Hibernation in *Duttaphrynus melanostictus*, a life in the cold lane

SUMAN PRATIHAR[1,2] and JAYANTA KUMAR KUNDU[1]

[1] *Molecular Biology Research Unit, Department of Zoology, Vidyasagar University, Midnapore, West Bengal, India.*

[2] Corresponding author: pratihar_vu@rediffmail.com

ABSTRACT - Hibernation occurs with exposure to low temperatures and, under normal conditions, during winter seasons when there are lengthy periods of low environmental temperatures. This investigation reports the effect of hibernation on the blood-plasma and biochemical parameters - plasma protein, plasma glucose, cholesterol, blood haemoglobin, bilirubin thyroid hormone and serum ion in the Indian common toad (*Duttaphrynus melanostictus*). Metabolic rate and blood cell count was also investigated to elucidate adjustments in hibernation. Adaptation during hibernation is crucial for the toad's winter survival.

HIBERNATION is a unique phenomenon of depressed metabolism that enables different animals, including anurans, to survive during hostile winter temperatures. Metabolic rate is strongly reduced during this time and the hypothalamic set point for core body temperature is lowered. Amphibians are a sensitive group of vertebrates adapting to small changes in temperature and moisture (Duellman & Trueb, 1986). Changes in global weather patterns (e.g. El Ninos or global warming) can alter their breeding behaviour, affect reproductive success, decrease immune function and increase sensitivity to chemical contaminants (Kats & Ferrer, 2003; Vredenburg 2004). Understanding natural reactions like hibernation to climate shifts is therefore important to further understanding amphibian life cycles.

Duttaphrynus melanostictus (Schneider, 1799) is the most common and widely distributed anuran species in the Midnapore region. It regularly hibernates and its breeding period ends in the middle of autumn. Heart rate, metabolic rate and haemoglobin concentration of hibernating toads can decrease significantly during this period. This study aimed to focus on the role of different biochemical parameters in metabolic depression and the regulatory controls that allow these energy-expensive processes to be suppressed when organisms enter a hypometabolic state.

MATERIALS AND METHODS

Ten adult *Duttaphrynus melanostictus*, each weighing between 80 to 100 g, were collected from a selected site at Midnapur (22015 N / 87039 E), West Bengal India in late October and mid November, 2010 (ambient air temperature 22.4°C) before the onset of hibernation. A second group (n = 10) were collected in mid January (7.4-9.2°C), from the same mud hole site, when hibernation was at its deepest. A third group (n = 10) was collected from the same site in late February (20.0°C) when the toads were aroused from hibernation. Other groups were collected from similar sites throughout the remaining months of the year (March, April, June, and July; n = 10 for all groups).

Blood samples were drawn immediately after animals were euthanased. Animal handling and euthanasia was performed following the ethical guidelines laid down by the Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) constituted by the Animal Welfare Division of Government of India on the use of animals in scientific research.

Blood samples were collected by cardiac puncture using a 21 gauge needle and a 5 ml syringe. These were transferred to EDTA coated vacutainer tubes for determination of protein, and in sodium fluoride coated vacutainer tubes for determination of glucose. Plasma was separated by centrifugation (1500×g) and the supernatant was taken for biochemical analysis. Plasma protein was estimated photometrically using a standard mathematical projection predicted against the known protein levels following the Lowry method

(Lowry et al., 1951). Plasma glucose was estimated photometrically by glucose-oxidase protocol using Merck diagnostic equipment (Merck-Diagonistica-PDLFT0879). Serum cholesterol was also analysed using standard laboratory equipment (Nice chemical; S20750). Serum T3 and T4 were analysed by using ELISA kit (Ranbaxy, Diagnova.1063062.1063073). Serum magnesium and calcium were analysed using Bioassay's Quantichrom™, Magnesium (DIMG-250) and Calcium (DICA-500) assay kit.

A cardiogram was used to record mechanical activities of the heart as it records the systole and diastole of the different chambers of the heart. The mechanical activities of the amphibian heart were directly recorded by connecting the heart to the writing lever with a thread which transmits the waves of contraction and relaxation from the heart to the liver. The cardiac activities were recorded on a moving drum. Metabolic rate was measured following the methods described by Navas (1996) with few modifications. Metabolic rate was measured within 10 days of capture, using only animals that appeared to be in good health. Animals were fed commercial crickets with vitamin and mineral supplements. To measure the resting metabolic rate, animals were placed in a metabolic chamber in a constant temperature for six hours to adjust the new environmental conditions. Air samples were then taken with a syringe and oxygen concentrations measured.

Haemoglobin was measured by using Bioassay System Quantichrom™ haemoglobin assay kit. This technique was based on the Triton/ NaOH method, in which haemoglobin is converted into a uniform colored end product. The intensity of colour, measured at 400 nm was directly proportional to the haemoglobin concentration in each sample. Direct Bilirubin reacts with the diazotized sulphanilic acid in aqueous solution to form Azobilirubin, a purple coloured compound, within one minute. The subsequent addition of methanol accelerated the reaction of unconjugated bilirubin in the serum, and a value for total bilirubin was obtained after letting each specimen stand for 30 minutes. Serum was used to quantitatively determine bilirubin in blood.

Total Count of Erythrocytes

4.0 ml of red cell diluting fluid (Trisodium citrate – 3.13 g, commercial formaldehyde – 1.0 ml, distilled water – 100 ml) was taken into an Erlenmeyer flask (25 ml). 0.2 ml of anti-coagulated blood sample was then added to the diluting fluid. Then a small drop of diluted blood was taken into the counting chamber of a haemocytometer. The counting chamber was placed under the microscope and numbers of red cells counted on a small square (0.2 x 0.2 x 0.04 sq. mm.) of the upper left of corner which was divided into 16 smaller squares to facilitate counting. Total cells were counted in five squares. The same was repeated on the other side of the chamber and an average of the two chambers was taken.

Total Count of Leucocytes

0.38 ml of white cell diluting fluid (glacial acetic acid – 2 ml, distilled water – 100 ml, aqueous methylene blue solution, 0.3% w/v – 10 drops) was placed into an Erlenmeyer flask (25 ml). 0.2 ml of anti-coagulated blood specimen was added into diluting fluid. The solution was mixed and kept for 2 minutes for complete haemolysis. Then a small drop of diluted blood was placed into the counting chamber of haemocytometer. The counting chamber was placed under the microscope and the numbers of white cells counted on the small square (1 mm^2) plate (16 x). Again total cells were counted in five squares. The same was repeated on the other side of the chamber and an average of two chambers was taken.

Differential White Blood Cell Count

A homogenous blood specimen was transferred with a pair of applicator sticks to a clean, grease free slide. A blood smear was drawn by a spreader slide at the angle of 300 to 450 between the two slides. The blood smear was dried quickly by air drying. Then the slide was stained with Leishman's stain for 2 minutes. The buffered water was added on the slide of about the volume of stain and was kept for 10 to 15 minutes. The stain was washed off and the slide was observed under oil immersion microscope. The cells were counted to the nearest 100 and the number was expressed as a percentage.

Serum Immunoglobulin

Nephelometry is a technique used in immunology to determine levels of IgM, IgG and IgA. It is performed by measuring the reduction in the intensity of the incident light after it passes through the sample being measured. It is based on the principle that a dilute suspension of small particles will scatter light (usually a laser) passed through it rather than simply absorbing it. The amount of scatter is determined by collecting the light at an angle (usually about 70 or 75 degrees).

Statistical analysis was performed using Microcal 6.0™ statistical analysis. Each biochemical experiment was replicated at least three times with 10 toads in each experimental group. A Student t-test was performed to compare the means of results to a significance of $P < 0.05$. Results herein are represented as mean ± SD unless mentioned otherwise.

RESULTS

Metabolic depression played a crucial role during the period of hibernation. During this phase metabolic rate decreased by 6% of the basal resting rate of the active animals. In active animals the metabolic rate was 0.117 ± 0.014 ml O_2g-1h-1 which decreased significantly to 0.110 ± 0.017 ml O_2g-1h-1 during the hibernating period. Body weight and heart rate also dropped during hibernation. Haemoglobin concentration was significantly reduced to 8.4 ± 0.18 g/dl from 9.2 ±.23 g/dl. On the contrary bilirubin concentration increased markedly from 0.89 ± 0.004 mg/dl to 0.93 ± 0.005 mg/dl during hibernating period. Plasma protein was significantly decreased in the hibernating toads compared with non-hibernating toads. Available reports indicate that cold exposure in winter inhibits protein synthesis in blood and causes changes in enzyme activities of *Lithobates pipiens* in the hibernating season (Churchill & Storey, 1993). Environmental cold stress increases protein catabolism. Sugar showed a significantly lower ($t = 12.21$, $P < 0.05$) concentration during the deep hibernation phase, compared to that in the non hibernating phase. Results showed, a marked increase in serum cholesterol during the hibernating phase (154 ± 2.56 mg/dl) compared with non hibernating (93 ± 2.26 mg/dl) periods. T3 levels changed little throughout the year but to the contrary T4 level varied significantly ($t = 15.53$, $P < 0.05$). TSH expression was found to reduce significantly ($t = 14.83$, $P < 0.05$) during hibernation. The number of total red blood cells (RBC) were less in hibernating toads compared with non hibernating toads. Increases in total count of white blood cells in hibernating individuals was possibly due to increases in first line defence during hibernation as has been shown for *Anaxyrus americanus* (Forbes et al., 2006). Eosinophil and lymphocyte number was greater in hibernating toads. Minimal changes were found in monocyte levels. Our results also indicate that IgM was significantly higher in hibernating individuals than non hibernating toads. It was clear from our findings that the serum magnesium ($t = 6.42$, $P < 0.05$) and calcium ($t = 5.25$, $P < 0.05$) differed significantly before hibernation and during deep hibernation.

DISCUSSION

The purpose of metabolic depression is to maximize the survival time of an individual when environmental conditions are unfavorable for normal life. Throughout episodes of hibernation metabolism must be sustained by endogenous fuel reserves alone and the problem of accumulated metabolic wastes must be addressed to prevent self poisoning. A new balanced body chemical state must be achieved that includes both a general reduction of rates of all cellular processes plus an induction of selected adjustments, specific to a depressed state. The mechanisms of metabolic depression appear to involve the coordinated reduction of a specific subset of key regulatory enzymes or proteins in the cell and activities of that process that are controlled.

For *Bufo spinulosus*, metabolic rate decreased during hibernation by 7.8% (at 50°C) and 13.6% (at 150°C) of summer value (Naya & Veloso, 2009). Heart rate and body weight of hibernating toads decreased significantly. Body weight reduction is a prominent feature throughout animals that experience hibernation. This is usually because hibernating animals solely depend on reserve foods that are stored in muscle and fat tissues under the skin. Milsom & Burlington (1993) also found that

in hibernating squirrels, vagal stimulation reduced heart rate in half of the animals and the amplitude of auricular and ventricular contraction decreased during hibernation.

In this study haemoglobin concentration was significantly reduced and this was probably due to reduced metabolic rate and oxygen consumption. No other haemoglobin deformities have been found in the band patterns of haemoglobin electrophoresis. To the contrary, bilirubin, the end product of haemoglobin metabolism, also significantly increased in the toads during hibernation. Baker & Breukelen (2009) also found similar results in the hibernating golden mantled ground squirrel. The recurring nature of metabolic rate depression as a survival strategy of animals suggests that the regulation of metabolic arrest has fundamental principles and mechanisms that are expressed not only in all cell types of an individual animal, but also, across phylogenetic lines.

Significant seasonal variations in plasma protein, plasma glucose and serum lipid profile have been found in hibernating *Duttaphrynus melanostictus* (Pratihar et al., 2006). The reason for this may be due to the qualitative and quantitative difference in the availability of food. The increased metabolic dependency towards lipids in hibernating toads may not only be in response to low temperature but is also a part of circanual homeostatic adjustment that is at least partly regulated by thyroid hormones (Pratihar & Kundu, 2009a). The decreased serum thyroxin in the toads suggested a state of hypometabolism and decreased metabolic rate that could allow further conservation of energy during hibernation. The decline in thyroid hormone concentration has a behavioural and physiological response that leads to an increased and sustained cholesterol level in serum, that seems to be the most important metabolite during the hibernating phase. Forbes et al. (2006) commented that a significant increase in total white blood cell count (WBC) occurs in response to an increase in bodily defense mechanisms during hibernation. Pratihar & Kundu (2010) found significant increases in WBC during hibernation for *D. melanostictus* compared to the non hibernating toads. Increased numbers of eosinophils also enables increased engulfment of antigen and antibody complexes. Lymphocytes were also more numerous in the hibernating individuals. The toads were producing γ-glubulin and an antibody that induces immune system response during hibernation. No significant changes in the number of monocytes however could be observed in the hibernating toads in this study. As such it appears that the migration of monocytes and formation of macrophage in different tissue play a rather insignificant role in the development of immunity during hibernation. IgM was significantly greater in hibernating individuals than non hibernating toads.

In this study it was clear that during hibernation the immunity of the organism was specifically antibody dependent. Cell mediated immunity plays a small role during that phase. Humoral immunity appeared to play a central role during hibernating period and cell mediated immunity also plays a smaller role during this period (Pratihar et al., 2008). The lower serum calcium levels indicated that during the entrance to hibernation period, and during the arousal from hibernation, the utilisation or excretion of calcium exceeded the mobilisation of calcium. It is therefore possible that during hibernation the circulation and the kidney function is not consistently adequate to meet the tissue demands for calcium and at the same time maintain the constant serum level of calcium. Serum chloride ion and bicarbonate ions do not play significant roles in temperature regulation throughout the hibernating phase (Pratihar & Kundu, 2009b). An investigation on the causes of changes in magnesium ions and passive alteration in water balance would further this study because such changes may be due to the excess water that is being removed by the renal tubules.

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