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Effect of keeping plasma frozen at −20°C on the concentration of blood metabolites

Javier Bustamante and Alejandro Travaini

Estación Biológica de Doñana, CSIC, Avda. María Luisa s/n, 41013-Sevilla, Spain

The stability of plasma metabolites under different storing conditions was tested in blood samples taken from black kites (*Milvus milvus*) and red foxes (*Vulves vulpes*). Keeping the plasma frozen at −20°C for 30 days had a significant effect on the concentration of urea, glucose and triglycerides but not on the concentration of uric acid and cholestrol. Concentration of plasma metabolites should be measured as soon as possible, without freezing the samples. Plasma can be stored at 4°C for up to 4 days without a significant effect in any of the metabolites tested.

Key words: Frozen plasma; *Milvus milvus*; *Vulves vulpes*.

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Introduction

In studies of blood metabolism of free ranging mammals or birds, it is not usually possible to measure the concentration of plasma metabolites shortly after the blood has been sampled. It is generally assumed that blood should be centrifuged as soon as possible (within 1 hr) and that the plasma, separated from blood cells, can be kept at 4°C for up to 4 days or frozen below −20°C for an unlimited time (M. Ferrer pers. comm.). We have not been able to find in the literature, tests of the validity of these assumptions, but we have found that storing the plasma at −20°C for an undefined length of time is the most habitual practice (Marsh, 1983; Minick, 1986; Puerta *et al.*, 1989a,b; 1990; 1992; Jenni-Eiermann and Jenni, 1991; 1992; Jenni and Jenni-Eiermann, 1992; Polo *et al.*, 1992). Other studies keep the samples frozen at lower temperatures (Mori and George, 1978; Reynolds, 1982; Saarela *et al.*, 1986) or do not give detailed information on how the plasma was kept before analysis (Jeffrey *et al.*, 1985; González and Hiraldo, 1991; Ferrer, 1992). We found only one study with wild animals in which plasma analyses were carried out within a few hours after extraction with a portable analyser (Bairlein and Totzke, 1992).

We studied the effect that keeping plasma for 4 days at 4°C, or frozen at −20°C for periods of 15 and 30 days had on the concentrations of five metabolites: urea, uric acid, cholesterol, glucose and triglycerides.

Materials and Methods

Blood was sampled from mammals—six red foxes (*Vulpes vulpes*)—and birds—12 black kite chicks (*Milvus milvus*). Blood was drawn under anaesthesia from the red foxes and by physical restraint from the black kites, in both cases by vein puncture. One to 5 ml samples were taken, and transferred to plastic vial with lithium–heparin. It was allowed to clot at room temperature (for 1 hr), and the plasma removed by centrifugation (3000 rpm, 10 min). The plasma was then divided into four aliquots. The first aliquot (control) was kept at 4°C and the concentration of all metabolites measured within 24 hr of the extraction. We assumed that the concentrations obtained in this first measurement corresponded with the concentrations of the metabolites in the blood. The second plasma aliquot was kept for 96 hr at 4°C, the third was frozen at −20°C for 15 days and the fourth frozen at −20°C for 30 days. A new

Correspondence to: J. Bustamante. Nationalparkverwaltung, Doktorberg 6, D 8240 Berchtesgaden, Germany.
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Table 1. Mean values (mg/dl) and SE of the plasma metabolites in the control samples

	Black Kite			Red Fox			
Metabolite	*N*	Mean	SE	*N*	Mean	SE	CV%*
Urea	6†	26.38	4.52	6	79.93	16.39	3.91
Uric acid	12	17.66	1.77	6	<2.0	—	1.83
Glucose	12	210.02	7.52	6	128.52	29.04	2.11
Cholesterol	8	260.16	34.96	5	142.82	8.01	3.08
Triglycerides	8	159.72	20.59	4	89.20	12.71	2.54

*Precision of the analyser, average Coefficient of Variation within measurements of the same aliquot. †Only kites with urea <20 mg/dl.

measurement of the concentration of the metabolites was taken from each of the aliquots after treatment.

Plasma analyses were performed with a REFLOTRON® (Boehringer, Mannheim) portable blood analyser. It measures the colorimetric change of a solid-phase reaction of 32 μl of plasma in test stripes specific for each metabolite. We used standard Reflotron-glucose (GOP method), Reflotron-triglyceride (esterase method), Reflotron-cholesterol (cholesterol esterase method), Reflotron-urea (urease method) and Reflotron-uric acid (uricase method) tests. Each measurement of concentration of a metabolite in a plasma aliquot was repeated 2–6 times (depending on the plasma available) to estimate the error of the analyser for each metabolite. The mean value of these 2–6 measurements was used in the statistical analyses. From the variation within aliquots, we estimated the error of the analyser for each metabolite.

Concentrations of all metabolites could not be measured in all individuals. Levels of uric acid in all the foxes were below the lower detection limit of the analyser (2 mg/dl), and only a few of the kites had urea levels over 20 mg/dl that could be measured. Small samples of blood obtained from some of the kites precluded determining the concentration of all metabolites in all the treatments. The mean values of all metabolites in the control samples are presented in Table 1.

To avoid the correlation in the values of the different treatments of each individual, the data were transformed before analysis by subtracting from each value the concentration of the control for that individual. Transformed values represented the difference of the concentration in each treatment with the real concentration of the metabolite in the blood. A nested ANOVA with three factors: species (two levels, foxes and kites), individuals (nested within species), treatment (four levels) and the interaction between species and treatment, was then performed on the transformed values. A Dunnetts two-tailed *t* test was performed *a posteriori* to compare the values of each treatment with that of the control. Nested ANOVAs were carried out with the procedure GLM of SAS (1988), which performs a nested ANOVA for unbalanced designs, and we used a type III sum of squares.

Results and Discussion

The way in which the plasma had been stored before the analysis had a significant effect on the concentrations of three of the metabolites: urea, glucose and triglycerides, while it did not have a significant effect on the concentrations of uric acid or cholesterol (Table 2). The ANOVAS did not show significant differences between foxes and kites, or a significant interaction between species and treatment. In the glucose there were significant differences among individuals, indi-

Table 2. Effect of keeping plasma under different conditions (treatments) on the concentrations of different metabolites. Two species are tested; the black kite and the red fox. Nested ANOVA on the differences with the control samples, procedure GLM of SAS (1988)

Factors	DF	SS	*F* value	*P*
Urea				
Species	1	14.337	2.48	0.127
Individual (species)	10	57.777	1.00	0.468
Treatment	3	57.958	3.34	0.034
Species*treatment	3	8.049	0.46	0.710
Uric acid				
Species	—	—	—	—
Individual (species)	11	4.661	1.55	0.165
Treatment	3	0.499	0.61	0.615
Species*treatment	—	—	—	—
Glucose				
Species	1	14.687	0.58	0.452
Individual (species)	16	942.288	58.89	0.014
Treatment	3	377.799	4.94	0.005
Species*treatment	3	130.618	1.71	0.179
Cholesterol				
Species	1	38.737	1.17	0.289
Individual (species)	11	755.500	2.07	0.060
Treatment	3	122.419	1.23	0.318
Species*treatment	3	126.152	1.27	0.305
Triglycerides				
Species	1	121.527	0.97	0.333
Individual (species)	10	1493.363	1.19	0.337
Treatment	3	3445.895	9.18	0.0002
Species*treatment	3	578.253	1.54	0.226

Individuals are considered a factor nested within species. — = missing value (data only for kites).
Species*treatment = species–treatment interaction.

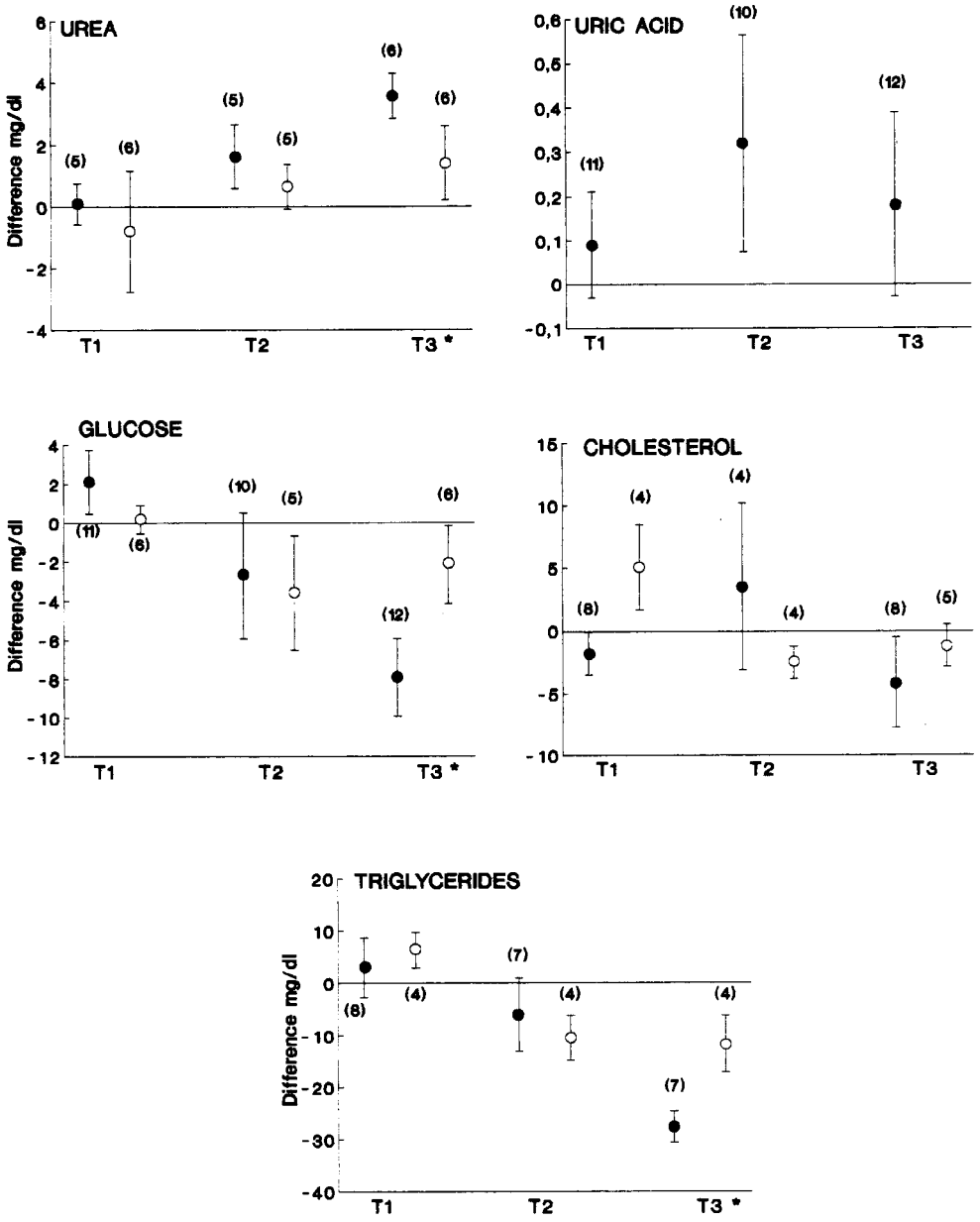

Fig. 1. Mean difference (±SE) between the treatments and the control samples. Treatments, T1: plasma 96 hr at 4°C; T2: plasma 15 days at −20°C; T3: plasma 30 days at −20°C. black kite = solid circles, red fox = open circles, * = $P < 0.05$ (two-tailed Dunnetts t test for effect of treatment $\neq 0$)

cating that the effect of the treatment differed between individuals. There was a significant negative correlation between the change in glucose in the plasma kept frozen at −20°C for 30 days and the glucose level in the control ($r = -0.568$; $P = 0.014$; $N = 17$).

The tests *a posteriori* indicated that concentrations of triglycerides and glucose obtained from samples that had been frozen at −20°C for 30 days significantly underestimated the real concentration of the metabolite in the blood, while the concentration of urea was significantly overestimated. Samples that had been frozen for 15 days showed the same trends but the differences were not statistically significant (Fig. 1). Samples that were kept at 4°C for 96 hr did not give concentrations significantly different for any of the five metabolites.

Our results show that it cannot be assumed that concentrations estimated from plasma that has been kept frozen at −20°C accurately represent the original concentrations in the blood. Not all metabolites are affected by freezing, and the effect of freezing can increase the concentrations of some metabolites while decreasing that of others. Our results also point out that the

time the samples have been kept frozen might have some effect on concentrations. Also, freezing the plasma had the same effect on the concentrations of blood metabolites of two very different species.

If possible, measurement of concentration of metabolites in plasma should be made as soon as possible without freezing the samples. Today, portable analysers permit the carrying out of plasma analyses in most field conditions (Bairlein and Totzke, 1992). It is better to store the plasma at 4°C for up to 4 days as this has no noticeable effect on the concentrations measured. If measurements cannot be carried out soon after taking the samples, and these have to be frozen, a test should be done on the effect of freezing and the time kept frozen on the metabolites of interest, to be able to correct the estimates. Our results suggest that if the tests cannot be done with blood of the species of interest, results from other species could be used.

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References

Bairlein F. and Totzke U. (1992) New aspects on migratory physiology of trans-Saharan passerine migrants. *Ornis Scand.* **23,** 244–250.

Ferrer M. (1992) Regulation of the period of postfledging dependence in the Spanish Imperial Eagle *Aquila adalberti*. *Ibis* **134,** 128–133.

González J. L. and Hiraldo F. (1991) Some hematological data from Marsh Harriers (*Circus aeruginosus*) in central Spain. *Comp. Biochem. Physiol.* **100A,** 735–737.

Jeffrey D. A., Peakall D. B., Miller D. S. and Herzberg G. R. (1985) Blood chemistry in food-deprived herring gulls. *Comp. Biochem. Physiol.* **81A,** 911–913.

Jenni L. and Jenni-Eiermann S. (1992) Metabolic patterns of feeding, overnight fasted and flying night migrants during autumn migration. *Ornis Scand.* **23,** 251–259.

Jenni-Eiermann S. and Jenni L. (1991) Metabolic responses to flight and fasting in night-migrating passerines. *J. comp. Physiol. B* **161,** 465–474.

Jenni-Eiermann S. and Jenni L. (1992) High plasma triglyceride levels in small birds during migratory flight: a new pathway for fuel supply during endurance locomotion at very high mass-specific metabolic rates? *Physiol. Zool.* **65,** 112–123.

Marsh R. L. (1983) Adaptations of the gray catbird *Dumetella carolinensis* to long distance migration: energy stores and substrate concentrations in plasma. *Auk* **100,** 170–179.

Minick M. C. (1986) Correlation of simultaneously determined plasma insulin and glucose levels in fasting and mildly or severly stressed raptors. *Comp. Biochem. Physiol.* **83A,** 301–305.

Mori J. G. and George J. C. (1978) Seasonal changes in serum levels of certain metabolites, uric acid and calcium in the migratory Canada Goose (*Branta canadensis interior*). *Comp. Biochem. Physiol.* **59B,** 263–269.

Polo F. J., Celdrán J. F., Peinado V. I., Viscor G. and Palomeque J. (1992) Hematological values for four species of birds of prey. *Condor* **94,** 1007–1013.

Puerta M. L., Huecas V. and García del Campo A. L. (1989a) Hematology and blood chemistry of the chilean flamingo. *Comp. Biochem. Physiol.* **94A,** 623–625.

Puerta M. L., Muñoz Pulido R., Huecas V. and Abelenda M. (1989b) Hematology and blood chemistry of chicks of white and black storks (*Ciconia ciconia* and *Ciconia nigra*). *Comp. Biochem. Physiol.* **94A,** 201–204.

Puerta M. L., Alonso J. C., Huecas V., Alonso J. A., Abelenda M. and Muñoz Pulido R. (1990) Hematology and blood chemistry of wintering common cranes. *Condor* **92,** 210–214.

Puerta M. L., García del Campo A. L., Abelenda M., Fernadez A., Huecas V. and Nava M. P. (1992) Hematological trends in flamingos *Phoenicopterus ruber*. *Comp. Biochem. Physiol.* **102A,** 683–686.

Reynolds J. (1982) Proteínas del plasma y grupos sanguíneos ABO y Rh del gorrión común (*Passer dometicus*) en dos localidades de Costa Rica. *Rev. Biol. Trop.* **30,** 73–78.

Saarela S., Hissa R., Etches R., Balthazart J. and Rintamäki (1986) Seasonal changes in some plasma hormones in pigeons from different environments. *Comp. Biochem. Physiol.* **84A,** 25–31.

SAS (1988) *SAS/STAT User's Guide*. Version 6. 4th Edition. SAS Institute Inc. Cary, NC