

Metabolic switching  
during the period of hibernation  
in *Duttaphrynus melanostictus* (SCHNEIDER, 1799)  
from India  
(Anura: Bufonidae)

Umstellungen im Stoffwechsel bei indischen Schwarznarbenkröten  
*Duttaphrynus melanostictus* (SCHNEIDER, 1799) während der Winterruhe  
(Anura: Bufonidae)

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KURZFASSUNG

Hibernation ist ein winterlicher Ruhezustand. Zwei Formen von Umweltstress - Nahrungsmangel und niedrige Umgebungstemperaturen - charakterisieren die Bedingungen, mit denen Amphibien zurecht kommen müssen, um diese Ruhephase zu überstehen. Die vorliegende Untersuchung berichtet über die Wirkungen der Winterruhe auf verschiedene biochemische Parameter des Blutplasmas, und zwar die Plasmaproteinfraktionen SGOT und SGPT, die alkalische Phosphatase, das Cholesterol und die Lipase bei der indischen Schwarznarbenkröte *Duttaphrynus melanostictus* (SCHNEIDER, 1799).

Die SGOT- und SGPT-Werte waren bei hibernierenden Kröten signifikant vermindert und die Cholesterol- und Lipasewerte des Blutes erhöht. Die während der Winterruhe verstärkte metabolische Abhängigkeit von Fetten ist nicht nur eine Wirkung der niedrigen Temperaturen sondern Teil der während des Jahres ablaufenden homöostatischen Regulationsvorgänge.

ABSTRACT

Hibernation is winter dormancy. Two different environmental stresses, a lack of food and low ambient temperatures, characterize the conditions amphibians must contend with in order to survive the winter dormancy. The present investigation reports the effect of hibernation on different blood-plasma biochemical parameters, viz. plasma protein's fraction, SGOT, SGPT, alkaline phosphatase, cholesterol, and lipase in the Indian Common Toad *Duttaphrynus melanostictus* (SCHNEIDER, 1799).

Results clearly indicate that the SGOT, SGPT decreased significantly in hibernating toads with a concomitant significant increase in blood cholesterol, and lipase. During hibernation, increased metabolic dependency towards lipids is not only a response to low temperature, but part of circannual homeostatic adjustment. Thus, during hibernation, metabolic switching appears to be essential for survival.

KEY WORDS

Amphibia: Anura: Bufonidae, *Duttaphrynus melanostictus*, hibernation, overwintering, physiology, metabolism, Midnapur, West Bengal, India

INTRODUCTION

To many amphibians and reptiles the ability to avoid seasonal and periodic environmental rigors by entering a state of metabolic inactivity is a crucial element in their survival. Specifically, winter dormancy and summer estivation — the usual context in which these terms are applied to ectotherms — permit these animals to survive and flourish, first, by reducing the impact of sea-

sonal extremes and, second, by significantly lowering the ectotherm's energetic costs during times that would not be favorable for activity (that is, when food is rare or not available).

Hibernation is a physiological and behavioral phenomenon of depressed metabolism, which enables different animals across phyla, particularly anurans, to survive

during hostile temperature during winter season. Metabolic rate is strongly reduced and the hypothalamic set point for core body temperature is lowered (PRATHAR & KUNDU 2010). Increased urea concentration in the blood acts as cryoprotectant agent, which helps the hibernating animals to sustain during winter (PRATHAR & KUNDU 2007). Increased metabolic dependency towards lipids in hibernating condition is not only response to low temperatures but part of the circannual homeostatic adjustment that is at least partly regulated by thyroid hormones (PRATHAR & KUNDU 2009). Available reports indicate that environmental cold stress in hibernating toads causes significant changes in plasma-protein and glucose

(CHURCHILL & STOREY 1993; EDWARDS et al. 2004; COSTANZO & LEE 2005) blood urea (COSTANZO & LEE 2005) and in SDS-PAGE (= sodium dodecyl sulfate polyacrylamide gel electrophoresis) pattern (DAS et al. 2004; BULBUL & KUTRUP 2007). Serum enzyme levels play a crucial role during hibernation.

*Duttaphrynus melanostictus* (SCHNEIDER, 1799) is a species of toad that is common in South Asia. It inhabits southwestern and southern China (including Taiwan and Hainan), and occurs throughout southern Asia, from northern Pakistan and Nepal through India to Sri Lanka, Andaman Islands, Sumatra, Java, Borneo and Bali. In this study, metabolic switching during hibernation was investigated.

## MATERIALS AND METHODS

Twenty adult Common Indian Toads *Duttaphrynus melanostictus* (SCHNEIDER, 1799), each weighing 100-120 g, were collected from a selected site in Midnapur (Midnapore), West Bengal, India (22°15'N 87°39'E) throughout the year as hibernating (January-March,  $n = 5$ ), in stages of arousal from hibernation (April-May,  $n = 5$ ), reproductive (June-August,  $n = 5$ ) and pre-hibernating phase (September-December,  $n = 5$ ). Blood samples were drawn via cardiac puncture at the time of euthanasia, using a sterile hypodermic syringe, and collected in tubes. Animals handling was in accordance with the ethical guideline laid down by the committee for the purpose of control & supervision of experimental animals (CPC-SEA) constituted by the Animal Welfare Division of the Government of India on the use of animals in scientific research.

Plasma protein concentration was estimated photometrically using a standard curve against a known protein (BSA, Bovine Serum Albumin) following Lowry's method for protein quantitation (LOWRY et al. 1951). For the photometric determination of alanine aminotransferase (ALAT) (= ALT - Alanine transaminase = SGPT = serum glutamic pyruvic transaminase) based on the reference method of the International Federation of Clinical Chemistry, the rate of NADH (= Nicotinamid-Adenin-Dinukleotid) con-

sumption, which is directly proportional to the ALAT activity in the sample, was measured photometrically (340 nm). The photometric determination of aspartate transaminase (AST = SGOT = serum glutamic oxaloacetic transaminase) was based on the reference method of the International Federation of Clinical Chemistry (Ecoline, Merck™ diagnostic kit). The rate of NADH consumption was measured photometrically (340 nm) and directly proportional to the AST activity in the sample.

Serum Cholesterol was also analyzed by using a standard kit (Merck™-Diagnostica PDLFT0189). Cholesterol reacts with ferric chloride in the presence of acetic acid. The optical density (extinction) of the red color thus produced was measured colorimetrically at 560 nm.

The QuantiChrom™ Alkaline Phosphate Assay Kit (DALP-250) was used to measure alkaline phosphate activity directly in the biological sample without pretreatment. This method utilizes p-nitrophenyl phosphate that is hydrolyzed by alkaline phosphate, resulting in a yellow colored product (maximal absorbance at 405 nm). The rate of the reaction is directly proportional to the enzyme activity.

Serum lipase was analyzed by using a Merck™ kit. Plasma protein fractions were analyzed after SDS-PAGE (sodium dodecyl

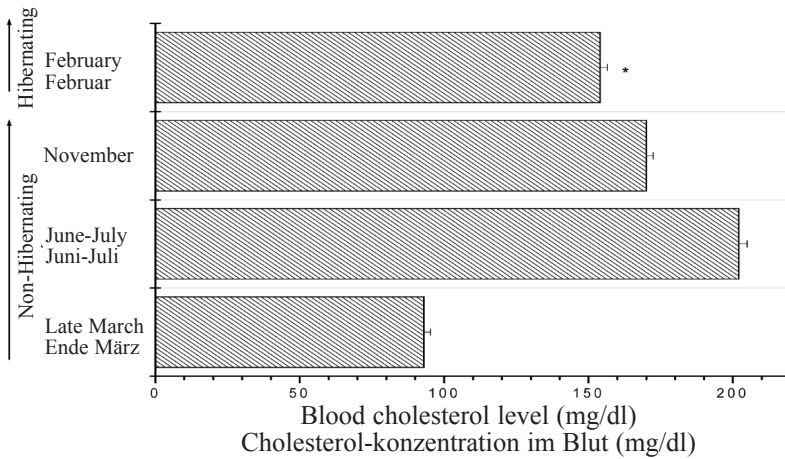


Fig. 1: Bar diagram showing changes in the blood cholesterol level (mg/dl) of *Duttaphrynus melanostictus* (SCHNEIDER, 1799) from India during different annual activity phases, hibernation included.

A significant ( $* P < 0.05$ ) difference was detected in the blood cholesterol levels of hibernating (February,  $n = 5$ ) versus non-hibernating ( $n = 15$ ) individuals.

Abb. 1: Balkendiagramm zur Darstellung der Veränderungen im Serum-Cholesterolspiegel (mg/dl) bei indischen *Duttaphrynus melanostictus* (SCHNEIDER, 1799) während unterschiedlicher Aktivitätsphasen im Jahresverlauf einschließlich der Winterruhe. Der Cholesterol-Wert im Blut der überwinternden (Februar,  $n = 5$ ) Individuen war gegenüber dem der nicht überwinternden ( $n = 15$ ) Individuen signifikant ( $* P < 0.05$ ) verschieden.

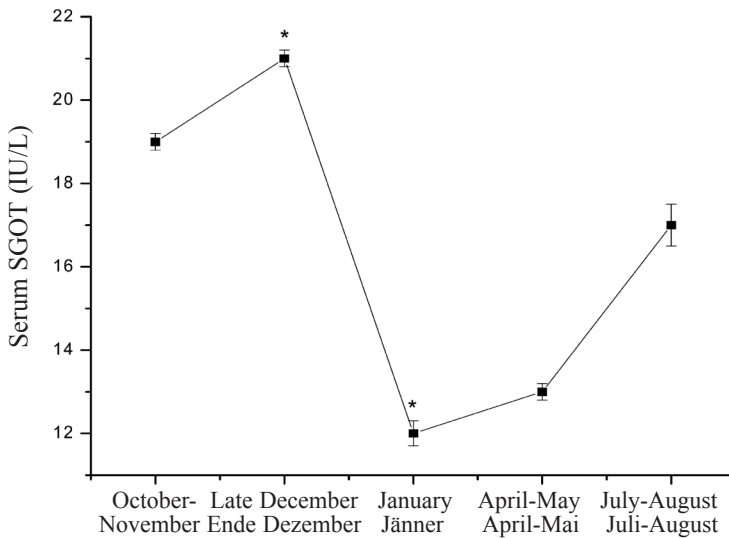


Fig. 2: Variation of serum SGOT concentration (IU/L) throughout the year in *Duttaphrynus melanostictus* (SCHNEIDER, 1799) from India. A significant ( $* P < 0.05$ ) difference was detected in the serum SGOT levels of hibernating (January,  $n = 5$ ) versus non-hibernating ( $n = 15$ ) individuals.

Abb. 2: Veränderungen im Serum-SGOT-Spiegel (IU/L) bei indischen *Duttaphrynus melanostictus* (SCHNEIDER, 1799) im Jahresverlauf. Der Serum SGOT-Wert der überwinternden (Jänner,  $n = 5$ ) Individuen war gegenüber dem der nicht überwinternden ( $n = 15$ ) Individuen signifikant ( $* P < 0.05$ ) verschieden.

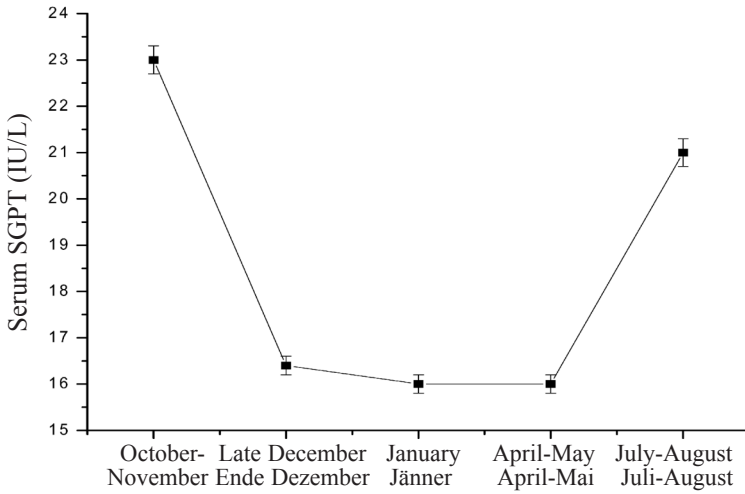


Fig. 3: Variation of serum SGPT concentration (IU/L) throughout the year in *Duttaphrynus melanostictus* (SCHNEIDER, 1799) from India. A significant difference in the serum SGPT level was not detected between hibernating (January,  $n = 5$ ) versus non-hibernating ( $n = 15$ ) individuals.

Abb. 3: Veränderungen im Serum-SGPT-Spiegel (IU/L) bei indischen *Duttaphrynus melanostictus* (SCHNEIDER, 1799) im Jahresverlauf. Der Serum SGPT-Wert der überwinternden (Jänner,  $n = 5$ ) Individuen war gegenüber dem der nicht überwinternden ( $n = 15$ ) Individuen nicht signifikant verschieden.

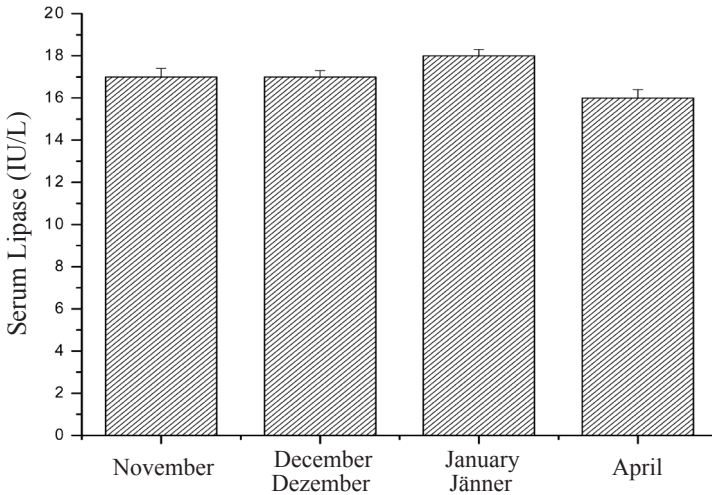


Fig. 4: Serum lipase (IU/L) expression in *Duttaphrynus melanostictus* (SCHNEIDER, 1799) from India during hibernating (January) and non-hibernating (November, December, April) phase. A significant difference in the serum lipase level was not detected between hibernating (January,  $n = 5$ ) versus non-hibernating ( $n = 15$ ) individuals.

Abb. 4: Die Konzentration der Serum-Lipase (IU/L) bei indischen *Duttaphrynus melanostictus* (SCHNEIDER, 1799) während (Jänner) und außerhalb (November, Dezember, April) der Winterruhe.

Der Serum Lipase-Wert der überwinternden (Jänner,  $n = 5$ ) Individuen war gegenüber dem der nicht überwinternden ( $n = 15$ ) Individuen nicht signifikant verschieden.

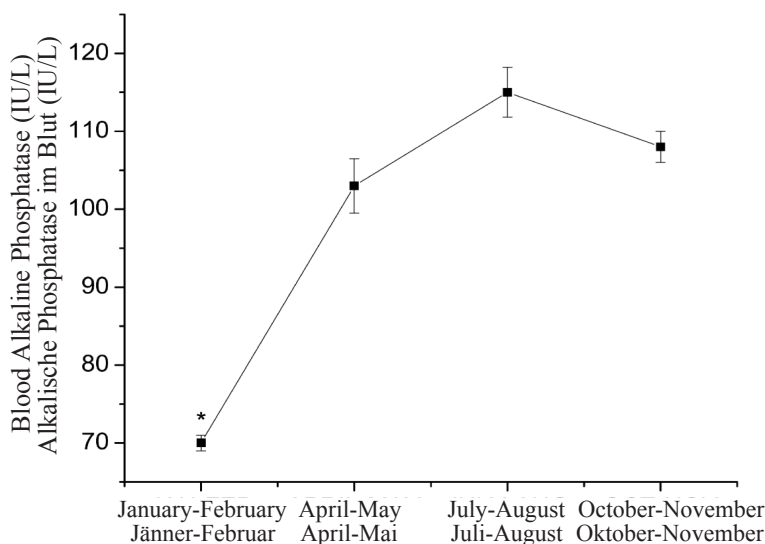


Fig. 5: Blood alkaline phosphatase (IU/L) levels throughout the year in *Duttaphrynus melanostictus* (SCHNEIDER, 1799) from India. A significant (\*  $P < 0.05$ ) difference was detected in the blood alkaline phosphatase levels of hibernating (January-February,  $n = 5$ ) versus non-hibernating ( $n = 15$ ) individuals.

Abb. 5: Die Konzentration der alkalischen Phosphatase (IU/L) im Blut von indischen *Duttaphrynus melanostictus* (SCHNEIDER, 1799) im Jahresverlauf. Der Wert der alkalischen Phosphatase überwinternder (Jänner-Februar,  $n = 5$ ) Individuen war von dem der nicht überwinternden ( $n = 15$ ) Individuen signifikant (\*  $P < 0.05$ ) verschieden.

sulfate polyacrylamide gel electrophoresis) and scanned through a UV-spectrophotometer.

Statistical analysis was done by using MicroCal™ Origin 6.0, statistical software

package. Each biochemical experiment was performed at least three times, with five toads in each experimental group. Student's t-test was performed to compare the means, with a level of significance of  $P < 0.05$ .

## RESULTS

During the pre-hibernation state in the month of November, the toads reserved fat materials and maintained the cholesterol level ( $170 \pm 2.43$  mg/dl). During hibernation they depleted the reserve fat, and during late hibernation in mid-February the cholesterol concentration was  $154 \pm 2.56$  mg/dl (Fig. 1).

SGOT (serum glutamic oxaloacetic transaminase = AST) was found to be significantly ( $P < 0.05$ ) different between early hibernation and deep hibernation (Fig. 2). Before onset of hibernation, SGOT was  $19 (\pm 0.2)$  IU/L, in the month of November, it was further increased to  $21 (\pm 0.2)$  IU/L in December when the animals had just entered hibernation. SGOT expression was

also significantly ( $P < 0.05$ ) decreased to  $12 (\pm 0.3)$  IU/L during deep hibernation in the month of January.

SGPT expression was low throughout the hibernation (Fig. 3). Serum lipase was expressed constantly throughout the year (Fig. 4). Expression of alkaline phosphatase was significantly low during the hibernating period (Fig. 5).

During hibernation the plasma albumin concentration was significantly reduced to 62% whereas the plasma globulin fractions significantly increased. It was also found that the gamma globulin (antibody) expression increased from 2.7% to 3.3% during the hibernating period.

## DISCUSSION

From a previous study, it is clear that during hibernation there is a significant reduction of plasma protein and blood sugar concentration (PRATHAR et al. 2006). At the late phase of hibernation, toads usually exploited their reserve fat as a main energy source. At that time of hibernation, toads generally did not eat or drink. As a result, the body weight was reduced almost exclusively due to loss of body fat. Serum lipid concentration was also low immediately after arousal from hibernation, but increased when feeding commenced. On the other hand, during the hibernating phase (January-February), urea concentration was 149.8 mg/dl. Urea level elevation plays a significant role in maintaining the toads' physiological status, particularly during the hibernating period. During hibernation, urea works as a cryoprotectant agent (PRATHAR & KUNDU 2007). The expression pattern of alkaline phosphatase confirmed that no liver dysfunction occurred during the period of hibernation. Behavior of SGOT and SGPT indicated some catabolism of the amino acids during the early

hibernation phase, which may be due to an excess of amino acids that were accumulated as a result of storage of nutrients prior to hibernation. After the initial phase of hibernation, SGOT declined significantly because of protein depletion, and metabolism was lipid dependent therefore, lipase activity was recorded unaltered throughout the hibernating phase. Similar observations were made by CHAKI et al. (2008) in aestivating snails. HOCHACHKA (2002) reported that during aestivation utilization of oxaloacetic acid occurs more than that of pyruvate. The plasma protein fraction is also altered significantly during the period of hibernation. Beta globulin is associated with LDL (Low Density Lipoprotein, also referred to as beta lipoprotein) which is the major carrier of cholesterol in the blood. In a generalizing analogy one can assume that increased gamma globulin during the period of hibernation is associated with the immune defense mechanism of the body during the phase of hibernation. All this metabolic switching helps the animal to adapt to harsh winter conditions.

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