Eur J Wildl Res (2008) 54:21–26
DOI 10.1007/s10344-007-0102-6

ORIGINAL PAPER

Variation in plasma biochemical parameters in captive adult red-legged partridges (*Alectoris rufa*) during daylight hours

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Received: 29 November 2006 / Revised: 25 March 2007 / Accepted: 2 April 2007 / Published online: 26 April 2007
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Abstract Blood biochemical parameters may provide useful information about the physical condition of the individual, making them a useful tool in ecological studies. However, to avoid biases, factors affecting circulating levels of plasma metabolites must be investigated. In this paper, we analyse the effect of daytime sampling hour on seven plasma metabolites in adult red-legged partridges (*Alectoris rufa*) with free access to food during the breeding season. We found that sampling hour affected circulating levels of glucose and triglycerides but not those of cholesterol, total protein, uric acid, urea or albumin. A sex effect was found only for glucose, uric acid, urea and triglycerides. Repeated sampling affected all the parameters studied. These results suggest that the effect of sex, sampling hour and repeated sampling should be carefully controlled for (methodologically or by statistical procedures) to avoid undesirable sources of error at least in some blood parameters.

Keywords Blood biochemistry · Circadian · Daily variation · Plasma metabolites

Introduction

Blood biochemical parameters provide a useful tool in many ecological studies, allowing evaluation of the physical condition of individuals (Ferrer 1993). However, circulating levels of plasma metabolites may be affected by factors such as age, weather, sex or season (Schalm et al. 1975; Kaneko et al. 1997; Alonso-Alvarez 2005) that should therefore be taken into account in data collection or controlled for by statistical methods to avoid undesirable errors or biased results.

One potentially influential factor is the hour of the day at which samples are collected, as many blood parameters show daily patterns of variation (e.g. García-Rodríguez et al. 1987; Ferrer et al. 1994) that may be attributable to circadian rhythms (Gwinner 1975). A recent study published in this journal (Rodríguez et al. 2006) analysed the variation in seven plasma metabolites during a 24-h period and in absence of an eat–fast cycle. The authors created four groups of birds (four males and four females by group). Each group was sampled at a different time: at 0630 (just after sunrise), 1200, 1700 (before sunset) and at 2300 hours. They found daily variations in glucose, cholesterol, uric acid, triglycerides and calcium, but not in total protein or creatinine. These results are interesting, although some issues should be considered. First, the study was performed at the interindividual level, whereas a repeated-measures design would be a more powerful approach to evaluate the existence of such daily variations, optimising the accuracy of the information obtained employing the same sample size. Furthermore, as birds were not individually housed, a pseudo-replication effect in the experimental design cannot be discarded (e.g. differences may be due to varying conditions between cages rather than those between sampling hours). Second, birds were sampled after 10–12 h of fasting. This experimental procedure is useful, as it diminishes the effect of the eat–fast cycle and its interference with internal rhythms. However, it reduces the applicability of the results to studies where feeding conditions cannot be controlled

Communicated by C. Gortazar

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(which is the case in most studies, especially in wild individuals). Therefore, the effect of feeding should be accounted for to obtain results with a more practical and methodological scope. Third, although daily variations were found in five of the parameters studied, only in levels of calcium were significant variations detected during daylight hours, the period of time when data collection is carried out in the great majority of studies. Fourth, this study was performed with juvenile birds and, as authors acknowledged, we cannot conclude that adult birds with active gonads show similar daily variations.

The aim of the present study is to confirm the existence of a pattern of variation in some plasma metabolites during daylight hours. For this purpose, we used a repeated-measures design and individually housed adult red-legged partridges during the breeding season with free access to food.

Materials and methods

Sample collection was carried out at the experimental facilities of the Instituto de Investigación de Recursos Cinegéticos (Ciudad Real, central Spain), during April 2006, the reproductive season of the species at this latitude. For the study, we randomly selected 14 male and 14 female 4-year-old red-legged partridges hatched in captivity (see Pérez-Rodríguez et al. 2007 for further details on rearing and housing conditions). Birds were housed in individual outdoor cages (1×0.5×0.4 m) at ambient temperature and natural photoperiod (13 daylight, 11 night hours). Individuals were fed ad libitum with commercial pelleted food (20% protein, 4.5% fat, 3.7% cellulose). During the experimental period, coincident with the mating–breeding season, males usually showed the typical calling behaviour of this species. Females, despite being individually housed, started egg laying 1 to 2 weeks after the experiment finished.

Each bird was sampled once a day at either 0815, 1215, 1630 or 2030 hours (close to sunset) on alternate days (6th, 8th, 10th and 12th of April, days 1, 2, 3, and 4, respectively, hereafter). Sunrise takes place at 0720 hours at this time of the year. Therefore, birds had approximately 1 h to break their fast before the first sampling. The study hence lasted 6 days and involved four samplings per bird. However, to distinguish between circadian variability and the effect of consecutive sampling (day effect), sampling hour was randomised at the individual level (e.g. bird A: day 1, 1215 hours, day 2, 2030 hours, day 3, 0815 hours, day 4, 1630 hours, and so on). One of the females escaped before the last blood sample was collected, reducing the sample size by one individual in the statistical analyses.

In all cases, a 500-μl blood sample was collected from the brachial vein in heparinised syringes. For each blood sampling, birds were extracted from the cage, handled and sampled within 2 min each to avoid short-term stress to affect the studied parameters (Wingfield et al. 1997). A 75-μl heparinised capillary was filled from the sample to measure the haematocrit, which would allow detection of possible haemodilution caused by repeated sampling. Blood samples and capillary tubes were kept cold until centrifugation (no more than 3 h later) at 10,000 rpm for 8 min. Plasma was stored at −80°C until analysis.

We studied the variations of seven parameters in each plasma sample: glucose, cholesterol, uric acid, urea, triglycerides, total protein and albumin. Plasma biochemistry was measured using an A25 BioSystems spectrophotometer autoanalyser (BioSystems S.A., Barcelona, Spain) with commercial kits purchased from BioSystems S.A. (see Vidal et al. 2006). All of these, except urea and albumin, were also analysed by Rodríguez et al. 2006. Glucose, cholesterol, uric acid, urea, triglycerides and total protein are parameters that appear to be related to nutritional status in birds (Alonso-Alvarez et al. 2002; Alonso-Alvarez and Velando 2003; Rodríguez et al. 2005) and are, therefore, a potentially useful tool for assessing individual body condition in ecological studies. Albumin may also be a health indicator, as its concentration may decrease in almost any disease and also malnutrition (e.g. Kawai 1973).

Statistics

Analyses were performed by using general linear mixed models (MIXED procedure and REPEATED statement from SAS software, Littell et al. 1998). Each plasma metabolite concentration was introduced as the dependent variable, whereas sex, hour and day of sampling, as well as all pairwise interactions were entered as fixed factors. Individual identity was included as a random factor, and non-significant terms were sequentially excluded from the model in a backwards stepwise manner. Moreover, to analyse haematocrit variability during the experience, a model including the same factors but testing haematocrit as a dependent variable was performed. Dependent variables met normal distribution and homoscedasticity requirements, except triglyceride concentrations, which had to be $\log_{10}$ transformed for the analysis. All tests are two tailed.

Results

Results of the general linear models for each blood parameter are shown in Table 1. We found a significant effect of the hour of sampling only in glucose and triglyceride levels. In both sexes, glucose values peaked after sunrise, decreasing afterwards, the levels being constant throughout the rest of the day (Table 1, Fig. 1). Triglycerides tended to increase as

Table 1 Result of the general linear model for each plasma metabolite

	Glucose		Cholesterol		Triglycerides		Total protein		Uric acid		Urea		Albumin	
	F(df)	*p*	*F*(df)	*p*	*F*(df)	*p*	*F*(df)	*p*	*F*(df)	*p*	*F*(df)	*p*	*F*(df)	*p*
Day	*7.10*	*<0.001*	*8.55*	*<0.001*	*5.24*	*0.002*	*11.0*	*<0.001*	*5.32*	*0.002*	*5.92*	*0.001*	*7.52*	*<0.001*
	(3,76)		(3,79)		(3,76)		(3,79)		(3,76)		(3,79)		(3,79)	
Hour	*4.05*	*0.010*	1.74	0.16	*6.47*	*<0.001*	1.63	0.18	2.29	0.13	2.53	0.11	1.37	0.24
	(3,76)		(3,76)		(3,76)		(3,76)		(1,75)		(1,78)		(1,75)	
Sex	*7.78*	*0.009*	0.81	0.37	*13.1*	*0.001*	0.82	0.37	*17.6*	*<0.001*	*5.28*	*0.029*	1.53	0.22
	(1,26)		(1,26)		*(1,26)*		(1,26)		*(1,26)*		*(1,26)*		(1,26)	
Day×hour	1.45	0.18	1.51	0.16	0.89	0.54	1.01	0.44	0.92	0.43	0.75	0.52	0.42	0.73
	(9,61)		(9,67)		(9,64)		(9,67)		(3,71)		(3,72)		(3,71)	
Hour×sex	1.24	0.30	0.75	0.52	2.33	0.08	0.68	0.56	0.47	0.49	0.09	0.76	1.55	0.21
	(3,70)		(3,64)		(3,73)		(3,64)		(1,74)		(1,71)		(1,74)	
Day×sex	1.61	0.19	0.14	0.93	0.53	0.66	0.62	0.60	*2.99*	*0.03*	1.41	0.24	1,37	0.26
	(3,73)		(3,61)		(3,61)		(3,61)		(3,76)		(3,75)		(3,76)	

Individual identity was entered as a random variable ($p<0.001$ in all cases), thus controlling for the repeated-sampling effect. *F* and *p* values for non-significant terms correspond to the step in which they were removed from the model. Significant effects in the final model (after a backward stepwise procedure) are marked in italics.

the day progressed, showing maximum values just before sunset in females. However, the pattern observed in males was slightly different, as revealed by the marginally significant interaction ($p=0.08$, Table 1) between sex and hour of sampling, males showing peak values at 1630 hours and a slight decrease afterwards (Fig. 1).

We found sexual dimorphism in the plasma concentration of four of the parameters studied (Table 1). Males showed higher levels of uric acid, urea and glucose, whereas females showed higher levels of triglycerides than males (Table 1, Fig. 1).

We found a significant effect of the day of sampling in all blood parameters (Table 1), which showed a common trend to decrease during the experiment. However, such an effect could be explained by haemodilution as a result of repeated sampling. This is supported by the fact that haematocrit levels decreased with increasing day of sampling ($F_{3,75}=7.39$, $p<0.001$, Fig. 2), but were neither explained by time of sampling nor by the day/time interaction term (both $p>0.1$). We also found a significant interaction between the effect of day of sampling and sex in uric acid levels, males showing a more marked decrease during the experiment than females (Fig. 2) that were also less affected by repeated sampling.

Discussion

We found that only two (glucose and triglycerides) of the seven blood metabolites studied showed significant variations during daylight in our sample of adult partridges during reproductive season. Rodríguez et al. 2006 found that these two parameters showed circadian variations, as did total protein, cholesterol, creatinine, calcium and uric acid. However, focusing on their results for daylight hours, the only parameter where they found significant differences was calcium concentration. This discrepancy may be due to the fact that we used a repeated-measures design, which may have been a more powerful approach to detect small changes in metabolite concentrations. Furthermore, our experimental birds had food ad libitum throughout the day, whereas those of Rodríguez et al. 2006 were sampled after a fasting period of 10–12 h, and therefore, our results may be a more reliable reflection of the natural patterns that researchers would expect to find in their field studies. Finally, it should be noted that we studied adult birds during the reproductive season (April), whereas Rodríguez et al. 2006 studied young (7-month-old) birds in February. It is not known whether circadian variations may differ between these 2 months or across ages. However, as plasma concentrations of several parameters have been shown to vary between ages in this species (Rodríguez et al. 2004), this possibility cannot be discarded. Another difference

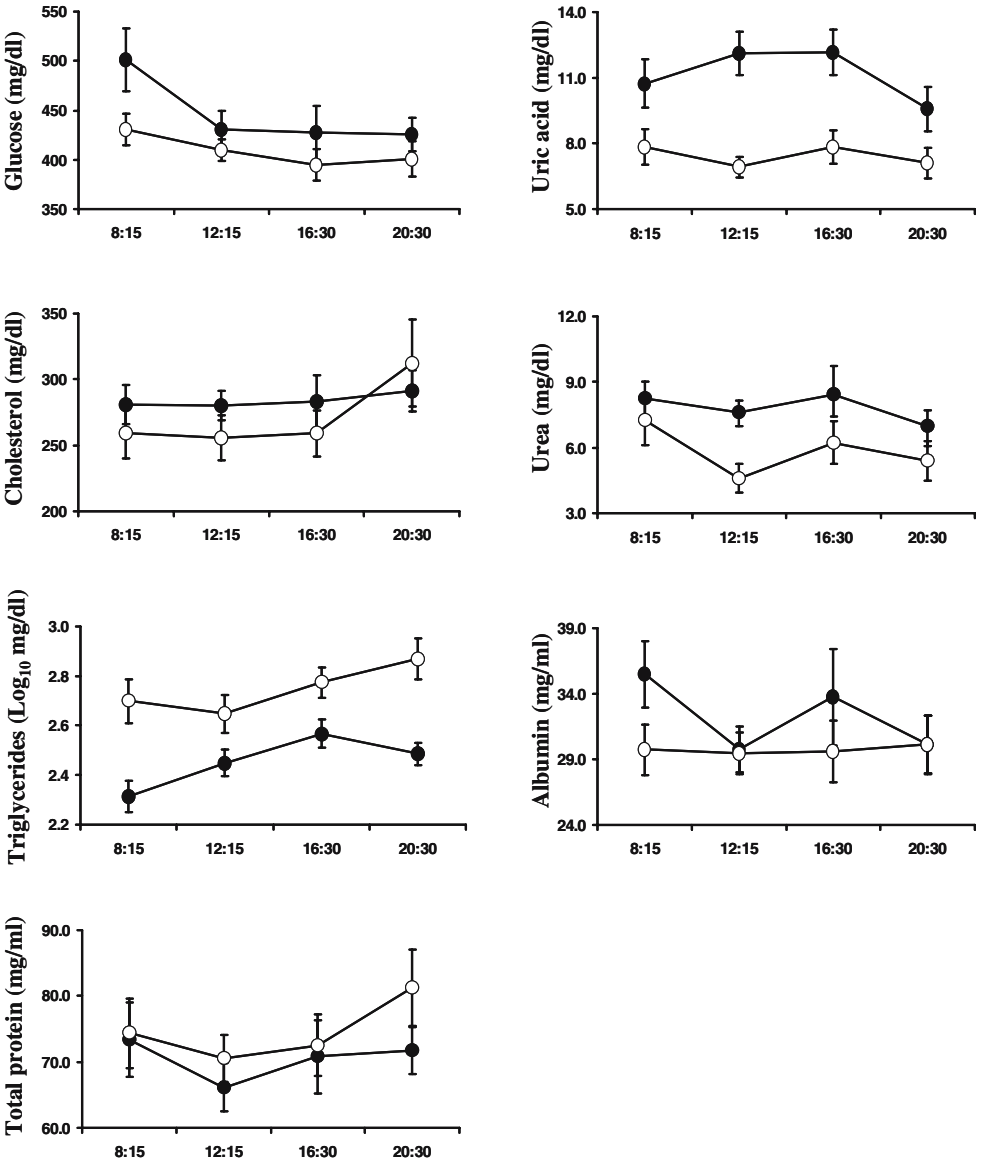

Fig. 1 Variation in plasma biochemical parameters of adult male (*closed symbols*) and female (*open symbols*) red-legged partridges during daylight hours. Values are means±SE

between the study of Rodríguez et al. 2006 and this one is that birds were fed with mixed cereals in the former but with commercial pelleted food in this. These differences in diet would lead to differences in baseline levels of several metabolites between both studies, but probably did not produce significant effects on the daily variations that are the focus of this study.

In our study, birds showed the higher values of glucose in the earliest sampling, just after sunrise. During the night, glucose levels are expected to be low due to nocturnal fasting. However, as we sampled the birds approximately 1 h after sunrise, they may have had time enough to break their fast and show this morning peak. Such a short-time glucose response to feeding has been reported in birds (e.g. Minick et al. 1996; Buyse et al. 2002). Nonetheless, studies published to date do not suggest a consistent pattern of diurnal change in circulating glucose (Jenni and Jenni-Eiermann

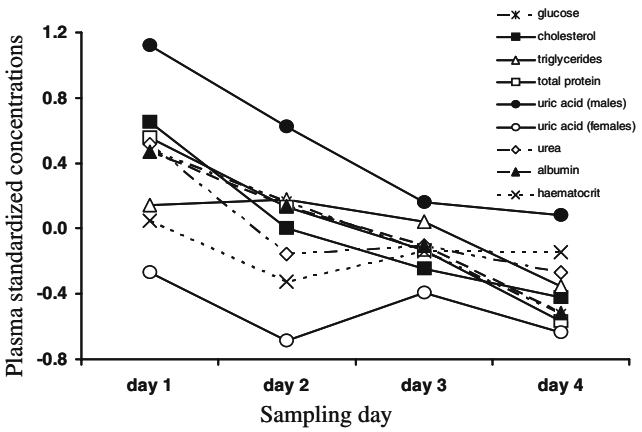

Fig. 2 Variation in plasma biochemical parameters and haematocrit levels in adult red-legged partridges during the experiment. *Values* are standardized means for each parameter. Error bars have been omitted for clarity

1996; Herichova et al. 2004, and references included in both articles), and hence, other potential explanations could be also plausible. Triglycerides showed a tendency to increase from dawn to dusk, with a slight decrease at the end of the day in males but not in females. Previous studies have shown that fasting values of triglycerides peaked at night hours (García-Rodríguez et al. 1987; Rodríguez et al. 2006). It is possible that the late afternoon peak found in our birds with free access to food correspond with the beginning of the increase towards higher night values. Another possibility is that plasma triglyceride concentration increased during the daytime in this study as a result of the slow metabolism of lipids ingested during the day (Stevens 1996). The recording of highest triglyceride values in females, but not males, at the end of day could be related to increased lipid metabolism associated with breeding and overnight egg formation, as most females started egg laying within a fortnight of the end of the experiment.

We detected sexual differences in plasma concentrations of glucose, uric acid, urea and triglycerides (males showing higher values in all parameters except triglycerides), but not in cholesterol, total protein or albumin. A previous descriptive study in this species reported sexual differences in cholesterol, triglycerides and total protein (females showing higher values than males in all these parameters), but not in glucose, urea or uric acid (Rodríguez et al. 2004). In contrast, Rodríguez et al. 2006 found sexual differences only in uric acid. The fact that Rodríguez et al. 2006 employed young birds at the onset of the breeding season whereas we used adult (4-year-old) birds at the middle of the breeding season seems to suggest that inconsistency between studies may be attributed to seasonal changes in plasma metabolites in relation to reproduction. Unfortunately, we cannot test this hypothesis against the results of Rodríguez et al. 2004, as they did not report the date at which samples were collected. However, the fact that triglycerides, total protein, urea and uric acid changed with the age of the birds seems to support the relationship of this sexual dimorphism with the maturation and activation of gonads. The higher metabolic rate of males due to sexual activity and the effect of androgens (Buchanan et al. 2001) may explain the higher values of uric acid and urea detected in this sex. Higher triglycerides in the plasma of females may be associated with fat mobilisation from body stores during ovulation and egg formation (e.g. Zaias et al. 2000).

A significant effect of the day of sampling on all plasma metabolites studied was detected. This effect could be due to haemodilution caused by repeated blood extraction, as reported by Pérez-Rodríguez et al. 2007. In birds, Campbell (1994) recommended a maximum blood sample of 1% of total body mass to avoid anaemia. In this paper, we extracted a total of 2 ml at the end of the experience (0.5% of a partridge body mass), which is well below the proposed limit. Nevertheless, at the end of the experiment, plasma haematocrit levels only decreased an average of 1.7 and 3.2% in males and females, respectively, which does not seem to suggest a serious impact for the health of the individual.

Alternatively, the decrease in haematocrit and, consequently, in all the plasma metabolites during the experiment could be attributed to an acute and/or chronic stress response as consequence of being disturbed/handled/sampled repeatedly. In fact, an increase in stress and corticosterone levels has been reported to affect plasma concentration of some plasma metabolites, but not others (e.g. Krasnodebska-Depta and Koncicki 2002; Remage-Healey and Romero 2002). However, it seems unlikely, as each blood sampling took less than 2 min per bird in a 48-h period, the cages were visually isolated, and we tried to minimise the general disturbance to birds to minimise the stress caused to them. Furthermore, the fact that all blood parameters (and haematocrit) tended to vary in the same way (decreasing throughout the study) seems to support the hypothesis that it was an effect of haemodilution rather than stress.

In summary, we conclude that some parameters, such as glucose and triglycerides, may show variations during daylight hours. As suggested by Rodríguez et al. 2006, we propose that researchers should avoid sampling at dawn or dusk, when peak values of some metabolites were detected. If the hour of sampling cannot be methodologically standardised, we recommend controlling for this variable is in statistical analyses by including it as a covariate. This statistical procedure may also allow one to control for possible variations caused by feeding patterns, a factor not taken into account in this study. Other factors, such as age or sex, should also be taken into account, as they may strongly affect the parameters studied. On the other hand, we also found that repeated sampling lead to a significant decrease in all plasma metabolites, which should be taken into consideration in studies involving such kinds of sample collection protocols. Whatever the mechanism involved in the observed decrease (e.g. haemodilution and/or stress), we recommend repeated sampling designs to be performed only when strictly necessary, minimising the volume of sample collected and establishing a reasonable period of time between samplings. Furthermore, sample volume should be kept constant between both individuals and consecutive samplings to avoid bias due to differences in haemodilution.

Acknowledgements We thank Elisa Pérez, Salvador Jesús Luna and Francisco Buenestado for assistance during sample collection and Daniel J. Morrish for reviewing the English. Financial support was provided by the Research Project PAI-02-006 of the Junta de Comunidades de Castilla-La Mancha. L.P.R. was supported by an FPU grant of the Spanish Ministerio de Educacion y Ciencia. C.A.-A. was supported by a Ramon y Cajal fellowship (Ministerio de Educacion

y Ciencia, Spain). M.M.-H. was supported by a postgraduate grant (I3P) from CSIC and the Research Proyect CGL2004-02568/BOS of the Spanish Ministerio de Educacion y Ciencia. This study was performed according to Spanish laws.

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