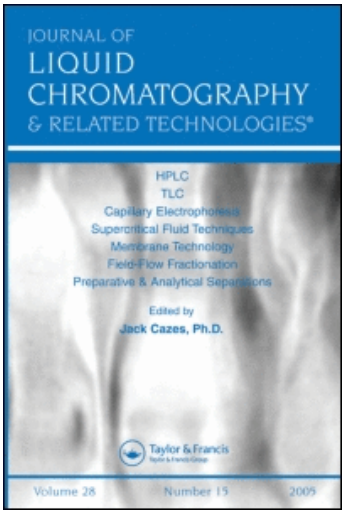

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EFFECTS OF TEMPERATURE ON THE NEUTRAL LIPID CONTENT OF *BIOMPHALARIA GLABRATA* AS DETERMINED BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY-DENSITOMETRY AND OBSERVATIONS ON SNAIL FECUNDITY

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□ *High performance thin-layer chromatography with densitometry was used to examine the effects of extreme temperature on the neutral lipid content of* Biomphalaria glabrata*, and observations on fecundity were made at these extreme temperatures of 32, 28, and 14°C. Fecundity results showed that egg laying at 32°C was higher than at 28 and 14°C. Free sterols, free fatty acids, and triacylglycerols were quantified at 2 and 4 weeks after the initiation of the experiment using Analtech channeled high performance silica gel plates with a preadsorbent zone. Plates were developed using the Mangold mobile phase, petroleum ether-diethyl ether-glacial acetic acid (80:20:1), and after treatment with 5% ethanolic phosphomolybdic acid spray reagent and 10 min of heating at 110°C, neutral lipids appeared as blue zones on a yellow background. Quantitative densitometric analysis was performed using a CAMAG TLC Scanner II with the tungsten light source set at 610 nm. Quantitative identification was based on correspondence of R_f values of sample and neutral lipid standard zones. At week 2 after the initiation of the experiment, there were significant differences in triacylglycerol content between all three temperatures, suggesting that at the higher temperatures snails were building reserves of depot fat.*

Global climate changes have led to concerns about the spread of infectious disease, including snail born infections such as schistosomiasis. Some of these concerns relate to the ability of vector snails to adapt to climate change, making studies on lipid use of vector snails at temperature extremes crucial to a better understanding of the potential spread of snail-borne disease.

Keywords *Biomphalaria glabrata*, densitometry, free fatty acids, free sterols, high performance thin-layer chromatography, HPTLC, snail fecundity, temperature effects, triacylglycerols

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INTRODUCTION

With global climate change there is concern in the increased spread of helminthic diseases, including snail borne infections such as schistosomiasis and echinostomiasis. Some of these concerns relate to the ability of the vector snails, particularly planorbid snails, to adapt to temperature changes as a factor of climatic changes. For these reasons, studies of temperature effects on vector snails are important to better understand the potential spread of snail borne infectious disease.

Previous studies have examined the effects of temperature on several biological characteristics of planorbid snails. For instance, Michelson[1] examined the effects of temperature, at 5°C intervals from 5 to 35°C, on the growth and reproduction of *Biomphalaria glabrata* (which was referred to as *Austrolorbis glabratus* in that paper). His study found that although 30°C was the optimal temperature for snail growth, that temperature had detrimental effects on snail fecundity (egg laying). El-Eman and Madsen[2] studied the effects of five temperatures (10, 18, 26, 28, and 33°C) on growth, reproduction, and survival of three snail species, including *B. alexandrina*, a common planorbid snail found in Egypt. They found that optimal temperatures for growth were 26 and 28°C, but the optimal temperature for reproduction was 26°C and no *B. alexandrina* survived for more than 4 weeks at 10 or 33°C. However, at 18°C *B. alexandria* showed increased survival compared to either *Helisoma duryi* or *Bulinus truncatus*, suggesting that *B. alexandria* is better adapted to cold temperatures in their natural habitats. Not only did *B. alexandrina* snails show good survival at 18°C, they also showed increased fecundity.

At present there are no studies that have examined the effects of temperature extremes on the lipid content of planorbid snails. Such information is important to obtain a better understanding of lipid metabolism in snails subjected to adverse temperatures. In addition to our high performance thin-layer chromatography (HPTLC) studies on neutral lipids in these snails subjected to extreme temperatures, we also observed fecundity of these snails at various temperature extremes, and our results are reported herein.

EXPERIMENTAL

Snail Maintenance

Sexually mature snails of an NMRI strain of *B. glabrata*, 10–15 mm in shell diameter, were obtained from Dr. Fred A. Lewis, Head, Schistosomiasis Laboratory, Biomedical Research Institute (Rockville, MD, USA) and used upon receipt. Two cultures, each with five snails, were maintained

at 14, 28, and 32°C. These are temperature extremes for *B. glabrata* as noted in Lewis et al.[5] The two high temperature cultures (28 and 32°C) were maintained in an incubator, and the low temperature culture (14°C) was maintained in a cold room. Cultures were maintained in 150×15 mm plastic Petri dishes in 100 mL of artificial spring water (ASW) as described in Ulmer.[3] Snails were fed *ad libitum* on boiled romaine lettuce. Water and food were changed every 2–3 days. All cultures were covered in aluminum foil and maintained in the dark. Joy[4] found that light affects the egg laying of *B. glabrata*, and because our experiment also measured snail fecundity, light was controlled as a variable as much as possible. Aluminum foil was used to keep the cultures in constant darkness, except for short light exposure (about 5–10 min per culture) when water and food were changed in the cultures.

Fecundity

Egg laying studies were done at 8, 10, and 12 days after the initiation of the snail cultures at the various temperatures. After an egg count was completed, the eggs were discarded. Table 1 reports total number of egg masses, eggs per mass, and total number of eggs for all snails at each egg count; it also lists a total egg count for all three counts combined. Cultures at 28°C had 9 snails, whereas those at 32 and 14°C each had 10 snails.

Sample Collection and Preparation

Snails were necropsied 2 and 4 weeks after the initiation of the study, i.e., from the time when the snails were placed in cultures at the three

TABLE 1 Number of Egg Masses, Mean ± Standard Error of Eggs Per Mass, and Total Number Eggs for Each Count and for All Counts Combined

No. of Days After Start of the Experiment		32°C	28°C[a]	14°C
8	Egg masses	11	11	9
	Eggs/mass	21 ± 6	26 ± 12	20 ± 9
	Total eggs	231	286	180
10	Egg masses	10	2	5
	Eggs/mass	34 ± 7	25 ± 5	22 ± 8
	Total eggs	340	50	110
12	Egg masses	16	12	3
	Eggs/mass	35 ± 9	36 ± 9	12 ± 8
	Total eggs	560	432	36
Total no. of eggs for all days		1121	769	325

[a]Cultures maintained at this temperature had 9 snails, whereas the cultures at the other temperatures each had 10 snails.

experimental temperatures. At necropsy, each snail shell was discarded to obtain the whole snail body, which was blotted dry and weighed. Each sample consisted of a single snail. The blotted wet weights of the snail bodies ranged from 63.6 to 191.8 mg; the shell diameters ranged from 14.8 to 17.7 mm.

A sample was homogenized in a 7 mL Pyrex Tenbroeck tissue grinder (No. 08-414-10B, Fisher Scientific, Pittsburg, PA, USA) with 3 mL chloroform-methanol (2:1). The homogenate was filtered through cotton wool, and Folch wash (0.88% KCl) was added in an approximate volume of 25% of the chloroform-methanol (2:1) used. The lipophilic layer was separated from the hydrophilic layer using a Pasteur pipet and dried in a warm water bath (approximately 40°C) under N_2 gas. Samples were reconstituted in chloroform-methanol (2:1) in a ratio of 10.2 μL/mg sample. Reconstitution volumes ranged from 645 to 1950 μL.

Thin-Layer Chromatography

Lipid identification and quantification were performed using HPTLC on Analtech (Newark, DE, USA) channeled plates with preadsorbent zone (Catalog # 61927). The mobile phase used was the Mangold solvent system, petroleum ether-diethyl ether-glacial acetic acid (80:20:1). A neutral lipid standard (Nonpolar Lipid Mix B, Matreya, Pleasant Gap, PA, USA) containing 20% each of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate was run with each sample to serve as markers for free sterols, free fatty acids, triacylglycerols, methyl esters, and steryl esters, respectively. Five percent ethanolic phosphomolybdic acid spray reagent followed by 10 min of heating at 110°C was used for detection of the neutral lipids as blue zones on a yellow background.

Quantitative densitometric analysis was performed with a Camag (Wilmington, NC, USA) TLC Scanner II with the tungsten light source set to 610 nm. To create a linear regression calibration graph, the CATS-3 software plotted the weights of the standard zones versus their peak areas. In the event that more than one aliquot in a sample was within this calibration graph, the value closest to the mean of the two middle areas was used to calculate the percent lipid. The percent lipid was calculated using the following equation:

$$\%\ \text{Lipid} = \frac{(\text{w})(\text{R})(100)}{\mu\text{g tissue}}$$

where w = mass in μg interpolated from the calibration graph and R = [(reconstitution volume(μL))/spotted volume(μL)].

Statistical Significance

Multiple *t*-tests were used for statistical analysis using Microsoft Excel's Student's *t*-test. Thus, the mean ± standard deviation for each neutral lipid fraction was compared at 2 weeks after the initiation of the experiment, matching group A vs. group B snails, group B vs. group C snails, and group A vs. group C snails. The same procedure was used to determine differences in the neutral lipid concentrations of these snails at 4 weeks after the start of the experiment. Because we were simultaneously comparing three *t*-tests in the interpretation of our results, we divided the accepted value of $P < 0.05$ by 3 to yield a significance value of $P < 0.017$.

RESULTS AND DISCUSSION

Fecundity

Egg counts were made on days 8, 10, and 12 after the initiation of the experiments, and the results are summarized in Table 1. They show that a temperature of 14°C inhibited egg production, since total egg production over these 12 days was markedly lower at this temperature. Egg laying was higher at 32 than at 28°C for this same time period. Our results with the NMRI strain of *B. glabrata* maintained in the dark showed that egg laying was not decreased at 32°C after 2 weeks of maintenance at this temperature. This result contradicts the findings of Michelson, who found that fecundity decreased at 30°C compared to 25°C.[1] The viability of the eggs, i.e., ability to hatch and develop into viable juvenile snails, was not studied.

The reasons for discrepancies between our study and that of Michelson[1] are not clear. One difference between our study and that of Michelson is that he used immersion heaters rather than incubators to achieve his higher experimental temperatures. This could possibly account for some differences between the two studies. Another difference in the two studies is that our study began with sexually mature snails with shell diameters of 10–15 mm, whereas Michelson used smaller snails that were sexually immature at the onset of his experiment. Perhaps the larger snails were more suited to the extreme temperatures used in our study. We did not study egg viability, and we did not determine if the eggs at the extreme temperatures in our study would have produced viable offspring.

Thin-Layer Chromatography

The major neutral lipid fractions that were quantified by HPTLC were free sterols, free fatty acids, and triacylglycerols. Each sample had neutral lipid zones that matched the R_f values of the standards, cholesterol

TABLE 2 Average Percent Lipid Values for the Lipid Fractions Studied

Group	Maintained Temperature	Free Sterols		Free Fatty Acids		Triacylglycerols	
		Week 2	Week 4	Week 2	Week 4[a]	Week 2[b]	Week 4
A	32°C	0.255 ± 0.169	0.272 ± 0.149	–[c]	0.329 ± 0.099	1.123 ± 0.238	0.351 ± 0.137
B	28°C	0.189 ± 0.110	0.152 ± 0.065	0.173 ± 0.099	0.144 ± 0.054	0.327 ± 0.157	0.285 ± 0.131
C	14°C	0.111 ± 0.053	0.103 ± 0.056	0.104 ± 0.057	0.035 ± 0.007	0.133 ± 0.086	0.175 ± 0.115

[a]There was a statistically significant difference ($P < 0.017$) between group A and B.
[b]There were statistically significant differences ($P < 0.017$) between all groups.
[c]Value had a standard error that was too large for inclusion.

(0.19), oleic acid (0.38), and triolein (0.57). When using the Smith et al.[4] solvent system, zones that we originally thought to be methyl esters and steryl esters did not comigrate with the methyl oleate and cholesteryl oleate standards; these experimental zones were relatively faint and were omitted from any further consideration in this study.

Quantitative results of the effect of temperature extremes on the neutral lipids of *B. glabrata* whole bodies are summarized in Table 2. There were no significant differences in free sterols between any groups at 2 or 4 weeks after the experiment was initiated. Likewise, there were no significant differences in free fatty acids at week 2 between groups B and C. The free fatty acid value for group A at 2 weeks had too large a standard deviation to be meaningful and was excluded from the table. At week 4, group A snails had a significantly greater concentration of free fatty acids than did group B snails.

Our most important HPTLC finding was at 2 weeks after the cultures were initiated at the temperature extremes. At this time, group A snails had a significantly greater amount of triacylglerols than either group B or C. Likewise, group B snails had a significantly greater concentration of triacylglycerols than did group C snails. By 4 weeks, no snail group showed any significant difference in triacylglycerol concentration when compared to the other groups.

The week 2 triacylglycerol results yield significant information about the adaptation these *B. glabrata* snails underwent in order to survive an extreme temperature range. The buildup of triacylglycerols at the higher temperatures of 28 and 32°C show that the snails were building reserves of depot fat. The fact that in week 4 these significant differences were gone could indicate that this depot fat had been used up, and that there was no longer any accumulation of fat. Triacylglycerols are mainly associated with fat reserves and are normally heavily concentrated in the digestive gland gonad complex (DGG) of the snail. Though whole snail bodies were used in this study, the triacylglycerol results still indicate the aforementioned conclusions.

Reasons for the statistically significant difference between samples maintained at 32 and 28°C at week 4 in the free fatty acid fraction are not clear. The accumulation of depot fat in the form of free fatty acids could possibly explain this result. However, unlike triacylglycerols, free fatty acids have diverse functions in snail physiology other than serving as fat depots, and the increase in this fraction in the whole bodies of snails maintained at 32°C remains unclear.

CONCLUSIONS

Fecundity results showed that at 32°C snails had increased egg laying capacity compared to these maintained at 28 and 14°C; this finding is

contradictory to that of Michelson.[1] Egg viability studies are needed at these extreme temperatures to determine the true effects of temperature on *B. glabrata* fecundity. HPTLC results for the triacylglycerol lipid fraction of whole snail bodies 2 weeks after these snails were placed in extreme temperatures showed the snails had increased mean percent lipid values at the higher temperatures; these results suggest that the snail had a buildup of depot fat to accommodate the increased temperature. Studies using only the DGG and the hemolymph (blood) of the snails could yield more conclusive results on the effects of temperature on lipid function in these snails.

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