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Profound changes in blood parameters during torpor in a South American marsupial

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ABSTRACT

Seasonal torpor or hibernation is a phenomenon characterized by a physiological transition to dormancy (torpor) during challenging periods in terms of energy availability or metabolic load. Extensive physiological reprogramming and changes in gene-expression, immune function, oxygen transport and intermediate metabolism, occur during eutherian hibernation. Here we studied the seasonality of blood parameters, and during daily torpor, in a South American marsupial (*Dromiciops gliroides*). Seasonal trends in blood parameters showed an increase in hematological parameters during winter, and increases in total proteins, albumin and globulin during autumn. In contrast, torpor induced a drastic drop during most blood parameters. PCV dropped significantly 60%, as well as RBC (58%), hemoglobin concentration (58%), WBC (79%), including neutrophils (51%), eosinophils (84%) and lymphocytes (82%). Biochemical parameters also showed reductions: triglycerides (81%), proteins (32%), albumin (24%), globulins (38%), albumin (24%), creatinine (48%) and glucose (42%). Our results confirm some patterns observed in hibernating eutherians, such as leukopenia, probably caused by sequestration of white blood cells in organs. However, red blood cells and hemoglobin concentration also were reduced, which is to the best of our knowledge has not been reported for marsupials. The observed reduction in biochemical parameters suggests that marsupials, as in eutherians, change from carbohydrate-based to lipid-based metabolism during hibernation. However, the absence of increases in beta-hydroxybutyrate is puzzling. Finally, we found an increase (although non-significant after statistical correction for multiple comparisons) of creatine kinase which together with an increase in neutrophil/lymphocyte ratio could be indicative of muscle lysis and inflammation. These results indicate profound changes in standard physiological processes during torpor.

1. Introduction

Physiological heterothermy (i.e., seasonal and daily torpor) is a widespread energy-saving strategy in birds and mammals, characterized by an interruption of the normal biological functions (McKechnie and Lovegrove, 2002; Carey et al., 2003). The most evident characteristic of an animal experiencing seasonal (=hibernation) or daily torpor is its low body temperature, which is in general, close to environmental temperature. However, during hibernation, animals also experience a drastic reduction in heart rate, respiration rate, and metabolic rate (Carey et al., 2003; Nespolo et al., 2010). The widespread use of hibernation in birds and mammals of relatively small size (McKechnie and Lovegrove, 2002; Melvin and Andrews, 2009) seems to indicate that this is a physiological adaptation for periods of small food offer and/or high-energy demands (i.e., cold). Systemic adjustments in hibernating animals, in addition to reductions of temperature, respiratory frequency and metabolic rate, have been mostly documented in eutherian mammals (Bouma et al., 2010a; Otis et al., 2011). During hibernation blood flow is re-distributed and blood-clotting time, leukocite and red-blood cells concentration are reduced to 10% of normal values (Yasuma et al., 1997; Boyer and Barnes, 1999; Bouma et al., 2010a,b). These reductions are probably related with cell sequestration in organs such as liver, lungs and spleen (Bouma et al., 2010a). Also, energy expenditure changes from carbohydrate-based in active animals, to lipid-based metabolism in torpor, with subsequent changes during plasma triglycerides and glucose (Cherel et al., 1995; Buck and Barnes, 2000; LeBlanc et al., 2001; Otis et al., 2011). Because of its role in maintaining membrane fluidity at low temperatures, cholesterol is released to the bloodstream during torpor (Geiser and Kenagy, 1987; Otis et al., 2011).

In vertebrates other than eutherian mammals, biochemical and haematological changes during hibernation are scarcely known. In hibernating echidnas, for instance, red-cell hemoglobin concentration appears to be increased (Andersen et al., 2000), whereas in reptiles, an increase in leukocytes has been observed during cold hardiness (Albadry et al., 1992). In contrast, Palenske and Saunders (2003) did not find hematological changes after comparing hibernating and non-hibernating bullfrogs. In marsupials, Wells et al. (2000) seasonally compared hematological patterns in the brushtail possum and found increased leukocytes during winter but no change in red blood cells. Also

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Hallam et al. (1995) studied dasyurid marsupials and found relatively high haematocrit and haemoglobin concentration, compared to eutherian values. However, we have been unable to find studies of blood changes during daily or seasonal torpor in marsupials.

Given that hibernation is seasonal, an appropriate experimental design aimed to determine physiological changes associated with hibernation needs to separate the effects of seasonality, from torpor itself. In fact, it is well described that many physiological parameters increase in winter but are reduced during acute torpor, such as leukocyte counts (Hissa et al., 1994; Bouma et al., 2010a). In this paper, we analyzed a suite of blood biochemical and haematological parameters (Table 1) in a South American marsupial (*Dromiciops gliroides*) that undergo seasonal as well as daily torpor (Bozinovic et al., 2004; Nespolo et al., 2010). We sampled active individual maintained at semi-outdoor conditions during the four seasons. In addition, in a different experiment we sampled active and torpid individuals in a repeated-measures configuration, during summer.

2. Materials and methods

Procedures associated with capture and animal handling were approved and authorized by the Chilean Agriculture and Livestock Bureau (Servicio Agrícola y Ganadero). Thirteen adult individuals of *Dromiciops gliroides* were captured near Valdivia, Chile (39°48′S, 73°14′W; 9 m) during the austral summer in December–January 2011, using Tomahawk-style live traps (30×10×10 cm, local manufacture). Traps were placed in trees and shrubs 1–2 m above ground and baited with bananas and checked daily at dawn, and captured individuals were transported to the laboratory on the day of capture. Animals were maintained as described before (Franco et al., 2012), in a climate controlled chamber at 20 ± 1 °C (standard error), with a 12:12 h photoperiod for two weeks. After this period, animals were placed in a semi-outdoor enclosure exposed to natural temperature and photoperiod as in Merritt et al. (2001). In brief, animals were enclosed in individual cages and sheltered from rain with an acrylic covering (without insulation) that maintained similar light and temperature conditions of their natural ambient. Ambient temperatures within the enclosures in summer were: 24.0 ± 2.8 °C (n = 68), autumn: 10.0 ± 0.6 °C (n = 85), winter: 11.8 ± 0.3 °C (n = 67), spring: 17.4 ± 0.6 °C (n = 57). Water and food (a mixture of peach compote, strawberry baby food and mealworms) were available ad libitum.

To determine the seasonal trends in blood parameters, we took samples in May, August, November and February, in adult, active (non-torpid) individuals. For the torpor experiment, blood was taken from a (different) sample of thirteen individuals, twice, during a period of two weeks: when active and during torpor. This experiment was performed the summer after the seasonal sampling.

To induce torpor, animals were exposed to 5 ± 1 °C and food was withdrawn for 12 hours. In this species, this treatment induces torpor in nearly 100% of the cases (Bozinovic et al., 2004; Nespolo et al., 2010). Torpid animals are readily identified as they look clearly lethargic and do not respond to handling, and their skin is unequivocally cold. Blood samples were taken either from the tail vein in active animals or directly via cardiac puncture in torpid individuals using 23-ga needles, and dividing it in micro-hematocrit tubes with EDTA and heparin-tubes for biochemical and hematological analyses, respectively. Samples were transferred and stored in Eppendorf ® tubes and kept cool on ice, until processing. The whole sampling process was completed in less than 10 minutes, usually 6–7 min. In two cases this time limit was surpassed and samples were discarded and sampling was repeated the day after.

2.1. Hematological analyses

Blood samples were placed in heparinized microcapillary tubes, sealed with wax at the bottom end and were centrifuged at 1500 *g* for 10 min, before being deposited directly in a microhaematocrit reader, to estimate hematocrit (or packed cell volume, PCV %). Hemoglobin concentration (Hb) was determined photometrically after hemolysis in Drabkin' solution (cyanohemoglobine method), using a Hitachi 4020 colorimeter (Roche, Germany) previous centrifugation at 400 *g* for 5 min to remove cellular detritus. Total erythrocytes (RBC) and leukocytes (WBC) were analyzed in an improved Neubauer chamber after dilution (1:200) in Natt and Herrick's dyeing solution (Natt and Herrick, 1952). RBC total number was obtained by counting the number of cells in the five small-squares of the large central square of the Nebauer chamber, and multiplying the raw data by 10,000 to obtain the final values. WBC total number was determined by counting all leukocytes in the chamber, and multiplying the raw data by 220 to obtain the final values. The proportion of different types of WBC was assessed on the basis of an examination of a total of 100 leukocytes under oil immersion. For this purpose, a drop of blood was smeared on one microscope slide, air-dried, fixed in absolute

Table 1
Haematological and biochemical parameters measured.

Parameter	Abbreviation	Meaning
Red blood cell concentration	RBC	Erythrocyte concentration in plasma. Measures oxygen-transport capacity.
Packed cell volume (haematocrit)	PCV	Volumetric proportion of erythrocytes in plasma. Oxygen-transport capacity.
Haemoglobin concentration	Hb	Total haemoglobin concentration in plasma. Oxygen-transport capacity.
Mean cell volume (in red blood cells)	MCV	Mean volume of erythrocytes, calculated from PCV and RBC (see methods). Inversely related with oxygen-transport capacity in small mammals (Rosenmann and Ruiz, 1993).
Mean cell Hb concentration	MCHC	Obtained from the ratio Hb/PCV. Oxygen-transport capacity.
White blood cell concentration	WBC	Total concentration of leukocytes, indicative of immune activity (increased during infection).
Neutrophil concentration	Neutrophils	The most abundant leukocyte. Levels increase during inflammation.
Lymphocyte concentration	Lymphocytes	Leukocyte type associated with specific immune response and viral infection. Its levels are reduced during inflammation.
Neutrophil: lymphocyte ratio	N/L	Index of inflammation.
Eosinophil concentration	Eosinophils	Leukocyte type associated with parasitic infection.
Monocyte concentration	Monocytes	Phagocytic leukocytes, the first line of response to pathogens. They can mature into macrophages.
β-Hydroxybutyrate	BHB	A ketone body, produced by lipid metabolism. It increases when animals use their lipid reserves.
Cholesterol concentration	Cholesterol	Indicator of circulating lipids (nutritional status). It maintains membrane fluidity at low temperatures in hibernators (Geiser and Kenagy, 1987)
Triglyceride concentration	Triglycerides	Main form of energy store in vertebrates. Indicative of nutritional status.
Total protein concentration	Proteins	Concentration of proteins in the plasma. Indicative of general nutritional status.
Albumin concentration	Albumin	Main binding protein in the plasma. Indicative of general nutritional status. It is increased in dehydrated individuals.
Globulin concentration	Globulins	The second most abundant protein in plasma. A broad family of proteins associated with general immune function.
Creatine kinase	CK	Muscle enzyme. It is increased after damage to skeletal and/or cardiac muscles.
Creatinine concentration	Creatinine	A by-product of muscle metabolism, indicative of kidney function. High levels of creatinine indicate damage in nephrons.
Glucose concentration	Glucose	Main form of usable energy in vertebrates. Indicative of nutritional status.