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EFFECTS OF AESTIVATION AND STARVATION ON THE NEUTRAL LIPID AND PHOSPHOLIPID CONTENT OF *BIOMPHALARIA GLABRATA* INFECTED WITH *SCHISTOSOMA MANSONI*

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ABSTRACT: The effects of aestivation or starvation on the neutral lipid and phospholipid content of *Biomphalaria glabrata* patently infected with *Schistosoma mansoni* were determined by high-performance thin-layer chromatography–densitometry. Infected–aestivated snails were maintained in a moist chamber at 24 ± 1 C and a relative humidity of 98 ± 1%. Infected–starved snails were maintained in artificial spring water (ASW) at 23 ± 1 C without exogenous food. Infected snails (the controls) were maintained in ASW at 23 ± 1 C and fed lettuce ad libitum. The 3 groups were maintained in the laboratory for 7 days, and then the lipids from the digestive gland–gonad complex (DGG) were extracted and analyzed by class. Infected–aestivated snails exhibited greater mortality rate and weight loss after 7 days than did the infected–starved snails. The steryl ester concentration in the infected–starved snails was significantly increased ($P = 0.010$) compared with the controls but not compared with infected–aestivated snails; the concentration of phosphatidylcholine in infected–aestivated snails was significantly decreased ($P = 0.007$) compared with the controls but not when compared with the infected–starved snails. Aestivation or starvation had a significant effect on the concentration of certain lipid classes in the DGG of *B. glabrata* infected with *S. mansoni*.

Biomphalaria glabrata serves as a host to the medically important digenean *Schistosoma mansoni*. This snail lives in a climate that frequently experiences prolonged dry seasons without standing water (Olivier, 1956). In the wild, *B. glabrata* snails can survive such droughts protected by ground-level vegetation, and the snails do not enter the soil to avoid desiccation (Olivier, 1956). These planorbids survive dry periods through a natural phenomenon referred to as aestivation, in which snails enter a state of dormancy out of water. During aestivation, snails do not eat, water loss occurs (Bezerra et al., 1999), and oxygen consumption is decreased (von Brand et al., 1957).

Ohlweiler and Kawano (2001) showed that immature larvae of *S. mansoni* survived and developed within aestivating *B. tenagophila*, although the survival of infected–aestivated snails was lower than that of uninfected–aestivated snails. Badger and Oyerinde (2004) noted that aestivation of *B. pfeifferi* did not affect the infectivity of *S. mansoni* cercariae, although the survival of cercariae from aestivated snails was reduced. Barbosa and Coelho (1953, 1955) found that the development of immature stages of *S. mansoni* was arrested in aestivating snails, but development resumed when snails were reimmersed in water with the larvae surviving aestivation for up to 90 days; fully developed larval *S. mansoni* in aestivating *B. glabrata* only survived up to 21 days. Dormant, immature *S. mansoni* larvae were found by Barbosa and Barbosa (1958) in naturally infected–aestivated *B. glabrata*.

Aestivation of *B. glabrata* results in decreased metabolic activity (Bezerra et al., 1999), which can be better understood by investigating the effects of aestivation on various analytes. Several studies have been done to show how aestivation affects various metabolites of planorbid snails. von Brand et al. (1957) determined that total polysaccharides, total lipids, lactic acid, and certain other volatile acids were depleted in uninfected aestivated *B. glabrata* compared with uninfected–nonaestivated *B. glabrata*. Bezerra et al. (1999) found, using high-performance liquid chromatography, changes in the organic acid content of uninfected–aestivated *B. glabrata* compared with uninfected–nonaestivated snails. White et al. (2006) investigated neutral lipid and phospholipids classes in uninfected–aestivated *B. glabrata* and found an increase in the steryl esters in the digestive gland–gonad complex (DGG) of starved snails compared with control snails, as well as a decrease in the triacylglycerols in the whole body of starved and aestivated snails compared with control snails. Studies have not been done on the effects of aestivation on the neutral lipid and phospholipid content of *B. glabrata* infected with *S. mansoni*. The DGG of *B. glabrata* is a major site of metabolic activity and is the complex of choice for the analyses described herein. Therefore, to determine how aestivation affects the neutral lipid and phospholipid content of the DGG of *B. glabrata* patently infected with *S. mansoni*, we used high-performance thin-layer chromatography (HPTLC)–densitometry. Because snails do not feed during aestivation, the lipid content of starved *B. glabrata* patently infected with *S. mansoni* was also determined and compared with the results of the aestivation studies.

MATERIALS AND METHODS

Snail maintenance

Biomphalaria glabrata snails were infected experimentally with *S. mansoni* by exposure en masse to approximately 8 miracidia/snail. Cultures of *B. glabrata* (NMRI strain) were maintained as described by Schneck and Fried (2005). Snails were maintained at 23 ± 1 C in aerated glass jars containing approximately 20 snails per 800 ml of artificial spring water (ASW), prepared as described by Ulmer (1970), under diffuse fluorescent light for a photoperiod of 12 hr per day. Cultures were fed boiled romaine lettuce leaf ad libitum, and the water in the cultures was changed 3 times a wk.

Aestivation, starvation, and control conditions

Groups of patently infected snails, obtained 6 wk after miracidial infection, were maintained under 3 conditions (aestivation, starvation, and control). For aestivation, infected snails were placed 4–5 per finger bowl (20 × 5 cm) lined with an ASW-saturated filter paper pad. Three Stender dishes, each with 5 ml of ASW, were placed in the chamber to maintain a high humidity; these dishes were covered with cheesecloth to prevent snails from migrating into the dishes. A second finger bowl was placed loosely on top of the chamber as a cover. This arrangement allowed for exchange of atmospheric air in the chamber. The temperature in the chamber was 24 ± 1 C, and the relative humidity was 98 ± 1%. Infected–aestivated (IE) snails were maintained without food for 7 days. Infected–starved (IS) snails were maintained 4–5 per 800 ml of aerated ASW at 23 ± 1 C without exogenous food for 7 days, and the water was not changed in these cultures. These snails did not feed on

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TABLE I. Weight percent (mean ± SE) of lipids in the digestive gland-gonad complex of aestivated, starved, and control *Biomphalaria glabrata* infected with *Schistosoma mansoni*; n = 3–5.*

Experimental Condition	FS	FFA	T	SE	PC	PE
Infected–aestivated (IE)	0.133 ± 0.041	0.0237 ± 0.0049	0.258 ± 0.060†	0.198 ± 0.055	0.372 ± 0.047‡	0.331 ± 0.11
Infected–starved (IS)	0.290 ± 0.053	0.0642 ± 0.019	0.447 ± 0.14	0.286 ± 0.042§	0.917 ± 0.20	0.341 ± 0.084
Control (CON)	0.256 ± 0.053	0.0363 ± 0.011	0.771 ± 0.26	0.0778 ± 0.010	1.76 ± 0.33	0.389 ± 0.061

* FS = free sterols, FFA = free fatty acids, T = triacylglycerols, SE = steryl esters, PC = phosphatidylcholine, PE = phosphatidylethanolamine.
† An outlier was found and eliminated by the *Q*-test (90% confidence interval).
‡ PC level in IE snails was significantly decreased compared with CON snails ($P = 0.007$) but not compared with IS snails ($P > 0.05$).
§ SE level in IS snails was significantly increased compared with CON snails ($P = 0.010$) but not compared with IE snails ($P > 0.05$).

their feces, suggesting that coprophagy did not provide these snails with a source of food. Control snails (CON) were maintained 4–5 per 800 ml of aerated ASW at 23 ± 1 C and fed romaine lettuce leaf ad libitum with the water changed 3 times a wk.

The maximum diameters of the snail shells were measured with a vernier caliper; snails were blotted dry with a paper towel and weighed on an analytical balance before the start of an experiment. A total of 29 patently infected snails (IE) were aestivated; the mean shell diameter of these snails was 11.2 ± 0.2 mm, and the mean weight was 0.2474 ± 0.0119 g. Twenty-five patently infected snails (IS) were starved; the mean shell diameter of these snails was 10.8 ± 0.2 mm, and the mean weight was 0.2312 ± 0.0125 g. Twenty-three patently infected snails (CON) served as controls; the mean shell diameter of these snails was 11.3 ± 0.2 mm, and the mean weight was 0.2390 ± 0.0117 g. All of the above measurements are reported as mean ± SE.

After 7 days, the IE snails were weighed again and revived by immersion in 3 ml of ASW in individual wells of a multiwell plate 30 cm from a 60 watt lamp (28 ± 1 C). After 7 days of starvation, the IS snails were weighed again to determine weight loss. To prepare the DGG sample for lipid extraction, the snail was gently crushed under a dissecting microscope; the shell was dissected from the body with forceps and discarded; the DGG was dissected from the body with fine scissors, rinsed with deionized water, and blotted on a paper towel to obtain a blotted wet weight of the tissue sample. The DGG were then extracted as described below within 2 hr of removal from the shells.

Sample preparation

Lipid analysis was performed on the individual DGGs of 3–5 snails for each experimental condition (Table I). Lipids were extracted from snail DGGs following the method of Folch et al. (1957). Samples were reconstituted with 175–375 μl chloroform–methanol (2:1), the exact volume being chosen to yield at least 1 sample zone that had a densitometric scan area bracketed within the scan areas for the standards during HPTLC analysis.

Thin-layer chromatography

Quantitative determination of neutral and polar lipids was performed by HPTLC on 10 cm × 20 cm Analtech (Newark, Delaware) HPTLC–HLF silica gel plates with 19 scored lanes and a preadsorbent zone.

The standard for neutral lipid analysis was Non-Polar Lipid Mixture-B (Matreya, Pleasant Gap, Pennsylvania), which contained equal amounts of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate to provide markers for free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl esters, respectively. The standard was diluted with chloroform–methanol (2:1) to make a solution containing 0.200 μg/μl of each component. To determine the free sterol, free fatty acid, and triacylglycerol content of the samples, the plates were developed with the mobile phase of Mangold (1969), petroleum ether-diethyl ether-glacial acetic acid (80:20:1). To determine the methyl ester and steryl ester content of the samples, the plates were developed with the Smith et al. (1995) mobile phase, hexane-petroleum ether-diethyl ether-glacial acetic acid (50:25:5:1). Lipids were detected as blue spots on a yellow background by spraying the plate with 5% phosphomolybdic acid (PMA) in absolute ethanol and heating at 110 C for 10 min.

The standard for polar lipid analysis was Polar Lipid Mix 1127 (Matreya), which contained equal amounts of cholesterol, phosphatidylethanolamine, phosphatidylcholine, and lysophophatidylcholine. The standard solution was prepared at a concentration of 0.250 μg/μl of each compound in chloroform–methanol (2:1). Plates were developed with chloroform–methanol–water (65:25:4), as described by Wagner et al. (1961). Lipids were detected as brown spots on a white background by spraying the plate with 10% $CuSO_4$ in 8% H_3PO_4 and heating at 140 C for 15 min.

Quantitative densitometric analysis was performed by use of a Camag (Wilmington, North Carolina) TLC Scanner II with the tungsten light source set at 610 nm for neutral lipids, and the deuterium light source set at 370 nm for polar lipids. The CATS-3 software was used to create a linear regression calibration graph relating the weights of the standard zones to their peak areas on each plate. From the calibration graph, the lipid content in sample zones was interpolated on the basis of their peak areas. Detailed descriptions of HPTLC methodology are available in White et al. (2006).

Statistical analyses were done using a single factor analysis of variance (ANOVA) to determine whether there was a significant difference in the lipid percentage of the DGG among IE, IS, and CON snails. If a significant difference ($P < 0.05$) was found, the data were subjected to the Bonferroni method to determine among which populations the difference occurred. The SPSS version 13.0 software was used for all data analyses.

RESULTS

A preliminary trial using *B. glabrata* patently infected with *S. mansoni* and aestivated for 14 days showed no surviving snails. The experimental period for all conditions was, therefore, reduced to 7 days. At the end of 7 days, 9 of 29 (31%) IE snails survived, 20 of 25 (80%) IS snails survived, and 20 of 23 (87%) CON snails survived. The revival time for the IE snails was 29 ± 4 min (mean ± SE). The revival period was considered to be the time it took the snail to extend its head foot region and attach to a substratum. After 7 days, IE *B. glabrata* had lost 26.2% of their weight, whereas IS *B. glabrata* had lost only 3.0% of their weight.

Calibration plots, relating the scan areas to the weights of neutral lipid (0.400–3.20 μg) and phospholipid (0.500–4.00 μg) standards, consistently gave linear regression coefficient (*r*) values of 0.97 or above.

The samples analyzed for neutral lipids in the Mangold (1969) mobile phase showed zones with comparable migration to the standards at R_F values of 0.17 (cholesterol), 0.28 (oleic acid), and 0.54 (triolein). The samples analyzed for neutral lipids in the Smith et al. (1995) mobile phase showed zones with comparable migration to the steryl ester standard at a R_F of 0.53 (cholesteryl oleate). Methyl esters were not present in any of the samples analyzed. The samples analyzed for phospholipids in the Wagner et al. (1961) mobile phase showed sample and standard zones at R_F values of 0.25 (phosphatidylcholine) and 0.48 (phosphatidylethanolamine). Lysophophatidylcholine in

the phospholipid standard was not detected with the cupric sulfate reagent at the concentration applied, and zones that might have been lysophophatidylcholine were not seen in the samples.

Table I presents data for the percentage (w/w) of neutral lipids and phospholipids in the DGG of IE, IS, and CON *B. glabrata*. The level of steryl esters in IS snails was significantly increased ($P = 0.010$) compared with CON snails, but not compared with IE snails ($P > 0.05$). The phosphatidylcholine level in IE snails was significantly decreased ($P = 0.007$) compared with CON, but not compared with IS snails ($P > 0.05$). No significant differences among any of the groups were found in the concentrations of free sterols, free fatty acids, triacylglycerols, or phosphatidylethanolamine.

DISCUSSION

White et al. (2006) found that 100% of uninfected *B. glabrata* survived 14 days of aestivation under the same conditions used in this study. Patent infection of *B. glabrata* with *S. mansoni* reduced the ability of this snail to survive aestivation under our laboratory conditions. This is consistent with previous studies (Barbosa and Coelho, 1953, 1955) in which patent *S. mansoni* infections in *B. glabrata* did not survive aestivation for long periods of time.

IE snails lost more weight during the experimental period than did IS snails; the increased weight loss in IE snails was probably due to loss of water, as reported previously (von Brand et al., 1957; Vianey-Liaud and Lancastre, 1986a, 1986b) for aestivated *B. glabrata* snails.

Although no previous study has examined the neutral lipids and phospholipids in aestivated *B. glabrata* infected with *S. mansoni*, the effect of aestivation on the total lipids in uninfected *B. glabrata* has been reported. von Brand et al. (1957) determined gravimetrically that the total lipid concentration as well as total polysaccharide concentration of the whole body of *B. glabrata* decreased with increasing length of the aestivation period. White et al. (2006) examined uninfected–starved and–aestivated snails and reported a significant decrease in the triacylglycerols in the whole body of uninfected–aestivated and–starved *B. glabrata* compared with uninfected–nonaestivated snails after 14 days. White et al. (2006), also found that after 14 days, the steryl ester concentration in the DGG of uninfected–starved snails was significantly higher than that of uninfected–nonaestivated snails but not higher than uninfected–aestivated snails. This finding is in agreement with that in the present study. The function of steryl esters in snails is not clear, so the importance of the increase in steryl ester concentration as a function of starvation is not clear. The present study also found that the phosphatidylcholine concentration significantly decreased during aestivation of the infected snails. Phosphatidylcholine has a structural role in cell membranes, so depletion of this lipid could indicate that the larval trematodes are somehow disrupting the snails' cell membranes.

This study examined the effects of aestivation on the lipid content of *B. glabrata* patently infected with *S. mansoni*. The en masse exposure method used in the study, i.e., approximately 8 miracidia/snail, precluded the possibility of determining the infection burden on a per snail basis. Some of our snails probably had a greater sporocyst burden than others. Heavy sporocyst infections probably have a greater impact on the effects of aestivation in snails than do light sporocyst infections. In our study, we could not determine the effects of heavy versus light sporocyst infection on the lipid content of aestivated snails because of our snail exposure method.

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