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Plasma triglyceride and β-hydroxybutyric acid levels in red-sided garter snakes (*Thamnophis sirtalis parietalis*) at emergence from hibernation

J. M. Whittier* and R. T. Mason[a]

Department of Anatomical Sciences, University of Queensland, Queensland 4072 (Australia), Fax +61 73 365 12 99, and [a]Department of Zoology, Oregon State University, Corvallis (Oregon 97331, USA)
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Abstract. Measurement of plasma levels of triglycerides and β-hydroxybutyric acid in females and males of the red-sided garter snake (*Thamnophis sirtalis parietalis*) suggest that the former may provide a useful physiological marker of condition. Levels of triglycerides at emergence from hibernation during a month of natural aphagia were significantly greater in females than in males and she-males, a subset of the male population that mimics females. Higher levels of triglycerides in the females may be attributed to their greater body mass per unit length, which was correlated with the level of triglycerides. Plasma triglyceride levels declined in females within one month of emergence, at the onset of feeding, and were unrelated to mating.
Key words. Triglycerides; lipids; body fat; reproduction; hibernation; snakes; reptiles.

In most snakes fecundity is dependent on body mass or size, with larger females producing more offspring than smaller ones[1,2]. Once a given size or age is passed and sexual maturity is attained, reproduction may be modulated by factors such as the nutritional condition of females. Nutritional condition has most often been measured by examination of relative body mass and stored fat reserves[2,3]. Such relationships have led to the hypothesis that stored energy reserves are limiting factors in the regulation of reproduction in snakes.

Few studies have investigated the components of fat that might serve as a metabolic signal that facilitates reproduction. Lipids present in fat storage depots (fat bodies) and plasma or serum in relation to egg development have been profiled in a few species of lizards and turtles[3,4]. Triglycerides make up the major lipid component of both of these tissues; for example, in the lizard *Sceloporous jarrovi*, 92.6 to 94.9% of lipids in fat body depots are triglycerides, with phospholipids, cholesterol esters, free fatty acids, diglycerides and cholesterol making up the minor lipid components[5].

Lipids in fat bodies in reptiles permit hibernation or estivation and vary seasonally in relation to activity[6,7]. The Canadian red-sided garter snake (*Thamnophis sirtalis parietalis*) utilizes stored fat reserves for both hibernation and reproduction. These reptiles feed for only three months of the year. Females use stored energy reserves to survive long periods of hibernation at low temperatures. After emergence, they rely on reserves while naturally aphagic, to reproduce[8,9]. As in other snakes, annual body mass fluctuations in *T. s. parietalis* from Manitoba are primarily a function of variation of body fat[10]. On emergence from hibernation 5–6% of female body mass is contained in fat bodies[8,11], and fat bodies decrease as a percentage of body mass to approximately 4–5% after ovulation. Body weight influences the incidence of reproduction in females of this species but we do not understand how information about body weight is discerned by the female's reproductive system[2].

In this study we examine plasma levels of triglycerides and β-hydroxybutyric acid (β-HBA) in *T. s. parietalis* in the spring of the year after emergence from hibernation. At this time animals are naturally aphagic (in May) and commence feeding (in June). β-HBA is a ketone body formed in mammals and birds when excess fat is oxidized by the liver and can accumulate in the plasma under conditions of fasting. We test the hypothesis that plasma levels of these metabolites would indicate the degree of reliance on the metabolism of stored body fat at emergence from hibernation. Furthermore, we evaluate changes in these metabolites over the period of resumption of natural feeding.

Materials and methods

Animals. Adult male and female red-sided garter snakes (39.0–70.0 cm SVL) were collected at snake dens in the Interlake region of Manitoba (Canada), in May of 1992. Animals were weighed to the nearest 0.5 g (BM), measured to the nearest 0.1 cm snout to vent (SVL), and individually marked by scute clippings. Males that elicited courtship behaviour from other males, termed she-males[21], were collected from mating aggregations in the field. Unmated females were collected as they first emerged from the ground and lacked a copulatory plug. Mated females were collected during copulation with

* Corresponding author.

males. Plasma lipid levels from three sets of animals were compared in the study:

1) Group I: Small unmated females (<64 cm SVL, n = 11), males (n = 8), and she-males (n = 12), bled in the field in May 1992, during a period of natural aphagia; these animals were selected from the population within a matched size range of SVL (40.0 to 64.0 cm).

2) Group II: Females sampled in the field in May 1992, during a period of natural aphagia (n = 9; 1 mated, 8 unmated) or in the laboratory in June 1992, once they had commenced feeding (n = 20; 5 mated, 15 unmated);

3) Group III: Mated females that were repeatedly sampled in the field in May 1992, and in the laboratory in June 1992 (n = 6).

Animals sampled in June 1992, had been captured in the field in May 1992, and were held in cloth bags at ambient temperatures in the field for 2–3 weeks prior to being transported by air to the laboratory. During this time the animals were naturally aphagic and were not offered food, although they were provided access to water every 2–3 days. On arrival in the laboratory animals were placed on summer-like conditions (28 °C, 14L:10D). Six to eight individuals were housed together in 20 gal aquaria illuminated with Durotest Vitalite bulbs. Animals were offered a diet of chopped smelt and canned mackeral fortified with vitamins (Petco Inc.), fed ab lib twice weekly. By June animals had commenced feeding. Water was provided ad lib. These conditions were sufficient to maintain a healthy colony of garter snakes.

Blood collection and processing. Blood samples were collected by cardiac puncture and were transferred to heparinized centrifuge tubes. Following centrifugation at 3000 rpm for ten minutes, the plasma was transferred to clear plastic tubes and frozen at −4 °C until analyzed.

Plasma lipid and ketone body analysis. Plasma levels of triglycerides and β-HBA were analyzed in two sets for each determination; one set included Group I animals, and the second set included group II and III animals. In some instances insufficient plasma was available for the analysis of both triglycerides and β-HBA.

Triglycerides were measured using a quantitative semi-enzymatic procedure that spectrophotometrically measures NADH oxidation (Sigma Diagnostics procedure 320-UV, Lot # 32H6121). A sample of 0.2 ml of plasma was analyzed for triglycerides; the absorbance of the mixture was determined in 1 cm disposable plastic cuvettes on a Varian DMS 80 UV/Visible Spectrophotometer. The final estimate of triglyceride concentration included multiplication by a constant (740) that included a factor using the average molecular weight of serum triglycerides in humans. The latter value was used as a standard value since no estimate of MW of triglycerides present in reptiles is available.

Using these methods, determination of a triolein standard (300 mg/dl) in triplicate yielded a mean (±1 SE) concentration of 318.94 (±7.5) mg/dl and a normal and an elevated serum (Lipid Control-N and Lipid Control-E, Sigma Diagnostics) control were 147.26 and 190.55 mg/dl, respectively, within expected ranges.

Plasma levels of β-HBA were measured quantitatively using a single enzymatic reaction with spectrophotometric determination of NADH oxidation as an end point (Sigma Diagnostic procedure 310-UV, Lot # 32H-6185). A sample of 0.05 ml of plasma was analyzed for B-HBA. Using this procedure we determined a calibration curve with four standard concentrations of β-HBA. These estimates were linear ($y = 0.951x + 0.908$) and significantly correlated ($r^2 = 0.9927$, $t = 16.47$, $p < 0.004$) with the known standard values over a concentration range from 10 to 75 mg/dl of β-HBA.

Statistical analysis. Triglyceride and β-HBA concentrations were compared within group I, group II and group III sets of animals. Plasma lipid concentrations in the first two groups were analyzed using nonparametric Kruskal-Wallis tests, with a test criterion, T, followed by a Dunn's test, with a test criterion, z, among treatments if significant differences were found. Differences in plasma metabolite levels in groups III, the females that were bled repeatedly, were analyzed using a Wilcoxon Signed Rank test, with a test criterion, T.

In addition, correlation and regression analyses were conducted to compare triglycerides and β-HBA to BM and SVL, as well as with each other, using a test criterion, t. Analysis of covariance was used to analyze the effects of BM and SVL on triglyceride levels in groups I animals, using a test criterion, F. A probability level of $p < 0.05$ was accepted as significant in all statistical tests.

Results

Significant differences in plasma levels of triglycerides were found among small female, male and she-male *T. s. parietalis* on emergence in May (group I animals; Kruskal-Wallis T, 6.410, 2 df, $p = 0.041$; fig. 1). The

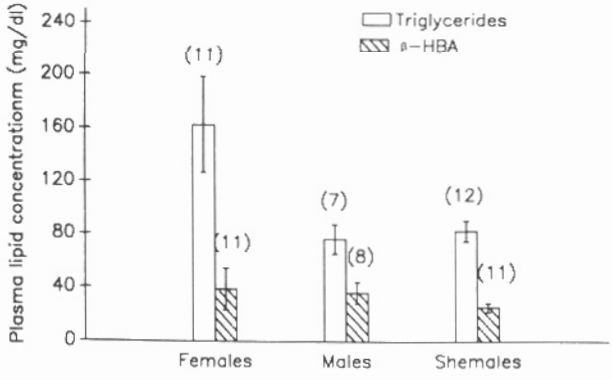

Figure 1. Plasma levels (mg/dl) of triglycerides (open bars) and β-hydroxybutyric acid (β-HBA, diagonal bars) measured in female, male, and she-male *Thamnophis sirtalis parietalis* in May at emergence from hibernation. Females had significantly elevated levels of triglycerides. Sample sizes are in parentheses.

Table 1. Mean (±1 SE) snout vent length (SVL) and body mass (BM) of size selected female, male and she-male *T. s. parietalis* sampled in May, 1992 (group I animals).

Sexual type	N	Mean ± 1 SE SVL (cm)	Mean ± 1 SE BM (g)	Mean ± 1 SE BM/SVL (g/cm)
Females	11	50.8 ± 1.6	50.4 ± 6.0*	0.963 ± 0.070*
Males	7	47.6 ± 2.0	35.1 ± 1.7	0.740 ± 0.022
She-males	12	46.9 ± 1.5	36.7 ± 2.5	0.773 ± 0.037

*Significantly different from other means, $p < 0.05$.

Table 2. Plasma levels of triglycerides and β-HBA in female *T. s. parietalis* in May and June (group II animals) after emergence from hibernation.

Month	Triglycerides (mg/dl) Mean ± 1 SE (N)	β-HBA (mg/dl) Mean ± 1 SE (N)
May	171.18 ± 37.74* (9)	35.24 ± 14.50 (11)
June	90.20 ± 12.72 (20)	37.10 ± 7.36 (16)

*Significantly elevated over levels in June, $p < 0.05$.

mean (±1 SE) triglyceride level of small females (162.79 ± 36.02) was significantly greater by twofold than that of both groups of males (males, 76.43 ± 11.22; she-males, 82.51 ± 7.81, Dunn's test $z = 0.0042$). Mean plasma levels of β-HBA in group I animals were relatively uniform (mean ±1 SE: females, 38.08 ± 15.48; males, 36.08 ± 8.01; she-males, 25.13 ± 3.23 mg/dl). These values were also low in comparison to plasma levels of triglycerides (fig. 1). No significant differences in plasma levels of β-HBA were detected (T, 1.988, 2 df, $p = 0.370$), and there was no significant correlation between individual levels of triglycerides and β-HBA ($r^2 = 0.0361$, $t = 0.99$, $p = 0.33$).

Group I individuals were selected from a size range (SVL) over which males and females overlap (40.0 to 64.0 cm), and there was no significant difference in mean SVL among males, females and she-males ($F = 1.72$, 2 df, $p > 0.1975$) in this sample. However, there was a significant difference in body mass (BM, $F = 201.33$, 1 df, $p < 0.0001$) among the groups (table 1). When BM was analyzed as a covariant of SVL, females had a significantly greater BM than males of similar lengths ($F = 201.23$, 1 df, $p < 0.0001$). She-males were intermediate in BM when BM was analyzed as a covariant of SVL, and their BM was not significantly different from either females or males.

Group I females had a significantly greater BM/SVL ratio, a gross measure of stoutness (table 1). There was also a significant correlation between BM, SVL and plasma levels of triglycerides among the group I animals sampled in May (BM $r^2 = 0.1313$, $t = 2.06$, $p = 0.0491$; SVL $r^2 = 0.1352$, $t = 2.09$, $p = 0.0456$). Similar results were found when all unmated females sampled in May, including groups I, II and III were included in the correlation analysis (BM $r^2 = 0.0337$, $t = -3.45$, $p = 0.0033$; SVL $r^2 = 0.1100$, $t = 3.78$, $p = 0.0016$). When the significant effect of BM of females, males and she-males was taken into account there were no significant differences in triglyceride levels among the three sexual types. Thus, analysis of covariance of triglyceride levels found no significant effect of BM ($F = 0.86$, 1 df, $p > 0.3616$), SVL ($F = 1.75$, 1 df, $p > 0.1974$) or sexual type ($F = 3.05$, 2 df, $p > 0.0655$). Similarly, no significant effect of sexual type was found when an analysis of covariance of triglyceride levels was conducted with the BM/SVL, or stoutness, measure, included as a covariate of triglyceride levels ($F = 2.17$, 2 df, $p > 0.1341$).

Plasma levels of triglycerides in group II females were significantly higher on emergence in May, during natural aphagia, than in June, once feeding commenced ($T = 179$, $p < 0.0381$; table 2). Plasma levels of β-HBA, in contrast, were not significantly different in the two months of sampling ($T = 137$, $p < 0.4015$; table 2). Moreover, there was no significant effect of mating status on plasma levels of either triglycerides or β-HBA ($T = 69.5$, 73, $p > 0.2697$, for triglycerides and β-HBA, respectively).

In group II females that were sampled repeatedly in May and June, plasma levels of triglycerides were also significantly higher in May than in June ($T = 10.5$, $p < 0.0464$; fig. 2). Further analysis of the triglyceride levels in females that were bled repeatedly (Group III) found that May and June levels in each female were significantly correlated ($r^2 = 0.8693$, $t = 5.16$, $p = 0.0067$). As was found in the group II females, plasma levels of β-HBA were not significantly different in the two months of sampling ($T = 33$, $p = 1.00$). There was no correlation between levels of β-HBA measured in the plasma of individual females in May and June ($r^2 = 0.0332$, $t = -0.56$, $p = 0.5921$). Unlike the results observed among females, males and she-males in group

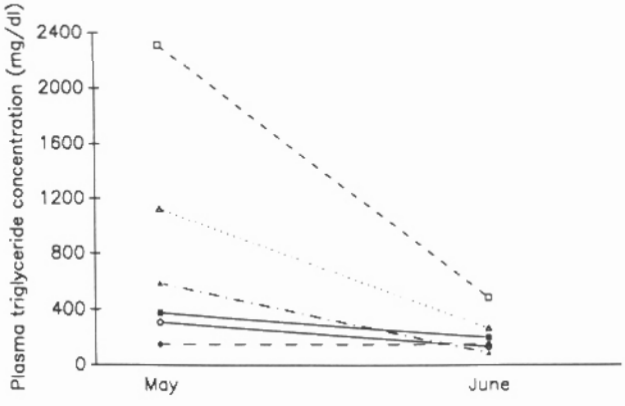

Figure 2. Plasma levels of triglycerides of individual female *Thamnophis sirtalis parietalis* measured repeatedly in May and June. Triglyceride levels decreased significantly from May to June, and May levels were correlated with June levels of each female.

I, there was no relationship among BM or SVL and plasma levels of triglycerides in group III females. There was also no significant correlation between BM or SVL and plasma levels of β-HBA in either month.

Discussion

This study found that during a period of natural aphagia plasma levels of triglycerides in female (*T. s. parietalis*) were significantly higher than in males or she-males of comparable body lengths. However, this difference among sexual types in the population may be attributed to the significantly greater body mass of females at a given length. Triglyceride levels were correlated with body mass and the greater levels observed in females were related to their greater body mass per unit of length. It is notable that with respect to this physiological parameter, she-males, the males of this species that elicit female courtship[12], apparently resemble males more than they do females. However, she-males had an intermediate mean relative body mass per unit length, falling between the mean values of males and females. Yet their levels of triglycerides in the plasma were more similar to males than to females. Previously, in all other measures of physiological function that have been examined, she-males have more closely resembled females than males[12,13].

Plasma triglyceride levels in this study were also found to drop dramatically in females over the six week period following emergence from hibernation. These changes in plasma triglycerides most likely reflect natural changes in metabolic state the females undergo at this time with the onset of eating in June after emergence from hibernation in May. We hypothesize that females use stored triglycerides in fat bodies after emergence in May prior to the natural onset of eating, and that the reliance on these stores drops in June by which time most females have begun feeding.

It is of interest that mating, which is accompanied by surges in circulating prostaglandin and estradiol in this species[14,15], apparently has no measurable effect on plasma levels of triglycerides in the short or long term. Females that mated did not have substantially different levels in 24 hours, and their plasma triglyceride levels dropped in 4 weeks time. From these observations we suggest that estrogen does not appear to modulate triglyceride mobilization in this species in vivo, as has been suggested in other reptilian species[16,17].

The correlation between triglyceride levels of females measured repeatedly in May and June suggests that triglyceride levels are consistent over time and are a useful measure of body condition. In contrast, β-HBA levels in all animals were relatively uniform and did not provide a useful physiological marker of condition. There was little change in plasma levels of β-HBA in females over the two months following emergence from hibernation. This suggests that in *T. s. parietalis* β-HBA may not be produced as a major metabolic byproduct of fat store oxidation, as it is in birds and mammals. Alternatively, snake tissues could more rapidly metabolize β-HBA so that it does not accumulate in the plasma to the extent that it does in other amniote vertebrates. Further study of lipid metabolism in reptiles is needed to help understand the role of these substances in hibernation and reproduction. Lipid deposition in developing follicles in reptiles is known to involve the uptake of vitellogenin, a large phospholipid produced by the liver, under the same regulatory mechanism as found in other vertebrates[18]. However, the role of other circulating plasma lipoproteins, such as the triglyceride-incorporating very low density lipoprotein (VLDL), implicated in avian yolk deposition[19], have not been investigated in reptiles. The recent finding of a putative apolipoprotein B-100-like molecule in the freshwater turtle (*Chrysemys picta*) supports the hypothesis that VLDL may play a central role in yolk deposition in reptiles[20] and should be investigated more fully.

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