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Full Length Research Paper

Biochemical evaluation of aestivation and starvation in two snail species

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There is resurgence in incidence of schistosomiasis in Nigeria with attendant socio-economic and health impact. The agents transmitting this disease are the *Bulinus* snails which employ aestivation to survive conditions of unfavourable weather such as lack of food and water. The mechanism of aestivation under aridity and drought is not clear. This study therefore investigated the effects of aestivation and starvation on endogenous metabolic reserves in haemolymph of two snail species namely: *Bulinus globosus* (Morelet) and *Bulinus rohlfsi* (Clessin). Aestivation, starvation and control experiments were set up for 30 days in the laboratory by placing three groups of snails collected from Oyan dam, Abeokuta in standard aestivation slope (30 *B. globosus* and 19 *B. rohlfsi*), aquarium (30 *B. globosus* and 23 *B. rohlfsi*) and control slope which had 20 *B. globosus* and 15 *B. rohlfsi*. Aestivation and control slopes contained water and mixture of sand and clay (3:1), while aquarium contained water only for starvation. All the snails were fed on lettuce *ad libitum* for 28 days during which water was completely drained out in the aestivation slope. The aestivation slope and aquarium were left for another 30 days without lettuce. Snails were thereafter sacrificed and haemolymph biochemical parameters were assayed. In aestivating and starving *B. globosus*, haemolymph creatinine, urea, total protein, glucose, alanine transferases (ALT) and aspartate transferases (AST) were significantly decreased, while haemolymph total cholesterol, triglyceride and α-amylase concentrations and activity increased significantly (p < 0.05). In *B. rohlfsi*, creatinine, urea, ALT and AST were significantly decreased when compared with controls (p < 0.05). *B. globosus* and *B. rohlfsi* possess ability to survive unfavourable conditions by economical utilization of stored metabolites, thus enabling them to carry infection from one season to the next. Our findings suggest that *B. globosus* is a better aestivator than *B. rohlfsi*.

Key words: Aestivation, enzymes, *Bulinus globosus, Bulinus rohlfsi, schistosomiasis.*

INTRODUCTION

Fresh water snails constantly face desiccation, occasioned by drying up of surface water from fresh water bodies either regularly in a seasoned manner or occasionally due to unusual rainfall. At such times, the snails enter a hypometabolic or dormant state (aestivation) and reduce their energy consumption, thereby extending the time that fixed reserves of endogenous metabolic fuel stores that can sustain life (Kenneth, 2001). However, little is known about the biochemical mechanism of aestivation required for alternate development between an intermediate snail host and the final mammalian host, both of which are indispensable for maintaining the parasite (WHO, 2005).

Aestivation is of epidemiologic importance since schistosome parasite is able to survive for long periods in its larval stage in aestivating snails, thereby maintaining its chances of returning to an aquatic environment to resume

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Abbreviations: ALT, Alanine transferases; **AST,** aspartate transferases; **LDL,** low-density lipoprotein**; BUN,** blood urea nitrogen; **UUN,** urine urea nitrogen.

its normal life cycle once favourable conditions reappear (Richards, 1967). The result is the spreading of the disease known as schistosomiasis which is a global health problem in the developing world where people are infected continuously and intermittently with the disease especially the poverty stricken people who live in conditions that favour the transmission of the disease (WHO, 2007). The pathological lesions of schistosomiasis emanate from inflammatory reactions to the large number of parasite eggs which are retained in host tissue rather than excreted with the faeces or urine to infect the snail (WHO, 2005; Saathoff et al., 2004). *Bulinus globosus* and *Bulinus rohlfsi* are the two intermediate hosts responsible for the transmission of infection from one season to another through the shelters they provide for *Schistsoma haematobium* that are the vector for the disease. However, the survival of these two snails especially during unfavourable conditions of weather is usually through the phenomenon of aestivation. The major factors involved in the epidemiology of this disease are: Human contact with water, the presence of the appropriate snail intermediate hosts, the presence of natural bodies of water which serve as potential breeding sites of the snail host, the unhygienic conditions in which people living in rural communities lead their lives and the socioeconomic status of the people (Schmidt and Robert, 2009). In Nigeria where urinary schistosomiasis is endemic, mainly school children in rural areas have been shown to be infected with the disease (Aladekomo et al., 2008; Chidozie and Daniyan, 2008).

During aestivation in *B. globosus* and *B. rohlfsi* snails, food uptake ceases, water loss occurs and the snails are not able to rid themselves of their excretion products in a usual way. All of these alterations exert an influence on the snail's metabolism which may be reflected in the concentration of metabolites present in the snails undergoing aestivation. During this period, freshwater snails require a metabolic strategy to cover their energy requirements which are generally decreased. In fresh water and land snails, adaptive changes in metabolism occur (Abou and Salwa, 2010). Since organic biomolecules such as lipid, carbohydrates and proteins play central roles in intermediate metabolism; tissue and hemolymph concentrations were studied in two aestivating snail species and compared with those of non-aestivating snails. In this context, they may serve as indicators of various metabolic reactions since they represent important components of energy and intermediate metabolism. Thus, they may indicate the use of carbohydrates as an energy source in the flow of aerobic and in the case of aestivating mollusks, anaerobic carbon and the replacement of glucose through acids or metabolism of lipids on a smaller scale via fatty acids and ketone bodies.

Using various clinical chemistry analytical procedures on samples, it was possible to determine the profile of various biomolecules and metabolite concentrations present in the haemolymph of aestivating snail species obtained from Oyan dam under Ogun/Osun river basin authority in Abeokuta, Ogun state. Using such data, one can then evaluate in a broader manner, the biochemical processes that permit the snails to survive under adverse environmental conditions. This experiment was therefore designed to evaluate possible utilization of biomolecules and metabolites and its effects on survival of the two snail species during the period of aestivation.

MATERIALS AND METHODS

Aestivation, starvation and control experiments were set up using the standard methods (ref).

Aestivation experiment

A soil slope consisting of 8.0 cm layer of a moist mixture of sand and clay (3:1) in a tray and filled with 250 cm^3 pond water to 10.0 cm mark was set up according to Oyeyi and Ndifon, (1990) and Rizzati. Thirty *B. globosus* and nine *B. rohlfsi* with mean shell length of 10.0 mm each were induced to aestivate after feeding on lettuce *ad libitum for* 28 days while allowing the water to drain gradually but completely over the 28 days period at room temperature.

The soil was left to dry for further 30 days without food to induce aestivation. The experiment was terminated thereafter. Aestivating snails were revived by the re-introduction of pond water. Revived snails were collected after 24 h and frozen until subsequent analysis.

For *B. globosus*, the aestivation experiment consisted of eleven snails, starvation eight snails and control which had twelve snails, respectively. The values for *B. rohlfsi* were seventeen, fourteen and twelve for aestivation experiment, starvation and control, respectively.

Haemolymph sample

Snail shells were cleaned with a filter paper to remove the adhering water. The haemolymph was collected with a Pasteur pipette inserted through a tiny hole made in the shell above the pericardial region. For each group or treatment, the haemolymph from snails was centrifuged at 120 g for 5 min at 2°C in order to remove the haemocytes and cell debris. The resulting supernatant was then used in the analysis described below.

Biochemical assays of serum analytes

Creatinine concentration was determined using jaffe reaction as described by the method of Bartels (1972). Urea was assayed based on kinetic UV assay using the method of Talke and Schubert, (1965).

Biochemical assay of lipid profile

Cholesterol profile was determined using the method of Allain et al. (1974) for the determination of $\Delta 4$ – cholestenone after enzymatic cleavage of the cholesterol ester by cholesterol esterase. Triglyceride profile was assayed using the method of Wahlefeld and Bergmeyer, (1974).

Low-density lipoprotein (LDL)

Cholesterol in haemolymph of the snails was determined using Roche automated analyzer. Glucose in haemolymph was estimated by the method of Schmidt (1961). Total protein was determined

Table 1. Serum concentration of some metabolites in the haemolymph of *B. globosus* under aestivation and starvation.

Parameter	Control (n = 12)	Starved (n = 8)	Aestivating (n = 11)
Creatinine (µmol/l)	12.55±0.30	10.58±0.15[a]	11.46±0.25
Urea (mg/dl)	14.77±0.32	3.463±0.26[a]	3.77±0.09[a]
Total cholesterol (mg/dl)	2.20±0.12	2.70±0.15	3.03±0.15[a]
LDL (mg/dl)	0.91±0.06	0.0±0.0[a]	0.0±0.0[a]
Triglycerides (mg/dl)	12.10±0.56	13.97±0.56	17.57±0.34[a]
Total proteins (g/dl)	2.2±0.06	0.96±0.03[a]	0.87±0.02[a]
Glucose (fasting) mg/dl)	88.90±0.38	42.21±0.47[a]	68.42±0.78[a]
Serum amylase (IU/l)	957.7±0.88	972.3±4.33	993±1.16[a]
ALT (IU/l)	27.83±0.18	13.96±0.08[a]	14.43±0.09[a]
AST (IU/l)	10.66±0.31	7.83±0.22[a]	6.83±0.14[a]

Values are mean ± SE.M of triplicate measurements. [a]P < 0.05 indicates a significant difference between the tests and the controls; n = no of snails in each group. The average shell diameter of the snails used in the experiments was 17 ± 2 mm.

Table 2. Serum concentration of some metabolites in the haemolymph of *B. rohlfsi* under aestivation and starvation.

Parameter	Control (n = 12)	Starved (n = 8)	Aestivating (n = 11)
Creatinine (µmol/l)	10.45±0.29	7.50±0.24[a]	9.20±0.15
Urea (mg/dl)	12.40±0.50±	2.00±0.28[a]	2.10±0.17[a]
Total cholesterol (mg/dl)	1.60±0.21	1.80±0.23	2.30±0.20[a]
LDL (mg/dl)	0.90±0.06	0.00±0.00[a]	0.00±0.00[a]
Triglycerides (mg/dl)	11.00±0.06	12.27±0.06	16.00±0.12[a]
Total proteins (g/dl)	1.00±0.00	0.31±0.01[a]	0.35±0.005[a]
Glucose (fasting) mg/dl)	80.50±0.03	47.30±0.30[a]	63.00±1.16[a]
Serum amylase (IU/l)	43.00±1.16	38.67±0.067[a]	59.00±2.89[a]
ALT (IU/l)	24.90±0.12	10.00±0.06[a]	12.80±0.46[a]
AST (IU/l)	9.66±0.15	4.85±0.08[a]	4.83±0.12[a]

Values are mean ± SE.M of triplicate measurements. [a]P < 0.05 indicates a significant difference between the tests and the controls; n = no of snails in each group. The average shell diameter of the snails used in the experiments was 17 ± 2 mm.

using Bradford (1976) method. Serum alanine and aspartate amino transferases (ALT and AST) activities were determined by the methods described by Bergmeyer et al. (1985, 1986) and Heins et al. (1995) Hamolymph amylase activity was determined by the method of Lorentz (1998) and Kurrle-Weittenhiller et al. (1996).

The data are presented as mean ± S.E.M and n represents the number of snails used in the experiment. Statistical calculations were based on analysis of variance (ANOVA) and Dunnet. The differences were considered to be significant when P < 0.05.

RESULTS

The concentrations of various biochemical metabolites in the haemolymph of aestivating, starved and non aestivating *B. globosus* and *B. rohlfsi* snail species are shown in Tables 1 and 2, respectively.

The concentration of creatinine in aestivating and starved *B. globosus* snails was significantly lower ($p < 0.05$) when compared to the control. Creatinine concentration was reduced after aestivation and starvation experiments. The concentration of urea in aestivating and starved *B. globosus* snails was significantly lower when compared to the control ($p < 0.05$), showing that urea concentration was reduced at the end of both aestivation and starvation. Similarly, the concentration of cholesterol in aestivating *B. globosus* snail was significantly higher ($p < 0.05$) when compared to the control, while there was no significant difference in concentration between starved and control snails, suggesting that cholesterol levels increased at the end of aestivation. LDL-cholesterol was absent in the *B. globosus* snails at the end of both aestivation and starvation experiment, while it was present in the control, showing significant differences ($p < 0.05$) between the tests and control. There was no significant difference ($p \geq 0.05$) in the concentration of triglycerides between control and starved *B. globosus* snails, while there was significant difference ($p < 0.05$) between aestivating and control snails. Triglyceride increased at the end of aestivation of *B. globosus* snails. There was significant difference ($p < 0.05$) in the level of total

proteins in aestivating and starved *B. globosus* snails when compared to the control. Total proteins decreased in aestivating and starved snails when compared to the control. There were significant differences ($p < 0.05$) in the glucose level between both aestivating and starved snails when compared to the control. Fasting glucose decreased in both aestivating and starved snails when compared to the control of *B. globosus* snails. There were significant differences ($p < 0.05$) in the activity of ALT between both aestivating and starved snails when compared to the control. ALT activity decreased in aestivating and starved snails when compared to the control of *B. globosus*. There were significant differences ($p < 0.05$) in AST activity between both aestivating and starved snails when compared to the control. AST activity decreased in aestivating and starved snails when compared to the control for *B. globosus* snails.

Similarly, both starved and aestivating *B. rohlfsi* snails had lower creatinine values when compared to the control. The differences in each case were significant ($p < 0.05$). Aestivating and starved *B. rohlfsi* snails contained significantly lower concentrations of urea when compared to the control ($p < 0.05$). There was no significant difference in the values of total cholesterol in aestivating *B. rohlfsi* snails when compared to the control and starved snails ($p < 0.05$).

There were significant differences between both the aestivating and starved *B. rohlfsi* snails when compared to the control ($p < 0.05$). A decrease in LDL-cholesterol in both aestivating and starved *B. rohlfsi* snails when compared to the control was recorded. Significant differences ($p < 0.05$) exist in the levels of triglycerides between aestivating and starved when compared with control of *B. rohlfsi* snails. There was an increase in the concentration of triglyceride in both aestivating and starved snails when compared to the control.

Significant differences ($p < 0.05$) were observed in the level of total proteins between aestivating and starved when compared to the control *B. rohlfsi*. Total proteins decreased in both aestivating and starved snails when compared to the control. Glucose levels showed significant differences between both aestivating and starved snails when compared to the control ($p < 0.05$). Fasting glucose decreased in both aestivating and starved *B. rohlfsi* snails when compared to the control.

There was no significant difference ($p \geq 0.05$) in serum amylase activity between the starved and the control snails, while there was significant increase ($p<0.05$) in aestivating *B. rohlfsi* snails when compared to the control snails. A significant increase in the activity of ALT in aestivating and starved *B. rohlfsi* snails when compared to the control ($p < 0.05$) was observed.

There were significant differences ($p < 0.05$) in the aspartate transferases (AST) activity in the aestivating and starved *B. rohlfsi* snails when compared to the control. AST activity decreased in aestivating and starved snails when compared to the control.

DISCUSSION

The trends observed in the profile of metabolite and biomecule concentrations in the haemolymph of aestivating and starved *B. globosus and B. rohlfsi* snails in the present study correlate well with the values reported by Jose et al. (1999). Under aestivation, there is a marked reduction in waste excretion. Aestivation reduces the snail's movements with a consequent decrease in their metabolic activity. The reductions of creatinine and urea concentrations at the levels observed in aestivating and starved *globosus and B. rohlfsi* snail's haemolymph when compared to the control may reflect an increase in anaerobic metabolism via raised glycolytic process or from an enhanced protein catabolism. The lower value of creatinine in both aestivating and starved *B. globosus and B. rohlfsi* is an indication of depletion in stored macromolecules during this period as the level of creatinine in organisms usually correlates with metabolic activities such as muscle and liver catabolism of ATP. Creatinine concentrations in urine can be used as reference values for the excretion of certain analytes such as albumin and α-amylase (Bartels, 1972).This correlation was observed in the values of total proteins (including albumin) with that of creatinine in this study. However, there was no correlation between creatinine concentration and α-amylase in this study.

The raised levels of urea in the aestivating and starved snails suggest that amino acid metabolism was increased during aestivation and starvation in these snails. Similar results obtained with other aestivating molluscs had been published (Ellersiek, 1976; De Jorge and Petersen, 1970; Horne., 1979). Urea, also known as cabamide is a good predicator of kidney function/disorder as urea level in the blood indicates kidney disorder. The blood urea nitrogen (BUN) and the urine urea nitrogen (UUN) tests which measure urea nitrogen levels in the blood and urine are often used to assess kidney's function (Marshall, 1913). In conjunction with the determination of creatinine, it is an indicator for water depletion, increased protein catabolism and other renal impairments. Urea concentration in starved and aestivating *B. globosus and B. rohlfsi* decreased significantly, confirming water depletion and significant muscle wastage during aestivation and starvation

It has been observed that marked reduction in waste excretion followed water loss from the body surface of *B. glabrata* under aestivation (Von-Brand and Mehlman. 1953). Elevation of the body fluid osmolality through production of high concentrate of solutes such as urea is an adaptive marker to reduce the impact of stressful environmental conditions on snails which helps to preserve the composition of body fluids (Mcclanhan, 1967; Storey and Storey, 1990). The opposite was the case in the present study.

Low density lipoproteins decreased to zero in both aestivating and starved snails. An elevated level of LDL is usually an indicator of increased lipogenesis (Naito et al.,

1995). There was a significant decrease in LDL level in the two species during aestivation and starvation indicating lipolysis rather than lipogenesis during aestivation and starvation in this study.

Triglyceride level increased in both aestivating and starved snail hosts in this study. This could be due to the conversion of other forms of lipid to this storable and easily accessible form of lipid for use during aestivation and starvation. The increase in total cholesterol and triglyceride levels may reflect an increase in anaerobic metabolism via intensified glycolytic processes and an enhanced protein catabolism leading to an increase in glucogenic amino acids (Hochachka, 1983; Hermes-Lima and Zenteno-Savin, 2002). This could be observed from the significant decrease in total protein content in both aestivating and starved snails. This could be responsible for the decreased in the glucose level also.

Total cholesterol increased in both aestivating and starved snail hosts in the study confirming earlier observation evidence of increased lipolysis under aestivation and starvation in which the levels of indicators of lipid metabolism, such as ketone bodies acetoacetate and β-hydroxybutyrate were decreased during aestivation in *Biomphalaria glabrata (*Jose et al., 1999*)*. Elevated cholesterol level is an indication of lipid and lipoprotein metabolism disorders (Pisani et al., 1995). It has been shown that non-fasting serum usually has slightly lower concentration of cholesterol when compared to fasting state which agrees with the results in this study.

It has been noted that serum level elevation of α-amylases is an indicator of diabetic keto acidosis (Salt and Schenker, 1976; Steinberg et al., 1986). Keto acidosis is a common occurrence during starvation. The significant increase during aestivation in the two species may suggest a state of oxidative stress occasioned by starvation. ALT and AST are elevated under conditions of muscle dystrophy, damage to internal organs and myocardial infarction (Tietz and Schubert, 1986). The opposite is the case in this study, suggesting that these two species develop perhaps, protective mechanisms during aestivation and starvation. Suseela (2005) observed that the activity of other enzymes involved in gluconeogenesis pathway increased significantly in organisms under stress and that this can lead to condition of accumulation of excess pyruvate, which in anaerobic conditions is converted to lactate.

Increase in the activity of serum amylase is an indication of increase carbohydrate degradation, while significant increase in the activity of alanine amino transferase in aestivating and starved snails might be an indication of increased protein catabolism through deamination which in turn may result in increase in glucogenic amino acids (Wilson, 2003; Suseela et al., 2007).

Depletion in the glucose level in a steady manner confirms earlier reports (Greiling et al., 1995) that glucose being an essential metabolite must be supplied at a steady rate to the sensitive organs such as brain, heart kidney and skeletal muscles in order to maintain their vital functions in all situations. The depletion can therefore be attributed to the utilization of this molecule by those sensitive organs and muscles at least to maintain basal metabolic rate.

In conclusion, it should be mentioned that aestivation being a biochemical and physiological condition might present regulatory mechanisms against oxidative stress. It has been observed that the augmented endogenous antioxidant capacity during aestivation is a mechanism of preparation for the oxidative stress that accompanies arousal (Hermes-Lima and Storey, 1995; Storey, 1996; Hermes-Lima et al., 1998). These include lipid peroxidation, oxidized protein products, cholesteryl ester markers, advanced lipoprotein, triglycerides and protein carbonates. It is probable that the analytes such as LDL-cholesterol, triglycerides and cholesterol assayed in this study might form part of endogenous antioxidant capacity building in these snails experiencing oxidative stress that could result from prolonged starvation and aestivation (depending on their concentrations as determined during aestivating and starvation) when compared to the aroused state. It is also probable that LDL-cholesterol is the most important molecule in the survival of these two snails when they are under the stress of aestivation. This could be as a result of LDL-cholesterol being a major transport molecule in the metabolism of lipids, which is known to be a major macromolecule reserve, utilized by starving or aestivating snails.

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