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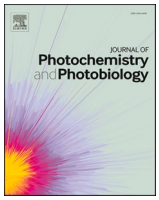

Different light/dark condition modulation of the daily rhythm of insulin and glucagon in dogs

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ABSTRACT

Considering the importance of glucose homeostasis and knowing that the alteration of its regulation is the cause of many dogs' pathologies, the present study aimed to investigate insulin, glucagon, and glucose daily rhythmicity in dogs subjected to a light/dark cycle (15/9 - L/D) and constant light (L/L). Five Beagle dogs (2 years old, male, 14.00 ± 0.50 kg) were housed in individual boxes, fed at 17:00, with water *ad libitum*; blood samples were collected every 4 h over 24 h for each photoperiod. A daily rhythmicity of insulin and glucagon was observed. Insulin acrophase was observed in the dark phase of the L/D cycle; glucagon acrophase was observed at the beginning of the light phase of the L/D cycle, for both parameters it was anticipated for about 4 h in the L/L cycle. The glucose oscillation trend remained constant during both photoperiods. Insulin and glucagon were positively correlated both in L/D and L/L cycles, the temporal difference of their oscillation of about 4 h persisted in both photoperiods. These findings contribute to the field of dogs' circadian physiology and should be taken into consideration during the dogs' clinical evaluation, particularly for the diagnosis and treatment of blood glucose homeostasis alterations.

1. Introduction

Many mammalian species are characterized by daily changes in their physiology and behavior. These variations are present regularly and cyclically, driven by an internal clock that allows living beings to anticipate and adapt to environmental changes to maximize their survival chances [1]. Some of these cycles have an oscillation range of 24–25 h and are defined as circadian rhythms (from Latin *circa-die*, almost a day) when this oscillation free-run under constant light conditions. These oscillations are synchronized within the 24 h thanks to the existence of environmental factors (*Zeitgebers*) that suggest to the organism how to synchronize their physiology with the external stimuli; principal *Zeitgebers* are alternance of light and dark, feeding and environmental temperature [1]. The circadian system is constituted by specific clusters of nerve cells located in the suprachiasmatic nuclei (SCN), within the anterior hypothalamus in the brain acting as pacemaker that synchronizes peripheral oscillators located in various tissues such as the liver, pancreas, adipose tissue, and skeletal muscle [2]. Whereas the principal synchronizer of the SCN has been demonstrated to be the light/dark cycle, in the peripheral tissue, the feeding time seems to entrain the peripheral oscillators (Food Entrainable Oscillators - FEOs) [3].

Among the physiological processes, energy expenditure and glucose homeostasis have been demonstrated to be regulated by the circadian clock [4]. In mammals, glucose modulation is ensured by the endocrine pancreas, which is organized in cell clusters called Langerhans islets. These islets secrete two fundamental hormones, insulin and glucagon. Beta cells secrete insulin, alpha cells are responsible for glucagon production. Insulin and glucagon can be considered antagonists in regard to their function; insulin ensures that glucose blood levels do not arise over a certain range, and to do so it acts as an inhibitor against hepatic gluconeogenesis as well as glucose secretion [2]. On the other hand, glucagon's function is the opposite, being secreted in response to decreased blood glucose levels, and it ensures that this level does not drop below a certain range. Its functions are stimulating glucose synthesis and secretion (gluconeogenesis) and increasing hepatic glycogenolysis. Together these two hormones ensure blood glucose homeostasis and are integral to understanding metabolic syndromes [5].

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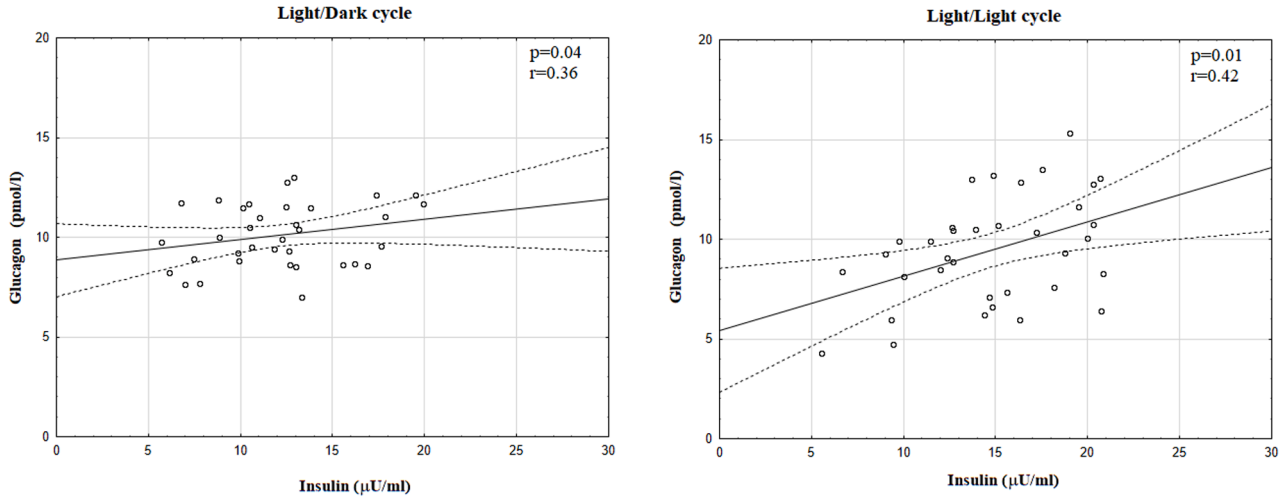

Fig. 1. Trend, mean ± standard deviation (SD) of insulin, glucagon and glucose expressed in their conventional units, recorded during the 24 h of L/D cycle and L/L cycle. Symbol indicates the acrophase.

Previous studies conducted on humans and mice have found that the secretion of these two hormones by alpha and beta cells of the endocrine pancreas showed daily rhythmicity [4]. The pancreas' peripheral clock is characterized by the presence of an autonomous circadian rhythm in the Langerhans islet cells [6]. In horses, it has been hypothesized that insulin secretion is endogenously generated and is not influenced by feed provided three times a day [7]. In dogs, a study investigated the glucose and insulin serum concentration after mealtime in the day and night [8], but any studies on the daily oscillation of insulin and glucagon have not been performed jet.

Disruption of circadian rhythmicity exacerbates metabolic syndrome, in particular, a misalignment between the peripheral pancreatic clock and the central clock has been associated with several illnesses, such as insulin resistance, and metabolic and cardiovascular diseases [9]. The bi-hormonal hypothesis proposed by Unger and Orci [10] back in 1975 emphasizes that the perturbation of glucose homeostasis in the context of diabetes mellitus is stemming from altering the balance between the glucose regulating counter hormones insulin and glucagon, rather than from the pathology of insulin alone [11]. Circadian misalignment is a risk factor for developing metabolic syndromes and Diabetes Mellitus (DM) is one of the most common endocrinopathies in dogs [12]. Thus, the objective of the present study was to investigate the daily rhythmicity of insulin and glucagon in dogs maintained under different light/dark conditions, as to identify the role of the L/D cycle on the daily oscillation of these two hormones involved in glucose homeostasis.

2. Materials and methods

2.1. Subjects and management

For this study five male Beagles dogs, 2 years old, 14 ± 0.5 kg, from the same private kennel, were subjected to the same management, fed with the same diet administered in the evening (17.00), water was available ad libitum. Before the start of the study, a clinical exam and a blood test was performed to ensure the health status of the dogs enrolled. Animals were free from internal and external parasites.

All dogs were kept in individual kennels (140 × 200 cm), separated by concrete walls but with screen doors, which allowed the subjects to hear and smell but not to see or contact each other.

General animal care was carried out by professional kennel staff not associated with the research team. Before the start of the study, all dogs were equipped with a catheter (16Gx51mm; Terumo, Roma, Italy) in the brachial vein for blood sampling, which was carried out every 4 h for 24 h, during two different photoperiods (light/dark cycle and light/light cycle), starting from 09:00 h of the day 1 and ending at 09:00 h of day 2. During the light/dark cycle (L/D) the natural illumination of the boxes was allowed by a opening window (50 × 100 cm). Dim red light (<3 lx, 15 W Safelight lamp filter 1A, Kodak Spa) was used for sample collections during the scotophase. Sunrise was at 05:05 h and sunset was at 20:55 h (L/D: 15/9). During the light/light cycle (LL), the windows were obscured, and illumination was provided by an artificial light (600 lx). Before the start of the study, dogs were subjected to a 3-days settling in period.

Thermo-hygrometric recordings were conducted during the entire study using a data logger (Gemini, Chichester, West Sussex, UK). Ambiental temperature was within the seasonal range of the period (27.5 ± 30 °C).

Department's Animal Ethics Council approved the study (058–2021) performed following the EU Directive 2010/63/EU about animal protection for scientific purposes.

2.2. Blood analysis

Blood samples were drawn into vacutainer tubes with clot activator and after standing at room temperature for 30 min were centrifuged at 3000 rpm for 15 min. The obtained serum was stored at −20 °C until analysis.

Elisa kits were used for the assessment of serum concentrations of insulin (Mercodia AB, Uppsala, Sweden), glucagon (Mybiosource Inc., San Diego, CA, USA), and glucose (BioSystems S.A., Costa Brava, Barcelona, Spain) following the manufacturer instructions.

2.3. Statistical analysis

Data were normally distributed ($p > 0.05$, Kolmogorov-Smirnov test). To detect statistical differences due to time of day and photoperiod two-way for repeated measure analysis of variance (ANOVA) was performed on the insulin, glucagon, and glucose serum values.

A trigonometric statistical model was applied to each time series, to describe the periodic phenomenon analytically, by characterizing the main rhythmic parameters according to the single Cosinor procedure [13]. Four rhythmic parameters were determined: mesor (rhythm-adjusted mean), amplitude (the difference between the peak, or trough, and the mean value of a wave), acrophase (the time at which the peak of a rhythm occurs), and robustness (strength of rhythmicity) using Cosinor Software. Rhythm robustness was computed as a percentage of the maximal score attained by the chi-square periodogram statistic for ideal

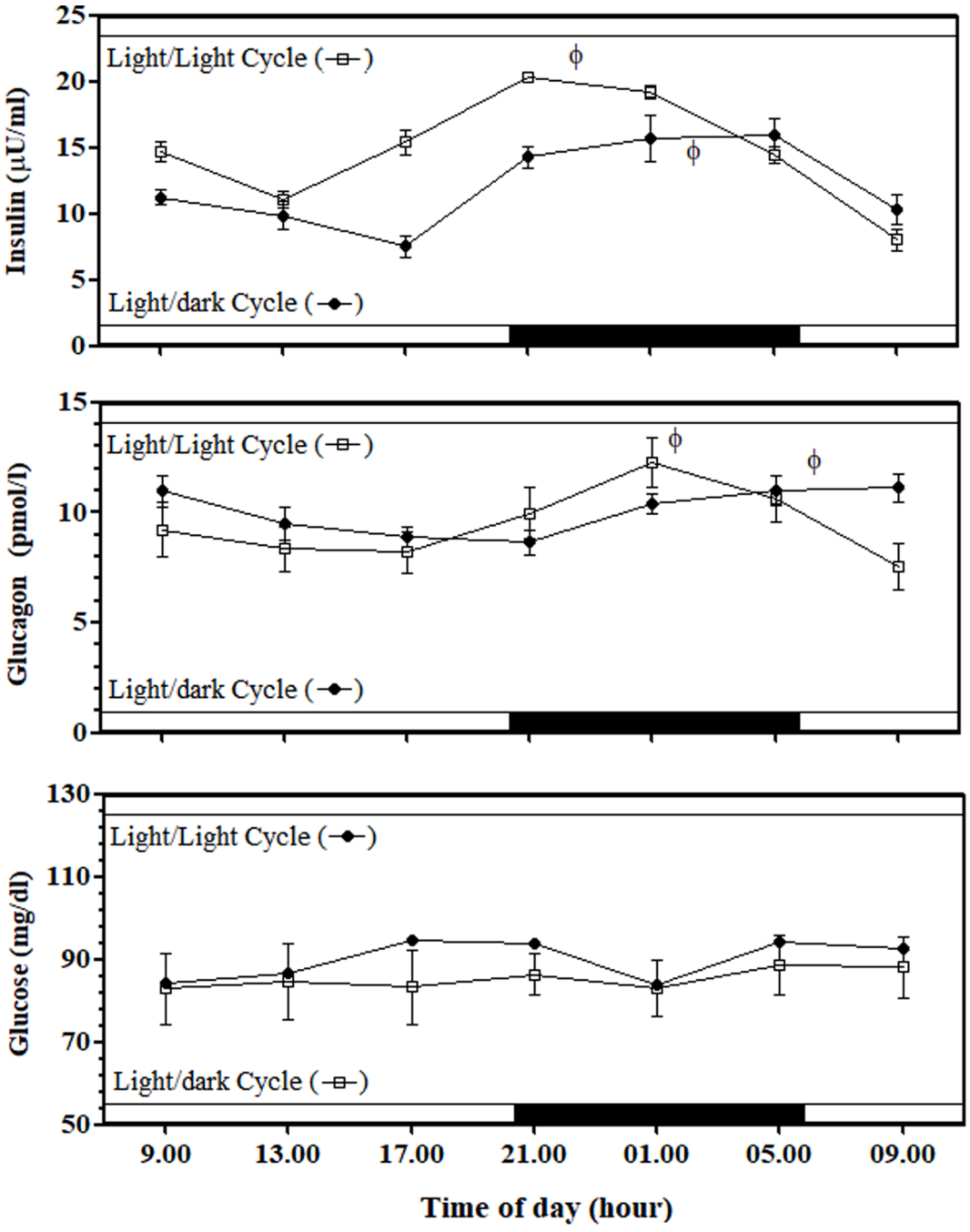

Fig. 2. Correlation between insulin and glucagon in the light/dark cycle and light/light cycle. Significant p and r values (Pearson coefficient) was shown.

data sets of comparable size and 24 h periodicity [13].

Also, the correlation coefficient (r) among the investigated parameters was determined. Regression lines, 95 % confidence interval for the different data recorded were determined. $p < 0.05$ was considered statistically significant. The data were analyzed with Statistica 7 (StatSofts, Inc, USA).

3. Results

The serum levels of the investigated parameters were within the physiological ranges reported for dogs [14–16]. The application of the two-way for repeated measures ANOVA showed a significant effect of time of day on insulin ($p < 0.0001$) and glucagon (time of day: $p < 0.0001$), and a significant effect of photoperiod on insulin ($p < 0.0001$). No effect of time of day and photoperiod was observed on glucose. Fig. 1 shows the trends of insulin, glucagon and glucose observed during the two photoperiods. The application of the single Cosinor method showed a daily rhythm of insulin and glucagon in both photoperiods. Insulin Mesor values were 12.27 µU/ml and 15.37 µU/ml in L/D and L/L, glucagon Mesor values were 9.88 pmol/l and 9.55 pmol/l, respectively in L/D and L/L. Insulin amplitude values were 4.22 µU/ml and 4.82 µU/ml respectively in L/D and L/L, glucagon amplitude values were 1.25 pmol/l and 2.05 pmol/l, respectively in L/D and L/L. During the L/D cycle, the insulin acrophase was observed during the dark phase (02:05), and it was anticipated of about 4 h (22:40) during the L/L cycle. Glucagon acrophase was observed in the light phase of the L/D cycle (06:30) and it was postponed of about 1 hour in the L/L cycle (01:34). Both serum parameters showed high robustness values (insulin L/D:

86.70 %, L/L: 78.80 %; glucagon L/D: 91.30 %; L/L 81.70 %). Insulin and glucagon showed a positive correlation ($r = 0.29$) in both photoperiods Fig. 2.

4. Discussion

In the present study, insulin and glucagon concentrations in dogs display variations in their concentrations throughout the day and that these oscillations followed daily rhythmicity.

The daily rhythmicity of insulin and glucagon was observed during the L/D cycle and persisted during the constant light, underlining the endogenous generation of these rhythms, having the ability to free run in constant light conditions.

Insulin and glucagon are secreted in different phases, at different times of the day; this way, the organism is capable of properly responding to the variations of blood glucose, which are caused by external factors such as feeding and fasting phases as well as the alternance of rest and activity [13].

Food intake has been found to be the main physiological stimulus for insulin secretion in humans and animals, but the amount of food intake may also depend on the time of day [6]. An experimental design should consider separately the effect of light and food on the secretion levels of hormones. We can exclude the role of feeding as a *Zeitgeber* having fed the dogs at the same h in both photoperiods. During the L/D cycle, insulin increased after the feed time (17:00) and reached its acrophase at 2:05, starting to decrease at the beginning of the light phase. During the L/D cycle, glucagon started to increase after 21:00, 4 h after the increase of insulin, and its acrophase was observed 4 h after the insulin acrophase (06:05). During the L/L cycle, insulin started to increase at 13:00, before the meal time, and reached its acrophase at 22:30. Also the daily rhythm of glucagon was anticipated of about 4 h respect to the daily rhythmicity observed during the L/D cycle. Its serum levels started to increase at 17:00, reaching the acrophase at 1:10. Insulin secretion and plasma insulin levels are subjected to circadian cycles in human beings, with an increase in the morning, a peak in the afternoon and a decline during the night [2]. The synchronization between the secretion of insulin and glucagon is confirmed by a positive correlation recorded in both photoperiods, characterized by the persistence of a temporal difference of their oscillation of about 4 h in both experimental conditions. As previously found by Chan et al. [4], we observed that blood glucose levels did not statistically change during the 24 h of monitoring in both photoperiods, suggesting that insulin and glucagon play a major role in maintaining this parameter stable during the 24 h.

The synergic activity of insulin and glucagon guarantees the constant glucose serum levels recorded in both photoperiods. Even though the effect of digestible carbohydrate and glucose uptake by the canine intestine has not been investigated, previous studies reported a potential individual up-regulation of glucose uptake by the small intestine in dogs [14]. In dogs fed with a commercial meal formulated for this species, glucose and insulin concentration did not differ in comparison with pre-meal values, in contrast to dogs fed with a commercial diet added with glucose, in which an increase of glucose and insulin values was observed [15]. Such as no changes in glucose concentrations have been observed between day- and night-time when animals were fed a commercial diet at 7:00 and 19:00 [8].

The data obtained in our study reveal a strong correlation between the two investigated hormones, confirming their synergic role in the homeostasis of glucose [4]. This is consistent with previous knowledge on this matter, confirming that insulin plays a hypoglycemic role, while glucagon is a hyperglycemic hormone. Consequently, the two work together to maintain blood glucose levels within physiological range values [4]. Even if it is known that the main stimulus for insulin secretion is the glucose blood level, multiple factors can modify it. Among these, glucagon plays an important role, along with the vagus nerve, amino acids, ketones, hypercalcemia, acetylcholine, long-chain fat acids, and prostaglandins. On the other hand, somatostatin, adrenaline, and the sympathetic system can be considered inhibitors of insulin secretion [1].

Despite the limited number of subjects enrolled and the 24-hour duration for each photoperiod, we can claim a daily rhythm in insulin and glucagon in dogs housed in an L/D cycle and in constant light, with a shift of the oscillation between the two photoperiods, whereas glucose serum concentration maintained its homeostasis in both experimental conditions [16–19].

Therefore, despite we acknowledge that our study is limited by the restricted sample size and the duration of monitoring of 24 h, we can claim that the present study contributes to the understanding of dogs' circadian physiology and glucose homeostasis in this species. The data obtained in this research may contribute to a better understanding of canine pancreatic physiology and could be found an application in the clinical practice for the diagnosis and the management of pathologies involving glucose homeostasis and for the application of correct chronotherapy. Further studies with L/B cycles with different amounts of light and times of feeding should be useful to better understand the circadian physiology of insulin and glucagon involved in glucose homeostasis.

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CRediT authorship contribution statement

Claudia Giannetto: Writing – review & editing. **Marilena Briglia:** Writing – original draft. **Francesca Arfuso:** Software. **Enrico Cancellieri:** Investigation. **Elisabetta Giudice:** Validation. **Giuseppe Piccione:** Methodology. **Maria Rizzo:** Supervision.

Declaration of competing interest

The authors declare no conflicts of interest.

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Data availability

Data will be shared upon reasonable request.

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