Influence of feeding schedules on the chronobiology of renin activity, urinary electrolytes and blood pressure in dogs*

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The contribution of the renin–angiotensin–aldosterone system (RAAS) to the development of congestive heart failure (CHF) and hypertension (HT) has long been recognized. Medications that are commonly used in the course of CHF and HT are most often given with morning food for the sake of convenience and therapeutic compliance. However, biological rhythms and their responsiveness to environmental clues such as food intake may noticeably impact the effectiveness of drugs used in the management of cardiovascular disorders. Only sparse information about the effect of feeding schedules on the biology of the RAAS and blood pressure (BP) is presently available. Two studies were designed to explore the chronobiology of renin activity (RA), BP, renal sodium (U_{Na,fe}) and potassium (U_{K,fe}) handling in relation to meal timing in dogs. In a first experiment (Study a), blood and urinary samples for measurement of RA, U_{Na,fe} and U_{K,fe} were drawn from 18 healthy beagle dogs fed a normal-sodium diet at either 07:00, 13:00 or 19:00 h. In a second experiment (Study b), BP was recorded continuously from six healthy, telemetered beagle dogs fed a similar diet at 07:00, or 19:00 h. Data were collected throughout 24-h time periods, and analyzed by means of nonlinear mixed-effects models. Differences between the geometric means of early versus late time after feeding observations were further compared using parametric statistics. In agreement with our previous investigations, the results indicate that RA, U_{Na,fe}, U_{K,fe}, systolic, and diastolic BP oscillate with a circadian periodicity in dogs fed a regular diet at 07:00 h. A cosine model with a fixed 24-h period was found to fit the variations of RA, U_{Na,fe} and BP well, whereas cyclic changes in U_{Na,fe} were best characterized by means of a combined cosine and surge model, reflecting a postprandial sodium excretion followed by a monotonous decay. Our data show that feeding time has a marked influence on the chronobiology of the renin cascade, urinary electrolytes, and BP. Introducing a 6- or 12-h delay in the dogs’ feeding schedule caused a shift of similar magnitude (05:06 and 12:32 h for Studies a and b, respectively) in the rhythm of these biomarkers. In all study groups, RA and BP exhibited a marked fall just after food intake. The drop in RA is consistent with sodium and water-induced body fluid expansion, while the reduction of BP could be related to the decreased activity of renin and the secretion of vasodilatory gut peptides. An approximately 1.5-fold (1.2–1.6-fold) change between the average early and late time after feeding observations was found for RA (p < 0.0001), U_{Na,fe} (p < 0.01) and U_{K,fe} (p < 0.05). Postprandial variations in BP, albeit small (ca. 10 mmHg), were statistically significant (p < 0.01) and supported by the model-based analysis.

In conclusion, the timing of food intake appears to be pivotal to the circadian organization of the renin cascade and BP. This synchronizing effect could be mediated by feeding-related signals, such as dietary sodium, capable of entraining circadian oscillators downstream of the master, light–dark-adjusted pacemaker in the suprachiasmatic nucleus.

Abbreviations: ACE, angiotensin-converting enzyme; AUC, area under the curve; AUC_{sat}, AUC for early time after feeding observations (first 12 h after food intake); AUC_{TAF}, AUC for late time after feeding observations (12–24 h after food intake); BP, blood pressure; CI, confidence interval; CVHD, chronic valvular heart disease; CWRES, conditional weighted residuals; d, day; DBP, diastolic blood pressure; EIA, enzyme immunoassay; OFV, objective function value; RA, renin activity; RAAS, renin–angiotensin–aldosterone system; RSE, relative standard error; SBP, systolic blood pressure; SCN, suprachiasmatic nucleus; U_{K,fe}, potassium fractional excretion; U_{Na,fe}, sodium fractional excretion

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INTRODUCTION

The circadian system is organized in a hierarchical scheme, in which a master pacemaker in the suprachiasmatic nucleus (SCN) regulates downstream oscillators in peripheral tissues and organs (Ko & Takahashi, 2006). This complex machinery ensures that biological activities, from gene expression to cellular and physiological manifestations, occur in the right sequence, and at the right time of day (Reppert & Weaver, 2002). Core clock components are defined as genes whose protein products are essential to the genesis and regulation of circadian rhythms within individual cells throughout the organism (Takahashi, 2004). Within the SCN, clock genes, such as Clock (circadian locomotor output cycles kaput) and Arntl (aryl hydrocarbon receptor nuclear translocator-like, also known as Bmal1) are responsible for the genesis and persistence of circadian rhythms (Dunlap, 1999).

In mammals, the circadian clock influences many physiological and behavioral variables including locomotor activity, body temperature, and heart rate (Reppert & Weaver, 2002; Van Esseveldt et al., 2000). Blood pressure (BP) and biomarkers of the renin–angiotensin–aldosterone system (RAAS) have been shown to oscillate with a circadian periodicity in humans (Cugini et al., 1981, 1984, 1985, 1986, 1987; Kawasaki et al., 1990), and more recently in dogs (Mochel et al., 2013). Our previous results showed that variables of the renin–angiotensin system and BP oscillate with significant day–night differences in dogs. For all endpoints but sodium elimination the levels were found to be higher at night than during the day. Our data further indicated that systolic and diastolic BP oscillate in parallel to the RAAS, such that, as opposed to healthy humans BP does not drop at night in dogs. Lastly, sodium intake was found to interact with the tonic and the phasic secretion of renin, suggesting that varying feeding schedules could also impact the chronobiology of the renin cascade.

The effect of food on the periodicity of the renin–angiotensin system has already received wide attention across species. Studies have been performed under (i) sodium restriction (Cugini et al., 1981, 1985), (ii) episodic versus continuous feeding (Blair-West & Brook, 1969; Clarke et al., 1978) and (iii) fasting conditions (Cugini et al., 1987). However, investigations on the influence of feeding time on the periodicity of the RAAS have led to conflicting results. In a study by Ikonomov et al. (1981), diurnal changes in food intake did not affect the rhythmicity of renin and sodium excretion. These results deviate from earlier findings by Kunita et al. (1976) in healthy volunteers where circadian changes in renin and aldosterone disappeared when meals were taken at night instead of the usual times of the day. In addition, experiments on the influence of meal timing on the chronobiology of BP revealed that reapportionment of daily food intake compared to ad libitum feeding could lead to a peak shift in rabbits and rats (Van den Buuse & Malpas, 1997), or to suppression of the rhythmicity (Van den Buuse, 1999). Few telemetry studies in dogs have suggested that food intake was accompanied by a rapid drop in BP and heart rate (Mishina et al., 1999; Miyazaki et al., 2002).

Accumulating knowledge on the chronobiology of the RAAS and BP provides a strong scientific rationale for determining the optimum dosing time, thereby making the best usage of available cardioactive molecules. Canine and human heart diseases share common neurohumoral features, such as activation of renin (Sayer et al., 2009; Watkins et al., 1976). Therefore, compiling data on the chronobiology of the renin–angiotensin system in dogs contributes to a better understanding of cardiovascular physiology in humans. This investigation offers a comprehensive description of the periodicity of renin activity (RA), BP, renal sodium and potassium handling in relation to feeding time in dogs, using a nonlinear mixed-effects modeling approach.

MATERIALS AND METHODS

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 Novartis 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), blood and urinary samples for measurement of RA, renal sodium and potassium exchanges were taken from 18 healthy adult (9 males and 9 females), non-neutered, 50–54 months
old beagle dogs weighing between 11.2 and 17.8 kg (Marshall Europe, Green Hill, Montichiari, Italy).

The relation of stress to elevated heart rate and BP is well known (Baumgart, 1991; Höglund et al., 2012). Therefore, sample collection for measurement of plasma and urinary variables, and telemetry recordings were performed in separate studies and groups of animals to preclude manipulation-related disturbances (e.g. venipuncture) on BP.

In the second experiment (Study b), systolic (SBP) and diastolic (DBP) arterial BP were continuously recorded from six telemetered male beagle dogs weighing from 10.5 to 15.0 kg (Marshall Europe, Green Hill, Montichiari, Italy).

Suitability for inclusion was evaluated by a physical examination and confirmed by the analysis of diverse hematological (red and white blood cells counts, Hb, Hct) and clinical chemistry (albumin, total protein, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, creatinine) parameters. The quality of telemetry signals was used as additional inclusion criterion in Study b.

**Housing conditions**

Prior to entering the experiment, dogs were offered a regular diet once daily between 07:00 and 09:00 h (Biomill Adult Medium, 0.5% sodium, Biomill SA, Herzogenbuchsee, Switzerland). They were then acclimatized to the experimental facility for 6 d (from D5 to D0; Figure 1), where they received a similar diet at 07:00 h. Housing conditions were similar to those described by Mochel et al. (2013). In brief, 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 from Study a 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 (Study b) were pair housed on days of BP monitoring. 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 Foodstuff, and was offered ad libitum. One week before and throughout the studies, water and food intake were recorded on a daily basis. Depending on the size of the ration, the individual daily sodium intake ranged from 42 to 80 mEq. The amount of food given per dog was kept constant throughout the experiments.

**Experimental procedure**

**Study a: Influence of feeding time on the periodicity of renin and urinary electrolytes**

Dogs were allocated at random to three study groups according to feeding time: (i) dogs fed at 07:00 h (Gr. 1, n: 6), (ii) dogs fed at 13:00 h (Gr. 2, n: 6) and (iii) dogs fed at 19:00 h (Gr. 3, n: 6), respecting a homogeneous distribution of bodyweight and sex between groups.
at 19:00 h (Gr. 3, n: 6) for 5 d (from D1 to D5, Figure 1), respecting a homogeneous distribution of bodyweight and sex between groups. Blood and urine samples were drawn on D5. The selection of the time period between the shift in meal timing and blood/urine collection was based on internal (undisclosed) data showing that it takes 4–5 d for the renin cascade to adapt to a change in feeding onset in order to reach a new steady state level.

Blood specimens were collected from the vena jugularis every 2 h (starting from 07:00 h) into 1.2 or 2.7 mL S-Monovette tubes (Sarstedt Inc., Newton, NC). Due to the known sensitivity of the renin-angiotensin cascade to posture and external stimuli (Muller et al., 1958), specific precautions were taken: (i) dogs were kept and maintained in the same position (up and standing) during blood collection, (ii) sampling was performed in a sound-protected room and (iii) 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 tubes, snap-frozen and stored at −80 °C. Urine samples were collected from the metabolism cage every 4 h (starting from 07:00 h) into cooled Erlenmeyer flasks and transferred into plain tubes, for the determination of (i) sodium (UNa), and (ii) potassium (UK) urinary concentrations (stored at 4 °C). Sodium and potassium fractional excretions (UNa,fe and UK,fe, respectively), were determined using the renal clearance of creatinine as described by Lefebvre et al. (2008).

Study b: Influence of feeding time on the chronobiology of BP

Dogs were allocated at random to two study groups according to feeding time: (i) dogs fed at 07:00 h (Gr. 1, n: 3), and (ii) dogs fed at 19:00 h (Gr. 2, n: 3) for 5 d (from D1 to D5, Figure 1), respecting a homogeneous distribution of bodyweight between groups. Telemetry recordings were performed on D5. 12 h apart feeding schedules (07:00 vs. 19:00 h) could be investigated due to the limited availability of telemetry-equipped dogs within our laboratory. The telemetry system consisted of: (i) an implantable transmitter (Chronic Use TL11M2-D70-PCT Implant, Data Sciences International, St. Paul, MN; DSI), (ii) cage receivers (RMC-1 General Purpose Receiver for Metal Cages, DSI), (iii) an ambient pressure monitor (APR-1 Ambient Pressure Reference, DSI), (iv) a data exchange matrix (Dataquest ART Data Exchange Matrix, DSI) and (v) an electronic data acquisition system (DQ ART 2.1 Dataquest Acquisition & 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. Transmitted signals were captured by the cage receivers and transformed by the data exchange matrix. Dogs recovered at least 1 month after surgery.

Beginning around 07:00 h and ending about 24 h later, ECG, arterial BP and body temperature signals were recorded continuously. Data from every third-quarter of an hour were analyzed and averaged to derive an hourly value.

The procedures described herein were in compliance with the recommendations of the BVAAWF/FRAME/RSPCA/UFAW Joint Working Group on Refinement regarding telemetry (Morton et al., 2003).

Analytical methods

RA 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) test (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 CV% below 25% were retained for statistical evaluation. Sodium and potassium concentrations were quantified using an ion selective electrode measurement method (Olympus AU 400; Beckmann Coulter International SA, Nyon, Switzerland). A colorimetric Jaffe reaction was used for quantitation of plasma and urinary creatinine concentrations.

Data analysis

Quantification of early versus late time after feeding differences

Comparison of AUCs. For rich data (RA, SBP and DBP), the individual area under the curve derived from early (AUCeTAF: first 12 h after food intake) versus late (AUClTAF: 12–24 h after food intake) time after feeding observations was estimated using the linear trapezoidal rule, and compared by paired t-tests using R version 2.15.1 (The R Foundation for Statistical Computing, Vienna, Austria). Homogeneity of variances between area under the curves (AUCs) was determined using the Bartlett’s test. p Values were adjusted for multiple comparisons using the Sidak procedure, and were reported for α: 0.05.

Comparison of urinary spot samples. For sparse urinary data (UNa,fe and UK,fe), spot samples drawn at early (+2, 4 and 6 h) and late (+14, 16 and 18 h) time after feeding onset were compared by paired t-tests. The Bartlett’s test was used for evaluating homoscedasticity between groups. p Values were adjusted for multiple comparisons using the Sidak correction, and were reported for α: 0.05.
**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 (3–5 model parameters). In nonlinear mixed-effects modeling of the OFV is derived as minus two times the logarithm of the likelihood of the data given the model, with a lower value indicating a better model (Sheiner & Ludden, 1992). This metric is used to draw statistical inference about the goodness-of-fit of the mathematical models, a negative OFV indicating that the likelihood is greater than 1.

The difference in OFV between two contending models follows an asymptotic chi-square distribution with degrees of freedom equal to the difference in the number of parameters between two models. A periodic rhythm was considered to be statistically significant for a drop in OFV superior to 5.9 for RA, $U_{K,fe}$, SBP and DBP (+2 parameters), and 9.4 for $U_{Na,fe}$ (+4 parameters), for a risk level $\alpha: 0.05$.

**Nonlinear mixed-effects (population) modeling.** Periodic variations of RA, BP and urinary variables were characterized by means of a nonlinear mixed-effects modeling approach, using the first order conditional estimation method with interaction of NONMEM version 7.2 (Icon Development Solutions, Ellicott City, MD). Individual model parameters were obtained post-hoc as empirical Bayes estimates.

Similar to Mochel et al. (2013), mathematical models were written using the following format (Equation (1)):

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

$$\phi_i = \mu \cdot \exp^{\eta_i}, \quad j = 1, \ldots, N,$$

where $y_{ij}$ is the observed variable (e.g. RA) 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 $t_{ij}$. In population modeling $f(\phi_i, t_{ij})$ is known as the structural model (error-free), while $g(\phi_i, t_{ij}, \beta)$ is the residual error model (combining unexplained variability and measurement noise). $\mu$ represents the typical value (population average) of a model parameter. The sources of variation between the individual parameters $\phi_i$ can be further explained by population characteristics (i.e. covariates) that can be included additively 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 $\mu$. The random variables $y_{ij}, \varepsilon_{ij}$ and $\phi_i$ were assumed to be normally distributed, while $\eta_i$ was log-normal distributed.

Search of population covariates was performed using the stepwise covariate model building tool of Perl-speaks-NONMEM (Lindbom et al., 2004) with forward inclusion based on $p: 0.05$ and afterwards backward exclusion based on $p: 0.01$. Analyzed covariates were: amount of dietary sodium, and sex.

Standard goodness-of-fit diagnostics, including population and individual predictions versus 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 2.15.1. Model selection was based on statistical significance between competing models using the OFV obtained from NONMEM, graphical evaluation and validity of parameter estimates. Residual error estimates from the mathematical models were used as supportive information for evaluation of lack of fit.

**RESULTS**

Quantification of early versus late time after feeding differences

**Study a: RA and urinary electrolytes**

As presented in Figure 2, RA, $U_{K,fe}$ and $U_{Na,fe}$ oscillated with circadian changes over the 24-h span in dogs fed a regular diet at different time instances.

In dogs fed at 07:00 h (Gr. 1), RA and $U_{K,fe}$ values were low in the morning (trough at 11:00 h), rose during the afternoon and peaked in the evening (around 21:00 and 19:00 h for RA and $U_{K,fe}$, respectively). In contrast, $U_{Na,fe}$ levels were high from morning to the middle of the afternoon, reaching a peak at 15:00 h, while decreasing from the latter half of the afternoon to the early morning.

For each of these variables, shifting the time of food intake by 6 and 12 h led to a phase shift of similar magnitude, with a peak delay of approximately 4–6 h, and 10–12 h for Gr. 2 and Gr. 3, respectively. Specifically, introducing a 12-h delay in feeding time (Gr. 3) produced an almost mirror biomarker profile of dogs fed in the early morning (Gr. 1). As a result, when data from the various feeding groups were pooled together and plotted against time after food intake in lieu of clock-time, RA, $U_{K,fe}$ and $U_{Na,fe}$ oscillated with a 24-h period (Figure 3). The relatively small variability (expressed as standard error of the mean) portrayed in Figure 3 is another indicator of the synchronization of RA and urinary electrolytes to feeding time. These findings were further supported by the statistical comparison of early versus late time after feeding observations. For RA, AUC$_{S_{TAF}}$ were on average 1.5 times greater than AUC$_{S_{TAF}}$ ($p<0.0001$), while an approximately 1.6- and 1.2-fold difference was found between early and late time after feeding measurements for $U_{Na,fe}$ ($p<0.01$) and $U_{K,fe}$ ($p<0.05$), respectively (Figure 3). Note that the $p$ values derived
In contrast, urinary sodium (middle pane) and potassium (right pane) fractional excretion (% change from baseline) in dogs fed a normal-sodium diet (0.5% sodium) at 07:00 h (continuous line) and 13:00 h (dashed line). (Bottom) Average plasma RA (left pane), urinary sodium (middle pane) and potassium (right pane) fractional excretion (% change from baseline) in dogs fed a normal-sodium diet (0.5% sodium) at 07:00 h (continuous line) and 19:00 h (dashed line). In dogs fed at 07:00 h (Gr. 1), RA and \( K_{fe} \) values were low in the morning (tough at 11:00 h), rose during the afternoon and peaked in the evening (around 21:00 and 19:00 h for RA and \( K_{fe} \), respectively). In contrast, \( U_{Na,fe} \) levels were high from morning to the middle of the afternoon, reaching a peak at 15:00 h, while decreasing from the latter half of the afternoon to the early morning. For each of these variables, shifting the time of food intake by 6 and 12 h led to a phase shift of similar magnitude, with a peak delay of approximately 4–6 h, and 10–12 h for Gr. 2 and Gr. 3, respectively.

**Study b: systolic and diastolic BP**

In all feeding regimes, systolic and diastolic BP oscillated in parallel throughout the observation span, with a substantial drop just after food intake (Figure 4). Similar to renin and urinary electrolytes, introducing a time-delay in the dogs feeding schedule triggered a shift in the rhythm of BP, such that variations of SBP and DBP in dogs fed in the morning (Gr. 1) were almost symmetric to those of individuals fed in the evening (Gr. 2). The amplitude of BP oscillations appeared to be greater in dogs fed at 19:00 h, compared to dogs fed in the early morning. Cyclic fluctuations of BP after food intake were further unveiled when pooling data from the two feeding groups (Figure 5). In doing so, a second dip in BP appeared approximately 12 h after food intake. AUCs\(_{STAF} \) for SBP \((p<0.001)\) and DBP \((p<0.01)\), respectively.

The \( p \) values derived from the Bartlett’s test were greater than the chosen \( \alpha \) level of 0.05, hence the null hypothesis that the variances of early and late measurements after feeding were identical could not be rejected.

**Chronobiological analysis**

**Chi-square statistics for testing the zero-amplitude hypothesis**

The cosinor fit of the data was statistically significant \((p<0.001)\) for RA, \( U_{Na,fe} \), \( U_{Na,fe} \), SBP and DBP, supporting the hypothesis of a periodic rhythmicity regardless of the feeding schedule (Table 1).

**Nonlinear mixed-effects (population) modeling**

A cosine model was found to fit the periodic nature of RA, potassium urinary excretion and BP, as shown by the standard goodness-of-fit diagnostics and the individual predictions in Figures 6 and 7.
The selected structural model was written as follows (Equation (2)):

$$f(t_{ij}) = M_i + A_i \cdot \left( t_{ij} - \psi_i - (k_i - 1) \cdot \Delta_i \cdot \frac{2\pi}{T_i} \right), \quad (2)$$

where $f(t_{ij})$ is the predicted RA, $U_{K,fe}$, SBP or DBP 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 $T_i$ is the fixed 24-h period of the cosine for that individual. A phase shift $\Delta_i$ for the $k$th feeding group ($k = 1, 2$ or $3$) was introduced in the model to quantify the effect of feeding time on the periodicity of $f(t_{ij})$.

A proportional error model was used to account for the residual noise in the measurement of RA, $U_{K,fe}$, SBP and DBP. Estimates of residual errors (CV%) from the mathematical models were 40%, 26%, 7% and 7% for RA, $U_{K,fe}$, SBP and DBP, 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. A combined cosine and surge model was found to describe best the cyclic variations of urinary sodium excretion.

The structural model was written as follows (Equations (3)–(5)):

$$g(t_{ij}) = \left( \cos \left( t_{ij} - \psi_{i1} - (k_i - 1) \cdot \Delta_i \cdot \frac{2\pi}{T_i} \right) + 1 \right) \cdot w_i, \quad (3)$$

$$h(t_{ij}) = \cos \left( t_{ij} - \psi_{i2} - (k_i - 1) \cdot \Delta_i \cdot \frac{2\pi}{T_i} \right), \quad (4)$$

$$l(t_{ij}) = B_i + A_i \cdot \left( g_{t_{ij}} + h_{t_{ij}} \right), \quad (5)$$

where $l(t_{ij})$ is the predicted $U_{Na,fe}$ level at time $t_{ij}$, $B_i$ is the baseline for the $i$th individual, $A_i$ is the amplitude of the rhythm, $\psi_{i2}$ is the acrophase of the surge function, $\psi_{i2}$ is the acrophase of the cosine, $T_i$ is the fixed 24-h period, and $w_i$ represents the width of the surge function for that individual. Similar to (2) a phase shift $\Delta_i$ for the $k$th feeding group ($k = 1, 2$ or $3$) was introduced in the model to quantify the effect of feeding
time on the periodicity of \( l(t_j) \). Standard goodness-of-fit diagnostics and individual predictions can be found in Figures 6 and 7.

The error model combined an additive and a proportional error term and estimated a residual error (CV\%) of 40%. 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.

Data from the various variables were fitted simultaneously, leveraging the richness of the plasma data (e.g. RA) to derive the acrophase of the (more sparse) urinary variables. Population parameter estimates, relative standard errors (RSEs), and 90% confidence intervals (CIs) are listed in Table 2. The precision of the final models parameters was considered satisfactory (RSE < 25%). The model estimated an approximately 4-h delay between the acrophase of RA and that of \( U_{K,fe} \), \( U_{Na,fe} \) oscillated with a relatively larger amplitude (50% of the baseline) than \( U_{K,fe} \), RA, SBP and DBP (20%, 16%, 3% and 9% of the mesor, respectively).

As reported in Table 3, including a phase shift (\( \Delta \beta \)) in the mathematical models (Equations (2)–(5)) led to a significant drop in OFV, indicating that feeding time had a substantial effect on the acrophase of RA, \( U_{K,fe} \), \( U_{Na,fe} \), SBP and DBP. In contrast, the introduction of an additional model parameter to account for the apparent greater amplitude of BP oscillations in dogs fed at 19:00 h did not reduce significantly the OFV, possibly due to a lack of statistical power related to the small sample size. The effect of varying feeding schedules on the periodicity of RA, \( U_{K,fe} \), \( U_{Na,fe} \) and BP can be appreciated in Figure 8, using predictions from the mathematical models. Estimates of mean value and standard error were finally used to compute the 95% CI of the phase shift parameter. This interval comprised the nominal value of 06:00 h (04:20–06:12) and 12:00 h (10:06–14:36) for Study a and Study b, respectively (Table 2). Hence, the hypothesis that the periodicity of RA, \( U_{K,fe} \), \( U_{Na,fe} \), SBP and DBP was delayed as much as the shift in feeding time could not be rejected (for an \( \alpha \) risk level of 0.05).

Effect of dietary sodium and sex on the periodicity of the renin cascade and BP

Results from the covariate analysis showed that the amount of sodium intake had a significant influence on the mesor and the amplitude of renin oscillations \( (p<0.001) \). Specifically, the mesor of RA for an average 15 kg healthy male dog fed around 380 g of dry food per day (i.e. 70 mEq Na/24 h) would be 75 pg/mL/h, with an
amplitude of 27 pg/mL/h. Increasing the portion size from 380 to 440 g (i.e. 80 mEq Na/24 h) per day would result in a decrease of both the mesor (from 75 to 40 pg/mL/h) and the amplitude (from 27 to 8 pg/mL/h) of RA.

Sex also had a significant effect on the amplitude of RA \( (p < 0.001) \). Similar to Mochel et al. (2013), an almost 4-fold increase in the amplitude of renin oscillations was observed in male compared to female beagle dogs.

In contrast, gender and dietary sodium were not found to have a significant influence on urinary electrolytes.

Sodium intake was not a significant covariate for BP model parameters either.

**DISCUSSION**

The renin–angiotensin–aldosterone system plays a pivotal role in the control of BP and fluid homeostasis by regulating sodium and potassium exchanges in the kidney tubules. Its contribution to the development of hypertension (HT) and congestive heart failure (CHF) has long been recognized (Kunita et al., 1976; Nicholls et al., 1993; Pedersen et al., 1995; Roche et al., 2002). Results from our previous research (Mochel et al., 2013) have shown that variables of the renin cascade and BP oscillate with a 24-h periodicity in dogs fed a regular diet at 07:00 h. In this earlier study, dietary sodium was found to have an influence on the chronobiology of RA and urinary aldosterone, indicating that varying feeding schedules could also impact the chronobiology of the renin cascade. Several authors have amply reviewed the effect of fasting (Cugini et al., 1987) and sodium restriction (Cugini et al., 1981, 1985; Van den Buuse, 1999; Van den Buuse & Malpas, 1997) on the chronobiology of the renin cascade and BP. However, the influence of meal timing has been little investigated experimentally. The present studies offer a...
FIGURE 6. Standard goodness-of-fit diagnostics. Standard goodness-of-fit diagnostics. Scatter plot of population (top panel) and individual predictions (middle panel) versus observations (log scale), and conditional weighted residuals (bottom panel, CWRES) of population predictions. From left to right: RA, $U_{\text{K,fe}}$, $U_{\text{Na,fe}}$, DBP and SBP. Solid black line: identity line. Dashed line: regression line. For CWRES, the x-axis represents time after feeding onset. Note: Population predictions are estimates of the average (plasma or urinary) concentration, or enzyme activity. A suitable model has the following features: (i) the line of identity is aligned with the regression line (for both individual and population predictions), while (ii) the residues (differences between observations and predictions) are centered on a mean value of 0, with (iii) a homogeneous dispersion around the mean.
FIGURE 7. Individual prediction time-course profiles based on individual parameter estimates (obtained as empirical Bayes estimates). Scatter plot of observed (open circles, log scale) and predicted (continuous line) individual data versus time after feeding onset (hour). Dashed line: population predictions. Out of clarity only a subset of three individuals per endpoint are represented herein (one per row). From left to right: RA, $U_{\text{K,fe}}$, $U_{\text{Na,fe}}$, DBP and SBP. Information sharing from RA is essential for modeling of the sparse urinary data.
TABLE 2. Parameter estimates of the mathematical models, RSEs of the mean, and 90% CIs.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Point estimate (unit)</th>
<th>RSE (%)</th>
<th>90% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RA</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>100 pg/mL/h</td>
<td>13%</td>
<td>78–121</td>
</tr>
<tr>
<td>Amplitude</td>
<td>16 pg/mL/h</td>
<td>16%</td>
<td>11.8–20.2</td>
</tr>
<tr>
<td>Acrophase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>24:30 h</td>
<td>5%</td>
<td>24:03–24:57</td>
</tr>
<tr>
<td>Phase shift</td>
<td>05:06 h</td>
<td>11%</td>
<td>04:27–06:06a</td>
</tr>
<tr>
<td>Potassium fractional excretion (U_{K,fe})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>12.6%</td>
<td>4%</td>
<td>11.7–13.4</td>
</tr>
<tr>
<td>Amplitude</td>
<td>2.5%</td>
<td>21%</td>
<td>1.6–3.3</td>
</tr>
<tr>
<td>Acrophase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>20:00 h</td>
<td>10%</td>
<td>18:46–21:14</td>
</tr>
<tr>
<td>Phase shift</td>
<td>05:06 h</td>
<td>11%</td>
<td>04:27–06:06a</td>
</tr>
<tr>
<td>Sodium fractional excretion (U_{Na,fe})</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>0.30%</td>
<td>7%</td>
<td>0.26–0.33</td>
</tr>
<tr>
<td>Amplitude</td>
<td>0.15%</td>
<td>18%</td>
<td>0.11–0.19</td>
</tr>
<tr>
<td>Acrophase of surge</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>08:44 h</td>
<td>16%</td>
<td>07:44–09:44</td>
</tr>
<tr>
<td>Phase shift</td>
<td>05:06 h</td>
<td>11%</td>
<td>04:27–06:06a</td>
</tr>
<tr>
<td>Width of surge</td>
<td>5:30 h</td>
<td>22%</td>
<td>03:30–07:30</td>
</tr>
<tr>
<td>Acrophase of cosine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>16:58 h</td>
<td>14%</td>
<td>15:58–17:58</td>
</tr>
<tr>
<td>Phase shift</td>
<td>05:06 h</td>
<td>11%</td>
<td>04:27–06:06a</td>
</tr>
<tr>
<td><strong>SBP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>141 mmHg</td>
<td>7%</td>
<td>125–157</td>
</tr>
<tr>
<td>Amplitude</td>
<td>4 mmHg</td>
<td>9%</td>
<td>3.4–4.6</td>
</tr>
<tr>
<td>Acrophase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>22:20 h</td>
<td>5%</td>
<td>21:10–23:30</td>
</tr>
<tr>
<td>Phase shift</td>
<td>12:32 h</td>
<td>9%</td>
<td>10:30–14:12a</td>
</tr>
<tr>
<td><strong>DBP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mesor</td>
<td>78 mmHg</td>
<td>8%</td>
<td>(68–88)</td>
</tr>
<tr>
<td>Amplitude</td>
<td>7 mmHg</td>
<td>17%</td>
<td>(5.0–8.9)</td>
</tr>
<tr>
<td>Acrophase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gr. 1</td>
<td>22:20 h</td>
<td>5%</td>
<td>21:10–23:30</td>
</tr>
<tr>
<td>Phase shift</td>
<td>12:32 h</td>
<td>9%</td>
<td>10:30–14:12a</td>
</tr>
</tbody>
</table>

*Point estimates and RSEs of the phase shift parameter were further used to compute the 95% CI. This interval comprised the nominal value of 06:00 h (04:20–06:12), and 12:00 h (10:06–14:36) for Study a (RA, U_{K,fe} and U_{Na,fe}) and Study b (SBP and DBP), respectively.

TABLE 3. Change in OFV when a phase shift \( \Delta \) for the \( k \)th feeding group (\( k = 1, 2 \) or 3) was introduced in the model to quantify the effect of feeding time on the periodicity of RA, U_{K,fe}, U_{Na,fe}, SBP and DBP.

<table>
<thead>
<tr>
<th></th>
<th>RA</th>
<th>U_{K,fe}</th>
<th>U_{Na,fe}</th>
<th>SBP</th>
<th>DBP</th>
</tr>
</thead>
<tbody>
<tr>
<td>OFV (without phase shift)</td>
<td>-486.9</td>
<td>-230.2</td>
<td>-5.3</td>
<td>-588.4</td>
<td>-467.1</td>
</tr>
<tr>
<td>OFV (with phase shift)</td>
<td>-521.2</td>
<td>-256.5</td>
<td>-24.5</td>
<td>-603.9</td>
<td>-487.1</td>
</tr>
<tr>
<td>Difference</td>
<td>-34.2</td>
<td>-26.3</td>
<td>-19.2</td>
<td>-15.5</td>
<td>-20.0</td>
</tr>
<tr>
<td>( p ) Value</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

comprehensive characterization of the chronobiology of the renin cascade, BP, sodium and potassium renal handling in relation to the timing of food intake in dogs, using a nonlinear mixed effects modeling approach. This methodology guarantees to make the best use of the data, leveraging available information from the densely sampled plasma values (e.g. RA) to estimate parameters of the more sparse urinary endpoints. Another value of the method lies in its ability to separate variability from measurement noise, taking into account 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.

From renin activation to urinary electrolytes and BP homeostasis: an integrated machinery

In agreement with our previous investigation (Mochel et al., 2013), the data indicate that RA, urinary electrolytes, and BP oscillate with a circadian periodicity in relaxed, healthy beagle dogs fed a regular diet in the early morning (07:00 h). A cosine model with a fixed 24-h period was found to fit the periodic variations of RA, U_{K,fe}, SBP and DBP well, as shown by the standard goodness-of-fit diagnostics and the individual predictions. In contrast, cyclic changes in U_{Na,fe} were best characterized by means of a combined cosine and surge model, reflecting an afternoon peak sodium excretion followed by a monotonous decay, rather than periodic oscillations around the clock. In early fed dogs, the activity of renin in the systemic circulation was low in the morning, in relation to sodium and water-induced body fluid volume expansion (Kaczmarczyk et al., 1980), and peaked in the evening (around 21:00 h). Time variations in U_{K,fe} mimicked those of RA (trough and peak around 11:00 and 19:00 h, respectively), reflecting RAAS-mediated excretion of potassium in the kidney distal tubules. Conversely, U_{Na,fe} peaked around 15:00 h and then decreased quite sharply to be maintained on low levels during the evening and night, consistently with the activation of sodium-conserving mechanisms (i.e. renin cascade) during time devoted to sleep. In dogs fed at 07:00 h, SBP and DBP increased during the first half of the night, peaked around midnight, while returning to baseline in the early morning, thereby showing parallel fluctuations to RA around the clock. The good agreement between the dynamics of RA and BP highlights the contribution of the renin cascade to BP regulation, mediated by the sodium-retaining effects of aldosterone, and the powerful vasoconstrictor action of angiotensin II. These variations are consistent with our previous findings, and support earlier observations from Piccione et al. (2005). The levels of SBP and DBP reported herein are similar to those from earlier experiments in dogs (Mishina & Watanabe, 2008; Miyazaki et al., 2002; Mochel et al., 2013).

Fluctuations of renin, BP, urinary sodium and potassium are synchronized to feeding schedules

The effect of feeding on the circadian clock has been scrutinized over the last decades. Food restriction has been shown to affect the circadian rhythmicity of several variables including: liver glycogen, serum glucose and serum corticosterone in rodents
In addition, Pauly et al. (1975) found that restricting the access to food to short time spans caused the acrophase of eosinophil counts to synchronize to the feeding schedule in mice. Similarly, research on the influence of feeding in rabbits has shown that changing the time of food presentation from the early afternoon to the early morning caused a complete and immediate shift of the peak BP and heart rate to the morning period (Van den Buuse & Malpas, 1997). However, the impact of feeding time on the periodicity of the renin cascade has not reached a consensus, as illustrated by the conflicting results between Kunita et al. (1976) and Ikonomov et al. (1981) outlined earlier in the introduction. Our data show that environmental triggers such as timed feeding have a marked influence on the chronobiology of RA, urinary electrolytes and BP. Precisely, introducing a 6- or 12-h delay in the dogs’ feeding schedule caused a shift of similar magnitude in the rhythm of these biomarkers, as confirmed by the model-based estimates of the phase shift parameter. This observation was supported by the statistical comparison of early versus late time after feeding observations. Food intake thereby provides cues and elicits physiological responses that are able to act as entrainment stimuli, known as ‘‘Zeitgeber’’ (synchronizers) (Mistlberger, 2011), to control the phase of RA, $U_{K,fe}$, $U_{Na,fe}$, SBP and DBP.

In all feeding groups SBP and DBP exhibited a marked fall just after food intake. The observed drop in BP, which is consistent with previous studies in healthy beagle dogs (Gelzer & Ball, 1997; Mishina et al., 1999; Miyazaki et al., 2002; Nakagawa et al., 2010), is likely related to the decreased activity of renin, and the secretion of vasodilatory gut peptides, such as neuropeptide and insulin (Shibao et al., 2007). Indeed, intravenous infusions of insulin at physiological levels have been shown to reduce BP in patients with autonomic disorders (Brown et al., 1989). In elderly hypertensives, postprandial hypotension is a common and clinically relevant disorder (Gelzer & Ball, 1997; Kohara et al., 1999), that is characterized by fall, syncope, stroke and increased long-term mortality (Jansen, 2005). Reducing the size while increasing the frequency of meals has been shown to be an effective therapeutic approach to this condition (Jansen, 2005). Results from our previous research (Mochel et al., 2013) suggested that BP does not drop at night in dogs fed in the early morning. However, these additional data indicate that it does so in individuals fed at 19:00 h. Alike healthy humans, lower SBP and DBP levels are therefore to be expected during sleeping hours in dogs fed in the evening.

![Graph showing predicted influence of feeding schedules on the chronobiology of RA, BP and urinary electrolytes in healthy dogs](image_url)
The postprandial increase in $U_{\text{Na,fe}}$ portrays the “impulse-response pattern” of sodium excretion (Boemke et al., 1995) that is characterized by a peak natriuresis 4–8 h after feeding onset. Finally, in addition to feeding time, meal size also has an influence on the chronobiology of renin. Similar to Mochel et al. (2013), results from the covariate analysis indicate that increasing the size of the ration (i.e. sodium intake) triggers a reduction of the mesor and the amplitude of RA oscillations. This confirms that the amount of 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.

**Circadian adaption to feeding schedule: a mechanistic view**

Light–dark cycles are one of the most influential external stimuli for synchronizing circadian oscillators. However, for many circadian processes (e.g. corticosterone, insulin and glucagon secretion), it is the timing of food intake that is predominant (Patton & Mistlberger, 2013). As opposed to *ad libitum* feeding, food restriction to short, pre-defined time intervals, elicits well-characterized physiologocal responses (e.g. food seeking behavior) to maintain metabolic homeostasis. Feeding schedules entrain circadian rhythms of clock-gene expression in many peripheral organs and brain regions, dissociating these from the SCN Zeitgeber, which remains synchronized to light–dark cycles (Patton & Mistlberger, 2013). This has been shown in several studies in which restriction to food access to a 2–6-h-time period shifted the periodicity of clock gene expression in most peripheral organs to realign with the expected meal time (Boulos & Terman, 1980; Dibner et al., 2010). Feeding-related signals that are capable of entraining peripheral oscillators include autonomic outputs from the central nervous system, dietary sodium, glucose and insulin (Mistlberger & Antle, 2011). Entrainment signals may also be provided by biochemical pathways affected by energy metabolism (Bechtold, 2008), such as the intracellular ratio of reduced to oxidized nicotinamide adenine dinucleotide cofactors (Rutter et al., 2001). In addition, timed feeding has the ability to synchronize the activity of central oscillators, as shown by Kurumiya & Kawamura (1991) in a rodent experiment where the peak activity of neurons located in the hypothalamus was driven by the time of food intake.

**Limitations**

First, the effect of a 6-h-delay in feeding time on the chronobiology of BP could not be investigated in Study b due to the limited availability of telemetry-equipped dogs within our facilities. Likewise, gender-related changes in BP could not be assessed, since telemetry recordings were only performed in male beagle dogs. Second, the statistical significance of certain findings was hampered by low statistical power related to the small study size. This is shown by the non-statistical significance of the apparent greater amplitude of BP oscillations in dogs fed at 19:00 h compared to 07:00 h. Finally, while our results indicate that meal timing drives the periodicity of BP and RAAS-related variables, the neural and molecular bases of such finding remain to be clarified.

**CONCLUSIONS AND CLINICAL IMPLICATIONS**

The present research provides the first chronobiological characterization of RA, BP and renal sodium–potassium handling, in relation to feeding schedules in dogs. Cosine and surge models were able to reproduce the time-variant changes of the experimental data with good accuracy, as suggested by the quality of the standard goodness-of-fit diagnostics and the individual predictions. Our data show that timing of food intake exerts a synchronizing effect (as confirmed by the model-based approach), such that a 6–12-h delay in the dogs’ feeding schedule triggers a shift of similar magnitude in the rhythm of these biomarkers. Because of the known sensitivity of the renin cascade and BP to changes in sodium intake, we hypothesize that the synchronizing effect of food could be mediated in part by dietary sodium. However, other feeding-related signals, such as insulin, are also capable of entraining circadian oscillators downstream of the master, light–dark-adjusted pacemaker in the SCN (Mistlberger & Antle, 2011; Patton & Mistlberger, 2013). Activation of the renin–angiotensin system is a well-characterized pathophysiological feature of heart failure and HT. Cardiovascular disorders, such as chronic valvular heart disease (CVHD), are highly prevalent in elderly dogs, affecting about 75% of subjects over the age of 16 (Guglielmini, 2003). Little attention is usually paid to the time at which cardioactive medications should be given. Vasodilators (e.g. angiotensin-converting enzyme (ACE) inhibitors), diuretics, and positive inotropes that are commonly used in the course of CVHD are most often given with morning food for the sake of convenience. Yet, biological rhythms and their responsiveness to environmental cues such as food intake may substantially influence the effectiveness of drugs used in the management of HT and heart failure (Nicholls et al., 1993). A growing amount of literature has pointed out the importance of administration time-dependent effects of treatment in the management of cardiovascular diseases. Results from Hermida & Ayala (2009) in hypertensive patients have shown that evening administrations of the ACE inhibitor ramipril significantly decreased BP at all times of the day, while the reduction was only transient with morning dosing. Besides physiological variations of RAAS-related variables, several circadian rhythms may affect the pharmacokinetics and pharmacodynamics of cardioactive medications. Administration–time differences in pharmacokinetics can result from day–night variations in gastric pH, motility, blood flow, liver enzyme activity...
and glomerular filtration (Dridi et al., 2008; Hermida et al., 2011). Differences in pharmacodynamics can be related to circadian variations in drug-free fraction, receptor number and conformation, and signaling pathways (Hermida et al., 2007, 2011; Smolensky & Haus, 2001). This information emphasizes the need for conducting further research on the chronobiology of the RAAS and BP in dog patients to determine the time of drug administration that would optimize efficacy while minimizing the occurrence of adverse effects of medications intended to treat HT and CHF.

ACKNOWLEDGEMENTS

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DECLARATION OF INTEREST

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

AUTHOR CONTRIBUTIONS

J.P. Mochel, M. Fink, M. Peyrou, C. Desevaux, M. Deurinck and M. Danhof conceived the experimental protocols. J.M. Giraudel, C. Bon, and B. Bieth contributed to the development of the hypothesis and reviewed the study protocols. J.P. Mochel, M. Fink, and M. Danhof participated in the analysis of the data, including the building of the nonlinear mixed-effects models.

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