Published January 20, 2015

Plasma concentrations of key metabolites and insulin in late-pregnant ewes carrying 1 to 5 fetuses

U. Moallem,[1] A. Rozov, E. Gootwine, and H. Honig

Department of Ruminant Science, Institute of Animal Sciences, Volcani Center, PO Box 6, Bet-Dagan 50250, Israel

ABSTRACT: Ewes bearing more than 1 fetus are more susceptible to pregnancy toxemia than those with a single fetus. Crossbreeding programs in Israel increased the occurrences of ewes bearing more than 2 fetuses; therefore, the aim was to assess the exacerbation in the metabolic status of ewes pregnant with several fetuses. Fifty ewes, genetically developed to achieve multiple-fetus pregnancies, were monitored, on average, from d 115 of pregnancy until lambing for plasma concentrations of several key metabolites and insulin. The numbers of fetuses were examined by ultrasonography at 35 d of pregnancy. Blood samples were collected weekly, and concentrations of glucose, β-hydroxybutyrate (BHBA), NEFA, triglycerides, cholesterol, total calcium, and insulin were determined. The average litter size was 2.75 (±1.1), and 1 (1F), 2 (2F), 3 (3F), and 4 or more (4F) fetuses were conceived, respectively, by 6 (12%), 17 (34%), 14 (28%), and 13 (26%) ewes. Total birth weights of lambs were 6.1, 9.5, 12.7, and 15.0 kg for 1F, 2F, 3F, and 4F, respectively ($P < 0.001$). Plasma glucose concentrations in 1F were greater than those in 3F and 4F ($P < 0.05$) and were similar among 2F, 3F, and 4F. Trends toward increasing plasma concentrations of BHBA and NEFA were observed as the number of fetuses increased and also as lambing approached. Plasma concentrations of BHBA and NEFA were, respectively, 3.7 ($P < 0.002$) and 2.1 ($P < 0.001$) times as great in 4F ewes as in 1F ewes. Trends toward decreased concentrations of triglycerides and cholesterol were observed as litter size increased. Insulin concentrations in blood decreased considerably as the numbers of fetuses increased and, on average, they were less by a factor of 5 in the 4F ewes than in the 1F ewes ($P < 0.001$). Moreover, insulin concentrations during the week before lambing were extremely low (e.g., 0.54 μIU/mL in the 4F ewes). Insulin concentrations were reduced in ewes bearing >3 fetuses, even 5 wk before lambing; this decline apparently began earlier than the last month of gestation. Therefore, it seems that insulin has a pivotal role in the etiology of pregnancy ketonemia in ewes carrying multiple fetuses. The present findings may suggest that the decline in insulin concentrations that apparently occurs in the earlier stages of pregnancy represents a homeorhetic control to spare glucose for the brains and fetoplacental units of the dams. The results clearly demonstrate the increased susceptibility to pregnancy toxemia of ewes carrying multiple fetuses. Appropriate nutritional strategies should be developed for ewes that conceive >3 fetuses, to meet the increased nutritional requirements of the fetoplacental unit.

Key words: hyperketonemia, insulin, multiple fetuses, pregnant ewe

©2012 American Society of Animal Science. All rights reserved.

J. Anim. Sci. 2012. 90:318–324
doi:10.2527/jas.2011-3905

INTRODUCTION

Pregnancy toxemia is the most frequent metabolic disorder of late-pregnancy ewes carrying more than 1 fetus (Henze et al., 1998; Sargison, 2007) and is a major cause of economic loss to the sheep industry (Scott et al., 1995). The frequency of pregnancy toxemia in ewes with a litter size of ≤2 is 0.005, whereas that in ewes with ≥3 fetuses is 0.19 (Zamir et al., 2009). About 60% of fetal growth takes place in the last part of gestation (Twardock et al., 1973), when approximately 33 to 36% of the circulating glucose of the ewe is directed into the fetoplacental unit (Hay et al., 1983).

The etiology of pregnancy toxemia in ewes is not completely clear. As lambing approaches, there is a decline in plasma glucose concentration, but the decrease is relatively modest compared with the increases in concentrations of ketone bodies (Henze et al., 1998). Schlumbohm and Harmeyer (2004) found that induced hyperketonemia suppressed endogenous glucose pro-

[1]Corresponding author: uzim@volcani.agri.gov.il
Received January 24, 2011.
Accepted August 19, 2011.

duction; therefore, the authors recognized hyperketonemia as a causative factor in the development of pregnancy toxemia.

As the number of fetuses increases, the metabolic demands of the gravid uterus increase and thereby affect the profile of maternal plasma metabolites. Only very limited information is available on the through-pregnancy metabolic profiles of ewes carrying 3, 4, or 5 fetuses. Generally, the symptoms of pregnancy toxemia and other metabolic failure occur during the last weeks of pregnancy in ewes carrying ≥3 fetuses; therefore, we hypothesize that during this period (the last 4 to 5 wk of gestation), there are gradual but dramatic changes in several key metabolites. To better define the metabolic needs of multiple-fetus ewes during late pregnancy, the objectives of the present study were to examine the association between carrying various numbers of fetuses, and plasma concentrations of glucose, β-hydroxybutyrate (**BHBA**), NEFA, triglycerides (**TG**), cholesterol, total calcium, and insulin hormone.

MATERIALS AND METHODS

The Volcani Center Animal Care Committee approved all the procedures involving animals in the present study.

Animals

The data in this study were derived from 50 multiparous Afec-Assaf ewes bred in the experimental flock at the Volcani Center, Bet Dagan, Israel. The prolific Afec-Assaf strain was developed by introgression of the *B* allele of the *FecB* (Booroola) locus to the Assaf breed (Gootwine et al., 2008). Ewes in the present study were bred in a few clusters from mid-November through mid-December and lambed from early the following April through mid-May (i.e., the spring season in Israel).

The flock was kept in open sheds under nondairy management, and pregnancy and litter size were determined on d 35 of gestation by transabdominal ultrasonography (Pie Medical, Maastricht, the Netherlands). All ewes were kept in 1 pen and group-fed the same diet. During the first 120 d of pregnancy, ewes were fed daily with an average of 0.44 kg of grain mix (containing 14% protein, and ME at 1.6 Mcal/kg of DM), 0.52 kg of corn silage, and 1.34 kg of good-quality oat hay (DM basis). During the last month of gestation, the ewes were fed daily with an average of 0.66 kg of grain mix, 0.35 kg of corn silage, and 1.34 kg of good-quality oat hay (DM basis). Diets were formulated for ewes weighing 70 kg and carrying 3 fetuses, according to NRC recommendations (NRC, 1985).

At lambing, the number of lambs born and the number of those born alive were recorded. Birth weights of lambs were recorded within a few hours after lambing. Gestation length for each ewe was deduced from mating and lambing records.

Blood Sampling

Each week, from d 115 of pregnancy on average and until lambing, at 0700 h (i.e., before feeding), blood samples (10 mL) were taken from a jugular vein into vacuum tubes (Becton Dickinson Systems, Cowley, UK). Additional blood samples (6 mL) were collected into tubes containing lithium chloride and L-iodoacetate (BD Vacutainer, Belliver Industrial Estate, Plymouth, UK) for glucose analysis. Blood samples were centrifuged immediately after the last sample collection (about 1 h from the first blood collection) at 3,000 × *g* for 20 min at room temperature, and plasma was stored at −18°C pending analysis.

Chemical Analysis

Plasma NEFA concentrations were determined with a NEFA C Test Kit (Wako Chemicals GmbH, Neuss, Germany). The intra- and interassay CV for the NEFA assay were 6.9 and 6.1%, respectively. Plasma insulin was determined by RIA (Diagnostic Products, Los Angeles, CA). The intra- and interassay CV for the insulin assay were 6.9 and 4.9%, respectively.

Plasma glucose was determined with a liquid glucose reagent set (Pointe Scientific Inc., Canton, MI) that used 2 enzymatic reactions with hexokinase and glucose-6-phosphate dehydrogenase to generate a UV emission that was correlated with the sample glucose concentration; the samples were examined at 340 nm with an optical density reader (Sunrise, Tecan, Austria), and results were calibrated against known glucose concentrations. The intra- and interassay CV for the glucose assay were 2.6 and 2.2%, respectively.

Cholesterol concentrations were determined with a liquid cholesterol reagent set (Pointe Scientific Inc.) that used 3 enzymatic reactions with cholesterol esterase, cholesterol oxidase, and peroxidase to generate a UV emission that was correlated with the sample cholesterol concentration; the samples were examined at 500 nm with an optical density reader, and results were calibrated against known cholesterol concentrations. The intra- and interassay CV for the cholesterol assay were 0.7 and 0.8%, respectively.

Triglyceride concentrations were determined with a liquid TG reagent set (Pointe Scientific Inc.) that used 4 enzymatic reactions with lipase, glycerol kinase, glycerol phosphate oxidase, and peroxidase to generate a UV emission that was correlated with the sample TG concentration; the samples were examined at 500 nm with an optical density reader, and results were calibrated against known TG concentrations. The intra- and interassay CV for the TG assay were 1.8 and 1.9%, respectively.

Plasma calcium concentrations were examined with a Konelab analyzer (Thermo Clinical Labsystems, Vantaa, Finland) by a method based on measuring the UV emission of the highly colored complex formed by cal-

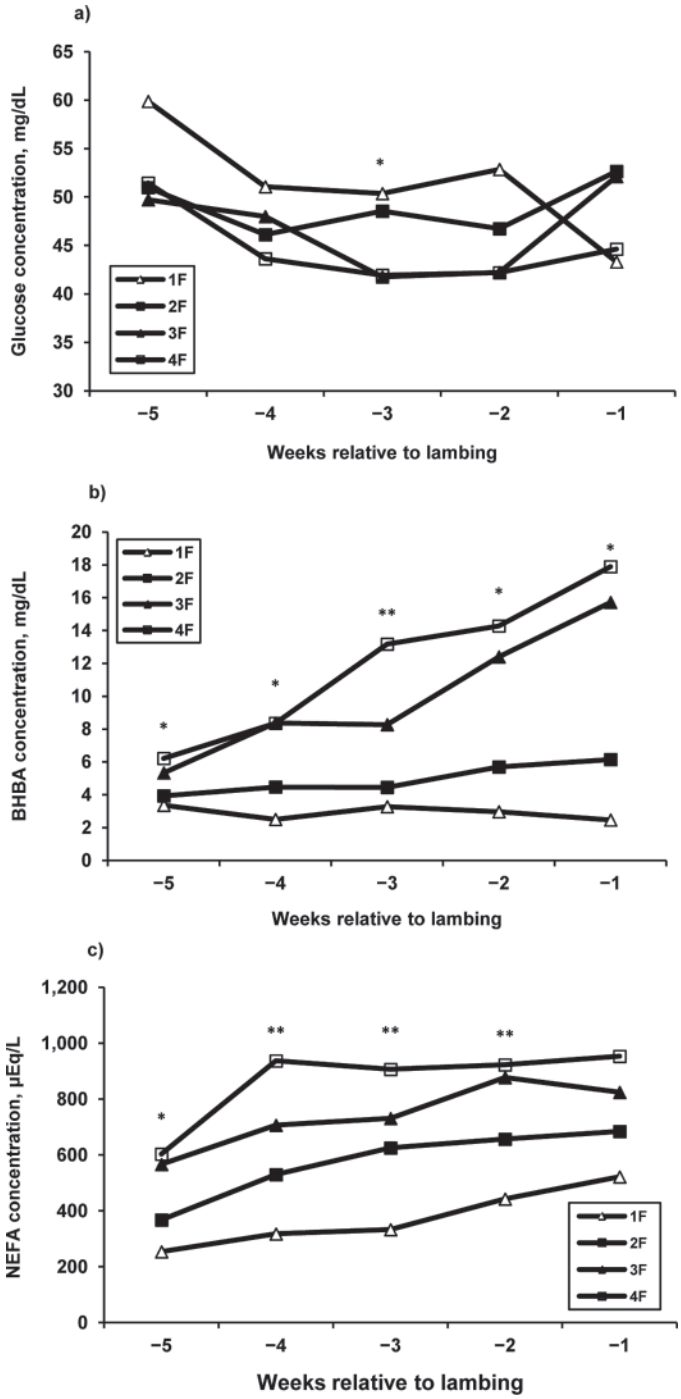

Figure 1. Temporal changes in glucose (a), β-hydroxybutyrate (BHBA; b), and NEFA (c) concentrations in plasma during the last 5 wk of gestation in ewes carrying 1 (Δ; 1F), 2 (■; 2F), 3 (▲; 3F), and ≥4 (□; 4F) fetuses. The pooled SEM values were 1.78 mg/dL, 1.61 mg/dL, and 62.04 µEq/L for glucose, BHBA, and NEFA, respectively. The statistical analysis was performed for every week separately, and the symbols used for the analysis each week were $^{**}P < 0.01$ and $^{*}P < 0.05$.

cium and Arsenazo III, which was examined at 660 nm; results were calibrated against known calcium concentrations. The intra- and interassay CV for the calcium assay were 0.97 and 1.5%, respectively.

Plasma BHBA concentrations were determined with a Ranbut D-3-Hydroxybutyrate Kit (Randox, Crumlin, UK), in which a reaction of 3-hydroxybutyrate and dehydrogenase generated a UV emission that was correlated with the sample BHBA concentration. The samples were examined at 340 nm with an optical density reader, and results were calibrated against known BHBA concentrations. The intra- and interassay CV for the BHBA assay were 1.3 and 1.6%, respectively.

Statistical Analysis

Pregnancy length, litter size, lamb survival rate, BW, and weekly plasma concentrations of glucose, BHBA, NEFA, and insulin (Figures 1 and 2) were analyzed with the GLM procedure (SAS Inst. Inc., Cary, NC); the model included the effect of parity. The final model used was

$$Y_{ijk} = \mu + LS_i + P_j + e_{ijk},$$

where Y_{ijk} is the dependent variable, μ is the overall mean, LS_i is the litter size effect (i = 1 to 4), P_j is the parity, and e_{ijk} is the random residual error.

Concentrations of metabolites and insulin in plasma during the entire study period were analyzed as repeated measurements by using the PROC MIXED procedure of SAS. The AR 1 (autoregressive order 1) was used as a covariance structure in the model. The effects of litter size, week before lambing, and their interaction were tested. The final model used was

$$Y_{ijklm} = \mu + LS_i + P_j + E(LS \times P)_{ijk} + WBL_{ijkl} = \text{week before lambing} + (LS \times WBL)_{il} + e_{ijklm},$$

where Y_{ijklm} is the dependent variable, μ is the overall mean, LS_i is the litter size effect (i = 1 to 4), P_j is the parity, $E(LS \times P)_{ijk}$ is ewes k nested in litter size group i and ewes nested in parity j, WBL_{ijkl} is the week before lambing as a continuous variable (l = −5 to −1); $(LS \times WBL)_{il}$ is litter size i × WBL l, and e_{ijklm} is the random residual error.

The PROC REG procedure of SAS was used for analyzing the correlation between BHBA and NEFA concentrations in plasma. The analysis was done on the means of each individual. Only ewes with at least 2 parallel BHBA and NEFA were included in the analysis. Least squares means and adjusted SEM are presented in Tables 1 and 2; $P < 0.05$ was accepted as significant unless otherwise stated, and tendencies were reported at $0.05 < P < 0.10$.

RESULTS

The average litter size for all ewes (n = 50) was 2.75 ± 1.1; of the 50 ewes, 6 (12%), 17 (34%), 14 (28%), 10 (20%), and 3 (6%), respectively, conceived 1 (**1F**), 2 (**2F**), 3 (**3F**), or 4 or 5 fetuses (**4F**; Table 1). Perinatal lamb mortality increased as the number of lambs born increased (Table 1); 20% of the lambs in the 4F group died, compared with 0% in the 1F group ($P < 0.01$). The average birth weight of the lambs decreased from

Table 1. Pregnancy length, number of fetuses, and lamb birth weight according to litter size

	No. of fetuses						
Item	1	2	3	4	5	SEM	*P*-value
No. of ewes	6	17	14	10	3		
Pregnancy length, d	146.6[ab]	145.1[ab]	144.8[b]	146.9[a]	146.0[ab]	0.64	<0.007
Litter size born alive, No.	0.97[d]	1.95[c]	2.66[b]	3.08[b]	4.31[a]	0.23	<0.007
Lamb survival rate, %	98.8[a]	97.5[a]	88.8[ab]	78.7[b]	86.0[ab]	1.04	<0.03
Total litter weight, kg	6.1[d]	9.5[c]	12.7[b]	15.0[a]	15.4[a]	0.66	<0.04
Average lamb BW, kg	6.1[a]	4.8[b]	4.2[bc]	3.7[cd]	3.0[d]	0.40	<0.01

[a–d]Within rows, means with different superscripts are statistically different.

6.1 kg in 1F litters to 3.5 kg in 4F litters ($P < 0.001$; Table 1); however, no significant differences were observed between 2F and 3F litters ($P > 0.13$), and the average birth weight in 4F litters tended to be lighter than that in 3F litters ($P < 0.09$). Total litter weight of lambs increased ($P < 0.04$) as the number of fetuses increased, and it was 2.4 times greater ($P < 0.001$) in the 4F group than in the 1F group (15.0 and 6.1 kg, respectively).

Average plasma concentrations of metabolites and insulin during the last 5 wk of pregnancy are presented in Table 2. Glucose plasma concentrations were greater in 1F ewes than in 3F and 4F ewes ($P < 0.02$; Table 2) and tended to be greater than those in 2F ewes ($P < 0.06$); however, no significant differences ($P > 0.44$) were observed between 3F and 4F ewes. No effects ($P > 0.20$) of days in pregnancy (**DIP**; Figure 1a) or the interaction of DIP × litter size on glucose concentrations were observed.

Average plasma BHBA concentrations during the 5 wk before lambing were 3.7 times greater in 4F ewes than in 1F ewes ($P < 0.005$; Table 2), but no significant differences ($P > 0.12$) were observed between 1F and 2F ewes, or between 3F and 4F ewes. The effect of DIP was significant ($P < 0.007$), but no interaction was observed. In F4 ewes, plasma BHBA concentrations increased ($P < 0.03$) from 6.1 mg/dL in wk −5 to 17.6 mg/dL in wk −1 (2.9 fold; Figure 1b).

Very similarly to BHBA, plasma NEFA concentrations were 2.1 times greater in 4F ewes than in the 1F ewes ($P < 0.001$; Table 2), and no significant differences in NEFA concentrations were observed between 1F and 2F ewes, or between 3F and 4F ewes ($P < 0.14$). The effect of DIP was significant ($P < 0.003$; Figure 1c), but there was no interaction ($P > 0.69$) of DIP × litter size. As shown in Figure 1c, increases in NEFA concentrations were prominent between wk −5 and −4, but later, the rates of increase in NEFA concentrations were moderate in all groups.

Triglyceride and cholesterol concentrations decreased ($P < 0.001$) as the number of fetuses increased, and TG concentrations in the 4F group were 42% greater than those in the 1F group ($P < 0.001$; Table 2). In both variables (TG and cholesterol), the effect of DIP was significant ($P < 0.01$ and $P < 0.007$, respectively), but no interaction ($P > 0.21$ and $P > 0.68$, respectively) of DIP × litter size was observed.

Plasma total calcium concentrations were greater in 1F ewes than in all other groups ($P < 0.009$; Table 2), whereas no differences ($P > 0.78$) were observed between 3F and 4F ewes. The effect of DIP and the interaction of DIP × litter size were not significant ($P > 0.17$ and $P > 0.93$, respectively).

Plasma insulin concentrations were 5.1 times greater in 1F than in 4F ewes ($P < 0.001$; Table 2). However, no significant differences ($P > 0.12$) were observed be-

Table 2. Metabolites and insulin plasma concentrations during late pregnancy in ewes that conceived various numbers of fetuses

	No. of fetuses					*P*-value[2]		
Item[1]	1	2	3	≥4	SEM	LS	DIP	LS × DIP
No. of ewes	6	17	14	13				
Glucose, mg/dL	51.5[a]	48.4[ab]	46.8[b]	44.7[b]	1.78	<0.01	<0.20	<0.40
BHBA, mg/dL	3.2[b]	5.1[b]	10.4[a]	11.9[a]	1.61	<0.002	<0.007	<0.14
NEFA, μEq/L	418.8[b]	567.0[b]	754.6[a]	875.1[a]	62.04	<0.001	<0.003	<0.69
TG, mg/dL	42.9[a]	31.4[b]	26.6[bc]	23.2[c]	2.34	<0.001	<0.01	<0.21
Cholesterol, mg/dL	88.5[a]	74.2[b]	76.3[ab]	70.4[b]	3.90	<0.05	<0.007	<0.68
Calcium, mg/dL	10.6[a]	10.1[ab]	9.8[c]	9.9[bc]	0.12	<0.003	<0.17	<0.93
Insulin, μIU/mL	15.2[a]	9.0[ab]	7.1[bc]	3.0[c]	2.08	<0.01	<0.001	<0.001

[a–c]Within rows, means with different superscripts are statistically different, $P < 0.05$.

[1]BHBA = β-hydroxybutyrate; TG = triglycerides.

[2]LS = litter size; DIP = days in pregnancy.

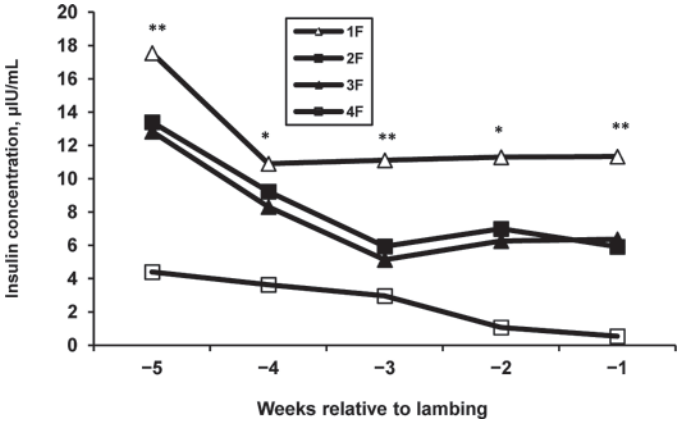

Figure 2. Temporal changes in insulin concentrations in plasma during the last 5 wk of gestation in ewes carrying 1 (Δ; 1F), 2 (■; 2F), 3 (▲; 3F), and ≥4 (□; 4F) fetuses. The pooled SEM value was 2.08 µIU/mL. The statistical analysis was performed for every week separately, and the symbols used for the analysis each week were $^{**}P < 0.01$ and $^{*}P < 0.05$.

tween 1F and 2F ewes, or between 3F and 4F ewes. The effect of DIP was significant (Figure 2; $P < 0.001$), as was the interaction of DIP × litter size ($P < 0.001$). There was a decline ($P < 0.01$) in insulin concentrations in all groups from wk −5 to −4 that continued until wk −3 in the 2F and 3F groups. However, the decline in plasma insulin concentrations in the 4F group continued until the last week of pregnancy and reached very small concentrations before lambing in these ewes.

DISCUSSION

In the present study, we demonstrated the increased susceptibility of ewes carrying multiple fetuses to hyperketonemia, which is the main indicator of pregnancy toxemia. We also showed that insulin plasma concentrations were reduced in ewes bearing more than 3 fetuses, and in the last week, they averaged 0.54 µIU/mL in this group.

Although the present study used a small sample, the decline in lamb survival with increasing litter size is consistent with the findings of large studies that investigated the association between litter size and mortality of lambs (Kleemann and Walker, 2005; Gootwine et al., 2008). In the present study, the average birth weight of lambs decreased as litter size increased (6.1 and 3.5 kg for the 1F and 4F groups, respectively). Lighter BW was associated with greater mortality in born lambs (Gootwine et al., 2007), which was also demonstrated in the present study (1.2 and 20.4% in the 1F and 4F groups, respectively). Nutrition appears to be the most critical factor in placental and fetal growth (Igwebuike, 2010), and the limitations in growth found in large litters might be related to a deficiency in supplies of nutrients and metabolites, similar to the observed low availability of glucose in ewes pregnant with multiple fetuses in the present study. The maternal-fetal gradient drives maternal glucose into the fetus, and if this gradient decreases because of either maternal hypoglycemia, as found in the present study, or because of fetal hyperglycemia, the flux of glucose into the umbilical circulation decreases (Battaglia and Meschia, 1988). This pattern of maternal-fetal glucose regulation apparently should protect the brain of the ewe from a glucose shortage in case of hypoglycemia of the dam. Moreover, the available glucose would be distributed among more fetuses in a multiple-fetus pregnancy so that each fetus would receive fewer energy resources than those in a pregnancy with 1 or 2 fetuses. These changes in metabolite availability might impair intrauterine growth, which would lead to reduced BW at birth and increase the perinatal mortality rate.

A trend was observed toward decreasing glucose concentrations with increasing litter size; surprisingly, however, concentrations were not significantly different among 2F, 3F, and 4F ewes. Moreover, the decrease in glucose concentration was relatively moderate compared with the increases in BHBA concentrations. These results are consistent with those of other studies, in which glucose concentrations were not especially reduced in ewes considered hyperketonemic (Henze et al., 1998; Kulcsár et al., 2006). Henze et al. (1998) found that only 40% of ewes with spontaneous pregnancy toxemia exhibited hypoglycemia on d 1 of the disease, whereas 40% showed normoglycemia and 20% hyperglycemia. Moreover, Henze et al. (1998) also found that glucose concentrations on the last day before death in ewes that died of pregnancy toxemia were not different from those in the ewes that survived. On the other hand, Scott et al. (1995) reported that plasma glucose concentrations in ewes that exhibited clinical signs of pregnancy toxemia were about 50% of those in normal ewes. It was suggested that insufficient glucostasis regulation might be responsible for the increased variation in glucose concentrations, rather than inadequate gluconeogenesis (Henze et al., 1998).

In the present study, concentrations of BHBA in plasma were greater as the number of lambs increased, as reported in other studies as well (Varnam et al., 1978; Harmeyer and Schlumbohm, 2006). Ketone bodies might be used as an alternative energy source for many tissues, including the placenta; however, although maternal ketonemia of multiple-fetus pregnancies was accompanied by increased uterine uptake of BHBA, no considerable amount of BHBA was transported to the fetus, so ketone bodies then become an important energy source for the placenta, but not for the fetuses (Battaglia and Meschia, 1988). Furthermore, Harmeyer and Schlumbohm (2006) demonstrated that BHBA utilization was reduced in the late-gestation ewe and that this reduction was greater in twin- than in single-bearing ewes, which led to increased plasma BHBA concentrations. Accordingly, it is plausible that the reduction in BHBA utilization might be greater in 3F and 4F ewes than in 1F and 2F ewes, which could explain the large difference in plasma BHBA concentrations between the latter and the former ewes: 3.2 and 11.9 mg/mL in 1F and 4F ewes, respectively. Collectively, it could be

concluded that the greater BHBA concentrations in the plasma of ewes carrying multiple fetuses might be attributed to increased production of BHBA in the liver, but also by underutilization of BHBA in these ewes, as was suggested by Harmeyer and Schlumbohm, (2006).

Endogenous glucose production in pregnant ewes was also found to be depressed by BHBA, with no effect on glucose utilization (Schlumbohm and Harmeyer, 2004). However, as mentioned above, in the present study the magnitude of decrease in glucose concentrations was relatively moderate compared with the magnitude of increase in BHBA. Moreover, the decreases in glucose concentrations of the dams in multiple-fetus pregnancies might be related to other factors, such as increased uptake of glucose by the fetuses, rather than to suppressed glucose synthesis. The incapability of fetuses to utilize BHBA as an energy source and the suppressing effect of BHBA on endogenous glucose production exacerbate the energy deficiency of fetuses and lead to accumulation of BHBA in the blood of late-gestation ewes.

Average NEFA concentrations in the blood were greater as litter size increased and also showed an upward trend as lambing approached. The increases in NEFA concentration in pregnant ewes and transition cows are due to increased mobilization of long-chain fatty acids from adipose tissues (Bergman, 1971). In 2 studies in which hyperketonemia was induced in sheep, NEFA concentrations decreased as BHBA concentrations increased (Heitmann and Fernandez, 1986; Harmeyer and Schlumbohm, 2006). In the present study, however, plasma NEFA concentrations increased in parallel with the increases in BHBA in all groups of ewes, except for the 1F group. Moreover, across-group analysis showed that NEFA and BHBA concentrations in plasma were positively correlated ($r = 0.81$). It might be that induced short-term hyperketonemia in experimental studies (Heitmann and Fernandez, 1986; Harmeyer and Schlumbohm, 2006) is different from that associated with naturally prolonged energy shortages, in which spontaneous, long-term hyperketonemia elicits adaptation of the metabolic system to energy deficiency, which leads to increases in NEFA concentrations rather than to a decrease, as in short-term induced hyperketonemia.

Ewes bearing more than 3 fetuses exhibited less blood calcium than those with 1 or 2 fetuses. Pregnancy toxemia in most cases has been accompanied by hypocalcemia. In a study by Schlumbohm and Harmeyer (2003), induced hypocalcemia in combination with normoketonemia or hyperketonemia elicited reduced endogenous glucose production. In the present study, however, the decreases in calcium concentrations were relatively moderate, and in all groups, they were within the physiological concentrations, although the BHBA concentrations increased dramatically. It was suggested by Schlumbohm and Harmeyer (2003) that hypocalcemia does not greatly reduce glucose turnover and might not result in pregnancy toxemia, but that it imposes additional metabolic stress on the glucose homeostasis system; this might be the case in ewes bearing >2 fetuses in the present study.

Plasma insulin concentrations diminished as litter size increased, and during the last week of pregnancy, they were extremely low in 4F ewes (e.g., averaged 0.54 μIU/mL). The decrease in insulin concentrations with increasing litter size has been reported in other studies as well (Henze et al., 1998; Kulcsár et al., 2006). The decline in plasma concentration of insulin, which is a lipogenic hormone, triggers lipolysis of the adipose tissue, which, in turn, results in increased plasma NEFA concentrations (Brockman and Laarveld, 1985). Nonesterified fatty acids originally are mobilized from adipose tissues and infiltrate the liver, mainly for mitochondrial oxidation processes. Incomplete oxidation of NEFA in the liver promotes the production of ketone bodies, and indeed, as mentioned above, the correlation between plasma BHBA and NEFA concentrations in the present study was positive and significant. Plasma insulin concentrations in the 4F ewes were about 25% of those in 1F ewes as early as 5 wk before lambing (4.4 vs. 17.6). Although this was not addressed in the present study, it is plausible to hypothesize that the reduction in insulin concentrations in this group started earlier than the last month of gestation and might precede other detrimental metabolic processes.

In the present study, NEFA concentrations increased during the last 5 wk of pregnancy; however, NEFA concentrations in the 4F group reached a plateau in wk −4 and remained constant until lambing. Moreover, insulin concentrations decreased to very low concentrations in 4F ewes, whereas BHBA concentrations were increased until lambing. It might be that the decrease in insulin concentration occurred early in pregnancy as a response to nutrition deficiency, but also as a homeorhetic control to save glucose for the brain and fetuses of the dam, but a side effect was mobilization of adipose tissue, which starts in the early stages of pregnancy. Accordingly, it also might be that the absence of an increase in NEFA concentration in 4F ewes during late pregnancy was due to a depletion of body reserves as a result of the massive mobilization earlier than the last month of gestation.

In general, epitheliochorial placentas are relatively less permeable to FFA than hemochorial placentas (Battaglia and Meschia, 1988), which means that the fetuses could not utilize NEFA originating from adipose tissues of the dam as an energy source. Furthermore, the fetuses could not utilize ketone bodies as a fuel source, and ketone body utilization is impaired in cases of pregnancy toxemia (Harmeyer and Schlumbohm, 2006). Collectively, it seems that reduced insulin concentrations, which apparently start to appear earlier than the last month of gestation, lead to adipose tissue lipolysis, which elicits increased concentrations of NEFA and, consequently, of BHBA in the blood of the ewes. Neither of these metabolites could be utilized to provide energy for fetuses; therefore, they accumulated

in the plasma of the pregnant ewe. Increases in these metabolites might elicit other detrimental effects that exacerbate the metabolic status of the ewe, such as depression of intake or of endogenous synthesis of glucose (Schlumbohm and Harmeyer, 2004). The present findings may suggest that the decline in insulin concentrations that apparently occurs in the early stages of pregnancy represents a kind of homeorhetic regulation that is a pivotal role in the etiology of pregnancy toxemia. It seems that reduced insulin concentrations lead to a sequence of metabolic events that lead to ketonemia and pregnancy toxemia in multiple-fetus gestations.

In conclusion, in the present study we have examined, for the first time, the concentrations of a few key metabolites in late-pregnancy Afec-Assaf 4F ewes. Dramatic changes in BHBA and insulin concentrations in ewes bearing $\geq$3 fetuses were observed. The decrease in glucose concentrations as litter size increased was relatively moderate compared with changes in BHBA and insulin concentrations. Insulin concentrations in ewes bearing >3 fetuses were decreased even at 5 wk before lambing, and it might be that the decline in insulin concentration started earlier than the last month of pregnancy as a response to nutrition deficiency, but also as a homeorhetic regulation to spare glucose for the brains and fetoplacental units of the dams. Therefore, we hypothesize that the decrease in insulin concentrations, followed by a sequence of metabolic events, has a pivotal role in the etiology of metabolic disorders in ewes pregnant with multiple fetuses. Exceptional nutritional strategies should be developed to cope with the increased demand for nutrients exhibited by the fetoplacental unit in ewes pregnant with multiple fetuses.

LITERATURE CITED

Battaglia, F. C., and G. Meschia. 1988. Fetal nutrition. Annu. Rev. Nutr. 8:43–61.

Bergman, E. N. 1971. Hyperketonemia-ketogenesis and ketone body metabolism. J. Dairy Sci. 54:936–948.

Brockman, R. P., and B. Laarveld. 1985. Effects of insulin on net hepatic metabolism of acetate and β-hydroxybutyrate in sheep (*Ovis aries*). Comp. Biochem. Physiol. A 81:255–257.

Gootwine, E., S. Reicher, and A. Rozov. 2008. Prolificacy and lamb survival at birth in Awassi and Assaf sheep carrying the *FecB* (Booroola) mutation. Anim. Reprod. Sci. 108:402–411.

Gootwine, E., T. E. Spencer, and F. W. Bazer. 2007. Litter-size-dependent intrauterine growth restriction in sheep. Animal 1:547–564.

Harmeyer, J., and C. Schlumbohm. 2006. Pregnancy impairs ketone body disposal in late gestation: Implications for onset of pregnancy toxaemia. Res. Vet. Sci. 81:254–264.

Hay, W. W., Jr., J. W. Sparks, R. B. Wilkening, F. C. Battaglia, and G. Meschia. 1983. Partition of maternal glucose production between conceptus and maternal tissues in sheep. Am. J. Physiol. 245:E347–E350.

Heitmann, R. N., and J. M. Fernandez. 1986. Autoregulation of alimentary and hepatic ketogenesis in sheep. J. Dairy Sci. 69:1270–1281.

Henze, P., K. Bickhardt, H. Fuhrmann, and H. P. Sallmann. 1998. Spontaneous pregnancy toxaemia (ketosis) in sheep and the role of insulin. J. Vet. Med. 45:255–266.

Igwebuike, U. M. 2010. Impact of maternal nutrition on ovine foetoplacental development: A review of the role of insulin-like growth factors. Anim. Reprod. Sci. 121:189–196.

Kleemann, D. O., and S. K. Walker. 2005. Fertility in South Australian commercial merino flocks: Source of reproductive wastage. Theriogenology 63:2075–2088.

Kulcsár, M., G. Dankó, C. Delavaud, C. Mircu, A. J. Nikolic, A. Gáspárdy, H. Cernescu, Y. Chilliard, S. Cseh, P. Rudas, and G. Y. Huszenicza. 2006. Endocrine characteristics of late pregnant hyperketonaemic ewes and their reproductive performance following the induction of ovarian cyclicity out of the breeding season. Acta Vet. Hung. 54:235–249.

NRC. 1985. Nutrient Requirements of Sheep. Natl. Acad. Press, Washington, DC.

Sargison, N. D. 2007. Pregnancy toxaemia. Pages 359–362 in Diseases of Sheep. I. D. Aitken, ed. Blackwell Publishing, Oxford, UK.

Schlumbohm, C., and J. Harmeyer. 2003. Hypocalcemia reduces endogenous glucose production in hyperketonemic sheep. J. Dairy Sci. 86:1953–1962.

Schlumbohm, C., and J. Harmeyer. 2004. Hyperketonemia impairs glucose metabolism in pregnant and nonpregnant ewes. J. Dairy Sci. 87:350–358.

Scott, P. R., N. D. Sargison, D. Penny, R. S. Pirie, and J. M. Kelly. 1995. Cerebrospinal fluid and plasma glucose concentrations of ovine pregnancy toxaemia cases, inappetant ewes and normal ewes during late gestation. Br. Vet. J. 151:39–44.

Twardock, A. R., H. W. Symonds, B. F. Sansom, and G. J. Rowlands. 1973. The effect of litter size upon foetal growth rate and the placental transfer of calcium and phosphorus in superovulated Scottish half-bred ewes. Br. J. Nutr. 29:437–446.

Varnam, G. C., M. K. Jeacock, and D. A. Shepherd. 1978. Hepatic ketone-body metabolism in developing sheep and pregnant ewes. Br. J. Nutr. 40:359–367.

Zamir, S., A. Rozov, and E. Gootwine. 2009. Treatment of pregnancy toxaemia in sheep with flunixin meglumine. Vet. Rec. 165:265–266.