogs

Our data document a clear circadian rhythmicity of RA, UA:C,U Na,U Na,fe, U K, and U K,fe in trained and relaxed healthy dogs, under standardized conditions. Our results are consistent with previous investigations in dogs (Corea et al., 1996), horses (Clarke et al., 1978, 1988), and humans (Cugini et al., 1981, 1985), which underlines the similarity of blood fluid homeostasis between mammalian species. The endogenous clearance of creatinine, used as estimate of the GFR, showed no periodic rhythmicity, which is consistent with other publications in dogs (e.g., Uechi et al., 1994).

In accordance with the usual feeding pattern in domesticated dogs, the animals in these studies were fed once daily at 07:00h. The effect of feeding time on the periodicity of the renin cascade remains unclear, as illustrated by the conflicting results between Kunita et al. (1976) and Ikonomov et al. (1981). Kunita et al. have reported significant differences in the periodicity of renin and aldosterone when meals were taken at night instead of the usual times of the day in five healthy volunteers. These results dispute data from Ikonomov et al. showing that diurnal changes in food intake do not affect the rhythmicity of renin and sodium excretion. Investigations in horses (Clarke et al., 1978, 1988) and sheep (Blair-West & Brook, 1969) have led to the conclusion that episodic feeding caused substantial variations of the RAAS, as opposed to continuous feeding. To the authors’ knowledge, no information is currently available in dogs.

**FIGURE 7.** Effect of sex and dietary sodium on the periodicity of renin activity in dogs. Predictions based on the covariate analysis. (Top) Renin activity (pg/mL/h) predictions from the population model for a male (left panel) and a female (right panel) dog with similar daily sodium intake (80 mEq Na), and similar body weight (13 kg). (Bottom) Renin activity (pg/mL/h) predictions from the population model in a 13-kg female dog fed a normal-sodium diet of increasing size, with daily sodium intake ranging from 34 to 80 mEq Na. As also shown in Table 4, results of the covariate analysis indicate that sex has a significant effect on the amplitude of RA, whereas sodium intake also has an influence on the mesor of RA oscillations (i.e., the greater the amount of dietary sodium, the smaller the mesor and the amplitude of RA).
A cosine model with a fixed 24-h period was found to fit the periodic variations of RA, UA:C, UK, and UK,fe well, as suggested by the quality of the standard goodness-of-fit diagnostics and the individual predictions. In contrast, circadian changes in U Na and U Na,fe were best characterized by means of a surge model, as described by Nagaraja et al. (2003), reflecting an afternoon peak sodium excretion followed by a monotonous decay, rather than periodic oscillations around the clock.

RA and UA:C measurements were low in the morning, rose through the afternoon, and peaked in the evening. The morning decrease in RA is assumed to be related to body fluid volume expansion secondary to water and sodium intake, referred to as “postprandial decrease of RA” in previous publications (Kaczmarczyk et al., 1980; Seeliger et al., 1999). The similitude of RA and UA:C signals, supported by the Pearson’s product moment correlation test, reflects aldosterone-stimulated secretion by the renin-angiotensin II pathway.

In contrast, U Na and U Na,fe were high from morning to the middle of the afternoon, and peaked around 15:00 h. This phenomenon has been termed “impulse-response pattern” of sodium excretion (Boemke et al., 1995), and is characterized by a peak urinary elimination 4–8 h after meal ingestion. During the evening and night, U Na was maintained on very low levels. The decrease in U Na,fe, together with constant GFR, suggests that tubular, rather than glomerular, events were primarily involved in the reduced elimination of sodium during night hours. This assumption is supported by the concomitant activation of known sodium-conserving mediators (i.e., UA:C) acting on the kidney tubules.

Variations in UK and UK,fe mirror aldosterone-mediated excretion of potassium in the distal tubules. Tubular transport is indeed the main modality of potassium exchange in the kidneys, whereas sodium reabsorption also occurs in the proximal kidney tubules, independently of aldosterone. This divergence is further supported by the positive correlation between UA:C and UK,fe, and the nonsignificant correlation between UA:C and U Na,fe.

**Dietary Sodium Influences the Tonic and the Phasic Secretion of Renin**

The relation of elevated RA levels to increased renin secretion has been established by Schricker et al. (1994). Specialized cells of the MD act as a sensing device monitoring changes in sodium chloride concentrations, so that granular cells of the JGA can adjust their net
secretion of renin to maintain an appropriate RA level (Laragh & Sealey, 2011). Herein, results from the covariate analysis show that dietary sodium interacts with the renin cascade, not only by influencing the tonic (i.e., mesor), but also the phasic (i.e., amplitude) secretion of renin (i.e., the greater the amount of sodium intake, the smaller the mesor and the amplitude of RA). Note that sex was also found to be a significant source of variability, with a 4.5-fold difference between the amplitude of male and female dogs.

The urinary aldosterone-to-creatine ratio has been validated as a reproducible measure of 24-h urinary aldosterone excretion in dogs (Gardner et al., 2007). U_{A:C} reflects aldosterone production over several to many hours, eliminating minute-to-minute variation, as seen with plasma aldosterone concentrations. From the covariate analysis, sodium intake had a significant effect on the mesor, but not on the amplitude of U_{A:C} oscillations, indicating that dietary sodium influences the tonic, but possibly not the phasic, secretion of aldosterone. This should, however, be interpreted with caution given the relatively small changes in dietary sodium investigated in our experiment.

Blood Pressure and Renin Activity Display Similar Fluctuations Around the Clock

The levels of DBP and SBP observed in this experiment were in agreement with previous investigations in dogs (Mishina & Watanabe, 2008; Miyazaki et al., 2002), and reference values reported in humans (Baumgart, 1991). Further, the high level of correlation between DBP and SBP was in the same order of magnitude as reported by Gavish et al. (2008) in ambulatory patients.

Circadian variations in DBP and SBP, albeit small (5 and 9 mm Hg, respectively), were statistically significant and supported by the model-based analysis. Blood pressure increased during the first half of the night, before returning to baseline in the early morning, thereby showing similar fluctuations to RA around the clock. Renin has been shown to play a pivotal role in BP regulation. In a study by Passo et al. (1971), increases in renin secretion were associated with a substantial rise in BP in 16 dogs. The main contribution of the RAAS to BP regulation is mediated by the sodium-retaining effects of aldosterone, and the powerful vasoconstrictor effect of angiotensin II. The role of RAAS activation in the development of hypertension in dogs has been elucidated in a renal failure model by Mishina & Watanabe (2008). In their study, RA, angiotensin II, and aldosterone were significantly elevated in association with a noticeable increase in BP, indicating that the RAAS was involved in the development of nephron loss-associated hypertension.

The nocturnal increase in BP observed in our experiment supports earlier findings from Piccione et al. (2005) in 10 healthy beagle dogs. These results are in contradiction with investigations in human healthy volunteers, where lower BP levels have been reported during periods of darkness allocated to sleeping (Fernández et al., 2009). In humans, Smyth et al. (1969) have shown that sleep is characterized by signs of parasympathetic predominance, with a noticeable reduction of heart rate and BP. According to Trinder et al. (2001), sleep displays a strong ultradian rhythm characterized by the regular occurrence of two fundamental states, alternating with a ca. 100-min period: the non-rapid (NREM) and the rapid (REM) eye movement sleep. NREM is associated with low BP and bradycardia, whereas REM is characterized by a substantial increase in heart rate and BP. Later investigations from Murali et al. (2003) have shown that sympathetic activity was increased to levels above awaking values during REM sleep.

Although dogs have a natural tendency to be crepuscular, they can easily become diurnal through human interactions. In essence, domesticated dogs are mainly active during the day, while sleeping at night. Similar to humans, REM sleep has been described in dogs (Hendricks & Morrison, 1981; Hendricks et al., 1989). In theory, nocturnal increases of BP could be a sign of REM predominance in this species. Differences in posture (dogs were monitored whilst freely moving allowing for different body positions, and not maintained recumbent as in human studies), may also explain part of the observed difference in BP dynamics (Mulier et al., 1958). In addition, the activation of sodium-conserving mechanisms (i.e., elevated U_{A:C}) may have been a prerequisite to increase the levels of SBP and DBP at night.

It has been argued that increased sodium excretion is preceded by an elevation of BP (Coleman & Guyton, 1969; Hall et al., 1980). This mechanism, referred to as "pressure natriuresis," would represent a powerful means of stabilizing BP via reduction of the extracellular fluid volume (Hall, 1986). Herein, an elevation of U_{Na} and U_{Na,fe} during daytime has been reported in the absence of increasing BP. Likewise, a reduction of sodium elimination was observed at night in spite of an increase in BP. These observations are entirely consistent with those of other studies in dogs (Andersen et al., 2000; Bie & Sandgaard, 2000; Sandgaard et al., 2000), indicating that the RAAS controls sodium homeostasis even when opposing changes in BP occur. According to Bie & Damkjaer (2010), in order for the pressure natriuresis mechanism to be involved, BP must change in response to varying sodium intake. This has not been reported in intact animals (Kjolby et al., 2005, 2008), as shown by the nonsignificant effect of dietary sodium on BP in our experiment.

Body weight and sodium intake were not found to have an effect on BP model parameters. However, the effect of sex on the chronobiology of BP could not be assessed in Study b, since only male dogs were included in the experiment.
**Tuning in to the Body’s Rhythms to Adapt Drug Dosing Schedules**

Deeper understanding of circadian rhythms can have a substantial impact on the therapeutic management of RAAS-related diseases by determining the time of drug administration that would optimize efficacy while minimizing the occurrence of adverse effects. This concept, referred to as chronotherapy, is currently being used for the treatment of human rheumatoid arthritis (Staessen et al., 1992), lung cancer (Mazzoccoli et al., 2012), and cardiovascular diseases (Nicholls et al., 1993). An increasing number of investigations on the use of angiotensin-converting enzyme inhibitors (ACEIs) in hypertension have shown a greater reduction of BP with bedtime administration as compared with morning dosing (Hermida & Ayala, 2009; Palatini et al., 1993). Sole & Martino (2009) have demonstrated that heart and vessel growth and remodeling were dynamic and occurred more actively during the period normally allocated to sleep. In mice, administrations of the ACEI captopril at sleeping hours significantly improved cardiovascular function and reduced adverse remodeling, whereas no effects were reported when the drug was given during active hours of the day (Martino et al., 2011). In a study by Nozawa et al. (2006), temocapril (another ACEI) prolonged the survival of spontaneously hypertensive rats, with a maximum effect after dosing during the resting period, and a minimum effect after dosing at the active period. The authors concluded that treatment with an ACEI at night may be a more effective dosing regimen in patients with hypertension.

Another therapeutic approach in the management of heart failure and hypertension is to continuously assess not only the medical response, but also the development of adverse effects. The optimal treatment time can vary considerably between patients, as shown by the recent work of Watanabe et al. (2013) in hypertensive patients under losartan/hydrochlorothiazide (L/H) (angiotensin II receptor blocker/thiazide diuretics) combination therapy. In their study, L/H taken few hours before bedtime in a 61-year-old man induced circadian hyperamplitude tension (CHAT), a condition associated with an increased cardiovascular disease risk. For yet another patient, CHAT was exacerbated when L/H was given during the day, but was alleviated when the same dose of treatment was taken in the evening. In all instances, optimization of therapy based on the most appropriate time of drug administration should be investigated on an individual basis (Cornelissen & Halberg, 2009).

**SUMMARY AND PERSPECTIVES**

This research offers the first chronobiological characterization of the RAAS in relation to BP and renal sodium-potassium handling in dogs. Our data demonstrate that BP oscillates in parallel to the RAAS, which is coherent with existing publications in dogs, but reflects a clear (and not elucidated) difference from humans. Cosine and surge models were able to describe and predict the time-variant changes of the experimental data with high accuracy, as shown by the quality of the standard goodness-of-fit diagnostics.

The use of nonlinear mixed-effects allowed borrowing information from the densely sampled plasma values (RA) to improve knowledge on the time variations of the urinary variables. The model-based approach provided insights into the relation of dietary sodium, sex, and body weight to renin and aldosterone secretion, which would have been impossible using standard statistical approaches. Specifically, sodium intake appears to be an important determinant of RA chronobiology, and we are currently investigating the influence of varying feeding time on the chronobiology of the RAAS. This would support earlier findings from Itoh et al. (1996) who showed that the effect of dietary sodium on BP depends on the circadian timing of its intake in humans.

Recent experiments in human patients have pointed out the importance of administration time-dependent effects of treatment in the management of RAAS-related diseases. These results suggest that additional investigations on the chronobiology of the RAAS and BP are required in diseased dogs under ACEI to determine whether it is possible to improve drug therapy of heart failure or hypertension by selecting the appropriate time of treatment.

**ACKNOWLEDGMENTS**

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**DECLARATION OF INTEREST**

With the exception of Pr. Meindert Danhof, the authors of this paper are Novartis employees. The experiments were supported by Novartis Animal Health, Basel, Switzerland.

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

**REFERENCES**


