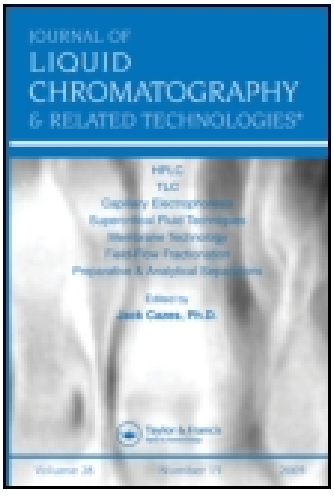

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Determination of the Effects of Estivation and Starvation on Neutral Lipids and Phospholipids in *Biomphalaria glabrata* (NMRI Strain) and *Helisoma trivolvis* (Colorado Strain) Snails by Quantitative High Performance Thin Layer Chromatography-Densitometry

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Abstract: High performance thin layer chromatography (HPTLC) was used to determine neutral lipids and phospholipids in *Biomphalaria glabrata* (NMRI strain) and *Helisoma trivolvis* (Colorado strain) snails during estivation and starvation. Control snails were maintained on a diet of Romaine lettuce leaves. HPTLC analyses showed a significant (ANOVA, $P < 0.05$) decrease in triacylglycerols in estivated and starved snails compared to the controls and a significant increase in steryl esters in estivated and starved snails compared to the controls.

Keywords: Thin layer chromatography, Lipids, *Biomphalaria glabrata*, *Helisoma trivolvis*, Estivation, Starvation

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INTRODUCTION

Biomphalaria glabrata is a freshwater planorbid snail that serves as an intermediate host of *Schistosoma mansoi*, a medically important human trematode that causes schistosomiasis.[1] *Helisoma trivolvis* is a ubiquitous freshwater planorbid snail in North America. The Colorado strain of *H. trivolvis* is refractory to infection with *Echinostoma trivolvis* miracidia and is an important laboratory model for studies on the immunology of larval trematode-snail host relationships as well as for neurobiological studies.[2]

In their natural habitats, planorbid snails are subjected to periods of drought for several months at a time. Estivation is a natural phenomenon that allows snails to become inactive and dormant in response to drought.[3] During estivation, snails do not eat and water loss occurs.[4] Oxygen consumption is also reduced.[5] Pulmonate snails, including the planorbids, retract into their shells during estivation, but they do not form a protective epiphragm or mucus membrane to close the opening of the shell.[3,4]

To understand how snails survive estivation, it is important to know how this condition affects the snails physiologically and biochemically. Studies of the digestive gland-gonad complex (DGG) and the whole bodies of snails provide good indicators of changes in the lipid content of snails as a function of diet, parasitism, starvation, and estivation.[6] No study has examined the effects of estivation on specific classes of lipids in the whole body and DGG of *B. glabrata* (NMRI strain) and *H. trivolvis* (Colorado strain). The purpose of this study was to determine the effects of estivation and starvation on the neutral lipid and phospholipid content of the whole body and DGG of these strains of *B. glabrata* and *H. trivolvis* by using high performance thin-layer chromatography (HPTLC).

EXPERIMENTAL

Maintenance of Snails

Stock cultures of *B. glabrata* (NMRI strain) and *H. trivolvis* (Co strain) were maintained as described in Schneck and Fried.[7] Snails were maintained at $23 \pm 1^{\circ}C$ in aerated glass jars containing approximately 20 snails per 800 mL of artificial spring water (ASW), prepared as described by Ulmer,[8] 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 three times a week.

Estivation, Starvation, and Control Conditions

Groups of snails of each species were maintained under three conditions (estivated, starved, and control) for 14 days. Control snails were fed

Romaine lettuce leaf *ad libitum* and maintained as described above. Starved snails were maintained as described above without exogenous food; the water was not changed in these cultures. Snails did not feed on their feces, suggesting that coprophagy does not provide a source of food for these planorbids. Snails were induced to estivate by placing 4–5 snails in a 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 25 ± 1°C, and the relative humidity was 98 ± 1%. Estivated snails were maintained without food for 14 days.

Snails were chosen from a sexually mature population with matched shell diameters. 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 prior to the start of an experiment. A total of 25 *B. glabrata* snails were used; the mean shell diameter was 15.0 ± 0.3 mm and the mean weight was 0.4767 ± 0.0279 g A total of 30 *H. trivolvis* snails were used; the mean shell diameter was 12.6 ± 0.2 mm and the mean weight was 0.4912 ± 0.0250 g. The above measurements are reported as mean ± standard error (SE).

At the end of the 14 day estivation period, the snails were weighed again and revived by immersion in 3 mL of ASW in individual wells of multiwell plates at a 30 cm distance from a 60 watt lamp (28 ± 1°C). At the end of the 14 day starvation period, the snails were weighed again to determine weight loss. In order to prepare the whole body sample for lipid extraction, the snails were gently crushed under a dissecting microscope; the shell was dissected from the body with forceps and discarded, and the body was placed in 50% Locke's solution. For DGG samples, the DGG was dissected from the body with fine scissors and saved; the rest of the body was discarded. Whole body and DGG samples were rinsed with deionized water and blotted on a paper towel in order to obtain a blotted wet weight of the tissue sample and then extracted as described below within 2 hr of the removal of the shells.

Sample Preparation

Lipids were extracted from the whole body and DGG samples by grinding the sample in a glass homogenizer with 20 mL of chloroform-methanol (2 : 1) per gram of sample. This homogenization was sufficient to completely dissolve the tissue sample, making a second extraction unnecessary. After homogenization, the homogenate was passed through a cotton filter and treated with a volume of 0.88% KCl,[9] one quarter that of the chloroform-methanol (2 : 1) used for lipid extraction. After refrigeration at 4°C for one hr, the upper, hydrophilic layer was removed with a Pasteur pipet and discarded, and the

lipophilic layer was dried under nitrogen gas in a water bath at 40°C. Samples were reconstituted with 175–875 μL chloroform-methanol (2 : 1), the exact volume being chosen to yield at least one 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 Whatman (Clifton, NJ) LHPKDF high performance silica gel plates with 19 scored lanes and a preadsorbent zone. Plates were prewashed by development to the top with dichloromethane-methanol (1 : 1) and subsequently dried in air in a fume hood. Standards were applied to the preadsorbent zone of adjacent lanes with a 10-μL Drummond (Broomall, PA) digital microdispenser in aliquots of 2.00 μL, 4.00 μL, 8.00 μL, and 16.0 μL and reconstituted samples in 1.00 μL to 16.0 μL aliquots by streaking a downward series of spots starting 2 mm below the silica gel/preadsorbent interface to within 5 mm of the bottom of the plate.

The standard for neutral lipid analysis was TLC Reference Standard 18-4A (Nu-Check-Prep, Elysian, MN), which contained equal amounts of cholesterol, oleic acid, triolein, methyl oleate, and cholesteryl oleate to provide markers for free sterols, free fatty acids, triacylglyerols, methyl esters, and steryl esters, respectively. The solid standard was weighed and 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 triacylglycerols content of the samples, the plates were developed with the Mangold[10] mobile phase, 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.[11] mobile phase, hexane-petroleum ether-diethyl ether-glacial acetic acid (50 : 25 : 5 : 1). In both mobile phases, petroleum ether with a boiling point range of 36–60°C was used. Plates were developed in a 3 mm deep pool of mobile phase to a distance of 9 cm from the bottom of the plate in a rectangular Camag (Wilmington, NC) TLC twin-trough chamber. Twenty-five mL of mobile phase was used for development and 25 mL for chamber saturation for 20 min in the other trough, which contained a saturation pad (Analtech, Newark, DE). Development time was approximately 10 min at 23°C and 50% humidity. Plates were dried in a fume hood with a steady stream of air from a hair dryer for 5 min, sprayed with a 5% solution of phosphomolybdic acid (PMA) in absolute ethanol,[12] and heated for 10 min at 115°C on a Camag plate heater to detect lipids as blue spots on a yellow background.

The standard for polar lipid analysis was Polar Lipid Mix 1127 (Matreya, Pleasant Gap, PA) which contained equal amounts of cholesterol, phosphatidylethanolamine, phosphatidylcholine, and lysophophatidylcholine. The

standard solution was prepared at a concentration of 0.250 μg μL^{-1} of each compound in cholorform-methanol (2 : 1). Plates were developed with chloroform-methanol-water (65 : 25 : 4),[13] as was described above for neutral lipid analysis. Development time was approximately 20 min. Dried plates were sprayed with 10% aqueous cupric sulfate solution, and heated for 10 min at 140°C to detect lipids as brown spots on a white background.

Quantitative densitometric analysis was performed by use of a Camag TLC Scanner II with the tungsten light source set at 610 nm for neutral lipids and 370 nm for polar lipids. Other scanning parameters were slit width 4, slit length 4, and scanning rate 4 mm s^{-1}. 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. Different volumes of sample solution were applied so that the area of at least one zone would be bracketed within the calibration graph. If the area of more than one aliquot of sample was bracketed, the mass of the aliquot having a scan area nearest to the average area of the middle two standards was used for calculation of results. Plates were photographed under white light using a Camag VideoStore Documentation System with a video camera.

The equation used to calculate the percent of the lipids in the whole body and DDG was:

$$\%\text{lipid } (w/w) = \frac{(\text{w})(\text{R})(\text{dilution factor})(100)}{\mu\text{g sample}}$$

where w = μg interpolated from the calibration graph and R = [reconstitution volume (μL)]/[spotted volume (μL)]. Some of the samples had to be diluted or concentrated to obtain a bracketed scan area within the calibration graph. An appropriate dilution factor was then included in the equation.

All statistical analyses were done using a single factor analysis of variance (ANOVA) to determine whether there was a significant difference in the lipid percent of the whole body or DGG among estivated, starved, and control snails of a given species. 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 AND DISCUSSION

All estivated, starved, and control snails of both species survived the 14 day period of the experiment. The revival times for estivated *B. glabrata* (n = 8) and *H. trivolvis* (n = 10) snails were 21.5 ± 3.4 min and 18.5 ± 3.1 min, respectively (mean ± SE). Revival time was considered to be the time it took the snail to extend its head foot region and attach to a substratum.

After the 14 day experimental period, estivated *B. glabrata* snails (n = 8) had lost 11.6 ± 1.6% of their weight, while starved *B. glabrata* snails (n = 9) had lost 7.8 ± 1.4% of their weight. Estivated *H. trivolvis* (n = 10) had lost 17.9 ± 3.2% of their weight, while starved *H. trivolvis* (n = 10) had lost 8.3 ± 2.8% of their weight. For both species, estivated snails lost more weight during the experimental period than did starved snails; the increased weight loss in estivated snails was probably due to loss of water. The fact that estivated *H. trivolvis* lost more weight than did *B. glabrata* suggests that *B. glabrata* were more resistant to water loss than *H. trivolvis*.

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[10] mobile phase showed zones with comparable migration to the standards at R_F values of 0.22 (cholesterol), 0.30 (oleic acid), and 0.58 (triolein). Figure 1 shows chromatograms developed in the Mangold[10] mobile phase. The samples analyzed for

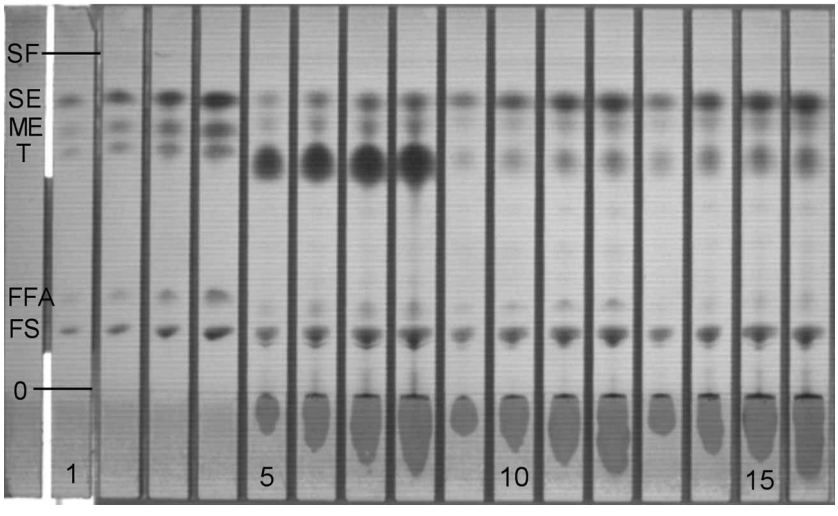

Figure 1. Photograph of chromatograms developed with petroleum ether-diethyl ether-glacial acetic acid (80 : 20 : 1)[10] on a laned, preadsorbent high performance silica gel plate showing relative neutral lipid concentrations in the DGG of *H. trivolvis* (Colorado strain). Lanes 1, 2, 3, and 4 contain 2.00, 4.00, 8.00, and 16.0 μL of mixed neutral lipid standard, respectively; lanes 5, 6, 7, and 8 contain 2.50, 5.00, 7.50, and 10.0 μL of reconstituted extract from a control snail, respectively; lanes 9, 10, 11, and 12 contain the same respective volumes of extract from starved snails; and lanes 13, 14, 15, and 16 contain the same respective volumes of extract from estivated snails. This plate was not used for quantitative analysis and is shown to illustrate the appearance of typical chromatograms. Abbreviations: O = origin and SF = solvent front. See Table 1 for compound abbreviations.

neutral lipids in the Smith et al.[11] mobile phase showed zones with comparable migration to the steryl ester standard at a R_F of 0.60 (cholesteryl oleate). Methyl esters were not present in any of the samples analyzed. Figure 2 shows chromatograms developed in the Smith et al.[11] mobile phase. The samples analyzed for phospholids in the Wagner et al.[13] mobile phase showed sample and standard zones at R_F values of 0.30 (phosphatidylcholine) and 0.55 (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. Figure 3 shows chromatograms developed in the Wagner et al.[13] mobile phase.

Table 1 presents data for the concentrations of neutral and polar lipids in the whole body of estivated, starved, and control *B. glabrata*. The only lipid class that showed a significant difference in bodies of estivated ($P = 0.049$) and starved ($P = 0.036$) versus the control snails was the triacylglycerol fraction. Table 2 presents data for the concentrations of neutral and polar lipids in the DGG of estivated, starved, and control *B. glabrata*. The only

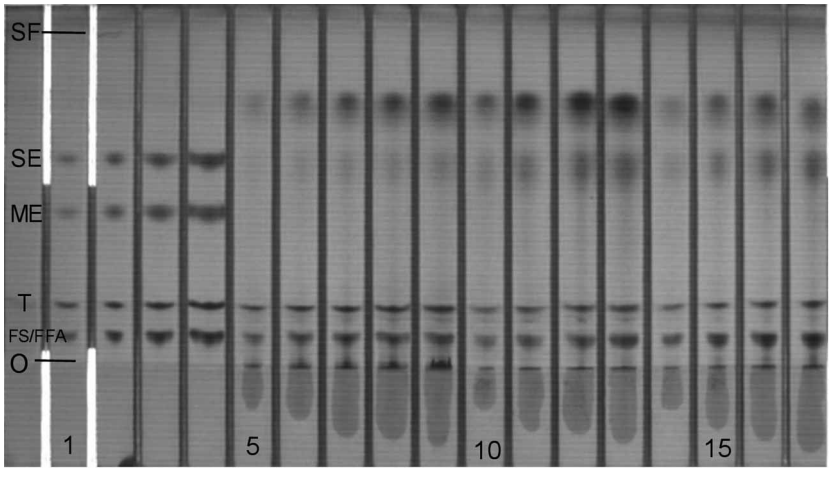

Figure 2. Photograph of chromatograms developed with hexane-petroleum ether-diethyl ether-glacial acetic acid (50 : 25 : 5 : 1)[11] on a laned, preadsorbent high performance silica gel plate showing relative neutral lipid concentrations in the DGG of *H. trivolvis* (Colorado strain). Lanes 1, 2, 3, and 4 contain 2.00, 4.00, 8.00, and 16.0 μL of mixed neutral lipid standard, respectively; lanes 5, 6, 7, 8, and 9 contain 2.50, 5.00, 7.50, 10.0, and 12.5 μL of reconstituted extract from a control snail, respectively; lanes 10, 11, 12, and 13 contain 2.50, 5.00, 7.50, and 10.0 μL of extract from starved snails; and lanes 14, 15, 16, and 17 contain the same respective volumes of extract from estivated snails. This plate was not used for quantitative analysis and is shown to illustrate the appearance of typical chromatograms. See Figure 1 for abbreviations.

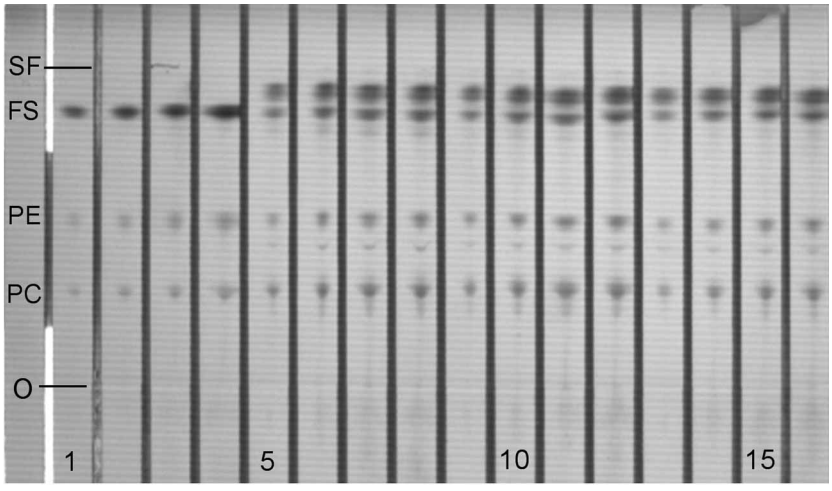

Figure 3. Photograph of chromatograms developed with chloroform-methanol-water (65 : 25 : 4)[13] on a laned, preadsorbent high performance silica gel plate showing relative phospholipid concentrations in the DGG of *B. glabrata*. Lanes 1, 2, 3, and 4 contain 2.00, 4.00, 8.00, and 16.0 μL of mixed polar lipid standard, respectively; lanes 5, 6, 7, and 8 contain 2.50, 5.00, 7.50, and 10.0 μL of reconstituted extract from a control snail, respectively; lanes 9, 10, 11, and 12 contain the same respective volumes of extract from starved snails; and lanes 13, 14, 15, and 16 contain the same respective volumes of extract from estivated snails. This plate was not used for quantitative analysis and is shown to illustrate the appearance of typical chromatograms. See Figure 1 for abbreviations.

significant difference found in the DGG was that of the steryl ester fraction. The steryl ester concentration in the DGG of starved *B. glabrata* was significantly increased above that of the control ($P = 0.04$), but not above the estivated ($P > 0.05$) snails.

Table 3 shows the concentrations of neutral and polar lipids in the whole body of estivated, starved, and control *H. trivolvis*. Triacylglycerols in the whole body of *H. trivolvis* significantly decreased in estivated ($P = 0.0001$) and starved ($P = 0.0001$) snails compared to the control. The level of steryl esters in the whole body of starved *H. trivolvis* was significantly increased above the level in estivated ($P = 0.046$) and control ($P = 0.002$) snails. Data for the concentrations of neutral and polar lipids found in the DGG of estivated, starved, and control *H. trivolvis* are shown in Table 4. The triacylglycerol levels in the DGG were significantly decreased in estivated ($P = 0.043$) and starved ($P = 0.035$) *H. trivolvis* compared to control snails. In addition, the steryl ester levels were significantly increased in the DGG of estivated ($P = 0.017$) and starved ($P = 0.004$) snails compared to control snails. Figures 1 and 2 show representative chromatograms for the neutral lipid content of the DGG of estivated, starved, and control *H. trivolvis*.

Table 1. Percent (mean ± SE) of lipids in the whole body of estivated, starved, and control *B. glabrata* with n = 4

Experimentals	FS[a]	FFA[b]	T[c]	SE[d]	PC[e]	PE[f]
Estivated	0.165 ± 0.016	0.00225 ± 0.0011[g,h]	0.0470 ± 0.021[g,i]	0.129 ± 0.017	0.397 ± 0.089[g]	0.403 ± 0.054
Starved	0.249 ± 0.025	0.00402 ± 0.0014[h]	0.0612 ± 0.020[i]	0.122 ± 0.029	0.920 ± 0.24	0.492 ± 0.20
Control	0.253 ± 0.043	0.000856 ± 0.00025[g,h]	1.63 ± 0.56	0.0515 ± 0.020	1.02 ± 0.32	0.557 ± 0.19

[a]FS = Free sterols.
[b]FFA = Free fatty acids.
[c]T = Triacylglycerols.
[d]SE = Steryl esters.
[e]PC = Phosphatidylcholine.
[f]PE = Phosphatidylethanolamine.
[g]An outlier was found and eliminated by the Q-test (90% confidence interval), leaving n = 3.
[h]Two of the four samples were below the limit of quantification. The values used to calculate mean and standard error were half of the limit of quantification.
[i]Triacylglycerol level in starved snails was significantly decreased in estivated ($P = 0.049$) and starved ($P = 0.036$) snails.

Table 2. Percent (mean ± SE) of lipids in the DGG of estivated, starved, and control *B. glabrata* with n = 4 estivated and control samples and n = 5 starved samples

Experimentals	FS[a]	FFA[b]	T[c]	SE[d]	PC[e]	PE[f]
Estivated	0.415 ± 0.075	0.0694 ± 0.022	0.0294 ± 0.0048	0.224 ± 0.067[g]	2.74 ± 0.56	0.786 ± 0.18
Starved	0.496 ± 0.098	0.0661 ± 0.0064[h]	0.0932 ± 0.032	0.240 ± 0.024	3.63 ± 0.60	1.02 ± 0.21
Control	0.225 ± 0.032	0.161 ± 0.065	0.672 ± 0.28[i]	0.0714 ± 0.024	2.16 ± 0.27	0.713 ± .048

[a–f]See Table 1 for definitions of abbreviations.

[g]Steryl ester level in starved snails was significantly increased above control ($P = 0.040$) snails, but not above estivated ($P > 0.05$) snails.

[h]An outlier was found and eliminated by the Q-test (90% confidence interval), leaving n = 4.

[i]An outlier was found and eliminated by the Q-test (90% confidence interval), leaving n = 3.

Table 3. Percent (mean ± SE) of lipids in the whole body of estivated, starved, and control *H. trivolvis* (Colorado strain) with n = 4

Experimentals	FS[a]	FFA[b]	T[c]	SE[d]	PC[e]	PE[f]
Estivated	0.240 ± 0.050	0.00536 ± 0.0018[g]	0.322 ± 0.080[h]	0.0348 ± 0.0091	0.797 ± 0.25	0.480 ± 0.19
Starved	0.330 ± 0.047	0.0171 ± 0.0072[g]	0.144 ± 0.024[h]	0.0628 ± 0.0067[i]	1.24 ± 0.47	0.825 ± 0.080
Control	0.241 ± 0.066	0.0213 ± 0.0076	2.77 ± 0.14[j]	0.0139 ± 0.0015	0.867 ± 0.20	0.519 ± 0.018[j]

[a–f]See Table 1 for definitions of abbreviations.

[g]One of the four samples was below the limit of quantification. The value used to calculate mean and standard error was half of the limit of quantification.

[h]Triacylglycerol levels were significantly decreased in estivated ($P = 0.0001$) and starved ($P = 0.0001$) snails.

[i]Steryl ester level in starved snails was significantly increased above estivated ($P = 0.046$) and control ($P = 0.002$) snails.

[j]An outlier was found and eliminated by the Q-test (90% confidence interval), leaving n = 3.

Table 4. Percent (mean ± SE) of lipids in the DGG of estivated, starved, and control *H. trivolvis* (Colorado strain) with n = 6 for neutral lipids and n = 4 for phospholipids

Experimentals	FS[a]	FFA[b]	T[c]	SE[d]	PC[e]	PE[f]
Estivated	0.347 ± 0.018[g]	0.0536 ± 0.014[g]	0.265 ± 0.014[h]	0.151 ± 0.025[i]	2.26 ± 0.50	0.430 ± 0.023
Starved	0.508 ± 0.067	0.0706 ± 0.0080[g]	0.217 ± 0.11[h]	0.179 ± 0.031[g,i]	1.13 ± 0.48	0.543 ± 0.12
Control	0.341 ± 0.060	0.0789 ± 0.021	1.66 ± 0.60	0.0503 ± 0.011	1.95 ± 0.26	0.455 ± 0.15

[a–f]See Table 1for definitions of abbreviations.

[g]An outlier was found and eliminated by the Q-test (90% confidence interval), leaving n = 5.

[h]Triacylglycerol levels were significantly decreased in estivated ($P = 0.043$) and starved ($P = 0.035$) snails.

[i]Steryl ester levels were significantly increased in estivated ($P = 0.017$) and starved ($P = 0.004$) snails.

Triacylglycerols are associated with depot fats in many invertebrates, including snails.[14] Therefore, it is not surprising that this lipid class was significantly decreased in the whole body and DGG of starved and estivated *H. trivolvis* and in the whole body of starved and estivated *B. glabrata*. Duncan et al.[15] and Conaway et al.[16] have previously reported decreased triacylglycerol levels in the whole body and DGG, respectively, of starved *B. glabrata*. During estivation snails do not consume food, and therefore are without exogenous nutrient.[4] Both estivated and starved snails probably use a considerable portion of their depot fats for energy. In accord with the marked decrease in the triacylglycerol levels in starved and estivated snails in our study, von Brand et al.[4] found by gravimetric analysis that the overall lipid content of the whole body of *B. glabrata* decreased during estivation. The gravimetric method of von Brand et al.[4] precluded determining the lipid class involved in the depletion. The use of TLC in our study allowed us to determine that the triacylglycerol fraction was the one that decreased during estivation and starvation.

The function of steryl esters in snails is not known and we cannot explain the significance of the increase in this lipid class in estivated and starved snails. An increase in steryl esters in the whole body or DGG of starved or estivated *B. glabrata* has not been reported previously, although Schneck et al.[17] noted such an increase in this lipid fraction in *H. trivolvis* (Colorado strain) fed hen's egg yolk compared to snails fed lettuce.

The finding that triacylglyerols significantly decreased and steryl esters significantly increased in whole body samples as well as DGG samples indicate that starvation and estivation affect lipids in the snail muscle and/or viscera and other unknown organs or tissue in addition to the DGG.

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