Effects of short-term administration of a glucogenic mixture at mating on feed intake, metabolism, milk yield and reproductive performance of lactating dairy ewes

# Effects of short-term administration of a glucogenic mixture at mating on feed intake, metabolism, milk yield and reproductive performance of lactating dairy ewes

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## A B S T R A C T

The effects of the intra-ruminal dosing of a glucogenic mixture including 70% glycerol, 20% propylene glycol and 10% water was studied on thirty late lactation dairy ewes of Sarda breed. The animals were divided in two homogeneous groups receiving by gavage either 200 mL of water (CTR group; body weight 40.9 ± 1.5 kg) or 200 mL of the above mixture (GLY group; body weight 39.4 ± 1.3 kg) twice daily from d 16 to d 19 of the oestrus cycle, synchronised by “ram effect”. The ewes were then mated and their reproductive responses to the synchronised mating evaluated by scanning on d 50 and at lambing. During the treatment, the ewes were housed in an open hut, machine milked twice daily and fed concentrate and hay to meet their nutrient requirements. During the treatment, concentrate intake was markedly reduced in GLY when compared with CTR ($P < 0.001$), without any effect on ewe body weight or body condition. The administration of the glucogenic mixture increased plasma osmolarity and blood volume as estimated by serum total protein concentration. Moreover, it increased plasma content of glycerol, glucose ($P < 0.001$) and insulin ($P < 0.01$) while decreasing plasma level of NEFA ($P < 0.001$) and urea ($P < 0.05$). Milk yield ($P < 0.01$) and milk lactose content ($P < 0.001$) were decreased by the glucogenic treatment, whereas milk protein and casein contents were increased ($P < 0.001$). As for reproductive performance, the glucogenic treatment numerically increased ewe’s conception rate, but the difference was not statistically significant. Prolificacy did not change between groups. In conclusion, the administration of a glucogenic mixture to late lactation dairy ewes caused significant changes both in

plasma and in milk composition during the treatment. However, reproductive performances were unaffected by the treatment.

## 1. Introduction

In the Mediterranean basin, dairy sheep breeding system typically implies one lambing per year, with the mating season starting in late spring for mature ewes and in early autumn for young ewes (Todaro et al., 2015). Mating of adult ewes usually starts when ewes are at the end of seasonal anoestrus period, while they are passing from mid to late lactation (5–6 months of milking). Nutritional plans applied during the mating period should meet the requirements of both the follicle and the mammary gland to increase the fertility of the flock while sustaining milk production. However, in this period pasture usually turns to reproductive phase which results in a decay of pasture nutritive value and a lower intake of nutrients (Pulina et al., 2006).

To cope with these adverse nutritional conditions and optimize reproductive performance, the short-term oral administration of a high dose glucogenic mixture can be useful. Glucogenic precursors such as glycerol and propylene glycol are rapidly absorbed by the rumen, reach the circulation, and serves directly as a substrate in the liver for glucose synthesis, thus causing a rapid and sustained rise of blood glucose (Nielsen and Ingvartsen, 2004). The administration of glycerol and/or propylene glycol has been also associated with an increase in insulin (Letelier et al., 2008) and insulin-like growth factor-1 (Porcu et al., 2017), and a decrease in NEFA and urea circulating concentrations (Habibizad et al., 2015). The follicle has a functional insulin-glucose-IGF-1 system (Scaramuzzi et al., 2010) which is affected by short-term nutritional treatments, and it is clear that components of this metabolic system are nutritionally regulated in the follicle (Dupont et al., 2014). Glucose, insulin and IGF-1 act synergistically to promote follicle growth and estradiol secretion (Downing et al., 1999). On the other hand, high circulating levels of urea and NEFA have been associated with lower fertility and negative energy balance (McEvoy et al., 1997; Aardema et al., 2011). Recent studies have shown that glucogenic-based flushing treatment improves oocyte quality in ewes submitted to ovum pick up (Berlinguer et al., 2012) and can increase ovulation rate in Manchega dry ewes (Letelier et al., 2008). These effects have been related to the modification of the plasma and follicular fluid composition during the treatment period (Porcu et al., 2017), modulated by ewe body condition score at mating (Williams et al., 2001).

All the above studies have focused on non-lactating ewes, thus the effects of short-term glucogenic dietary treatments in lactating dairy ewes have been overlooked so far.

The ovary uses glucose as its principal energy source and its well described positive effects on fertility have been related to its properties as a metabolic fuel (Scaramuzzi et al., 2010). During lactation however,

glucose requirements in the mammary tissue increase dramatically, competing with those of other tissues and organs, such as the ovaries.

Starting from these premises, the present study aimed at investigating the impact of the administration of a glucogenic mixture on feed intake, metabolism, milk yield and reproductive performance of lactating dairy ewes. The hypothesis underlying the study was that the glucogenic mixture administration could create in lactating dairy ewes a hormonal and metabolic milieu favourable for follicle growth, maturation, and ovulation resulting in an improvement of sheep reproductive performance.

## 2. Materials and methods

The experiment was carried out from June 11$^{th}$ to July 31$^{st}$ 2015 (experimental period) at Bonassai research station of Agris, located in north-western Sardinia, Italy (40 °N, 8 °E, 32 m a.s.l.). Weather conditions during the experiment were assessed using an on-farm weather station and temperature humidity index (THI) was calculated according to (Johnson and Kibler, 1963).

The animal protocol and the implemented procedures described below are in accordance with the ethical guidelines in force at Agris and the University of Sassari (CIBASA 21.01.2014), in compliance with the European Union Directive 86/609/EC and the recommendation of the Commission of the European Communities 2007/526/EC.

### 2.1. Animals and treatments

On June 11 (d 0 of the experimental period), thirty Sarda dairy ewes were selected from the farm flock, homogeneous for age (mean ± SE 3.3 ± 0.26 years) and lactation stage (mean ± SE, 155 ± 5 days in milk [DIM]). The ewes were weighted, and their body condition was scored. On the same day, sheep milk yield (MY) was measured at two milkings and milk samples were collected and analysed. Thereafter, the ewes were randomly assigned to two experimental groups, homogeneous for body weight (BW; CTR group 40.9 ± 1.5 kg, GLY group 39.4 ± 1.3 kg; P = 0.43), body condition score (BCS; CTR group 2.78 ± 0.05, GLY group 2.71 ± 0.05; P =0.30), MY (CTR group 1088.2 ± 54.8 g, GLY group 1016.8 ± 51.2 g; P = 0.57), milk fat (CTR group 6.65 ± 0.20%, GLY group 6.95 ± 0.19%; P =0.89) and protein (CTR group 5.10 ± 0.17%, GLY group 5.40 ± 0.16%; P =0.79) concentrations. One ewe was discarded because of an acute trauma. Each group was further divided in two subgroups, used as replicates. During the experimental period, the ewes were housed overnight in an open hut, where they were machine milked twice daily.

Ram effect was used to synchronise ewes' ovulation. For this reason, the ewes were kept isolated from rams for 6 weeks before starting the experiment. At d 0 of the experimental period, vasectomised rams were introduced in the flock at the ratio of 2 rams per 15 ewes and were left in until the presumptive

starting of oestrus (d 16). Thereafter, the vasectomised rams were replaced with nonvasectomized rams at the same ram per ewe ratio. The rams were removed from the flock on d 30.

Coincidentally with the onset of the follicular phase, from d 16 to d 19 (treatment period), one experimental group (glucogenic treated ewes n= 15; GLY) received, orally twice daily 200 mL of a glucogenic mixture. The glucogenic formulation contained (v/v) 70% glycerol, 20% propylene glycol (both reagent grade (> 99% purity) chemicals from Sigma Chemical Co., St. Louis, MO, USA), and 10% water. The second group (control ewes: n =14; CTR) received 200 mL of water twice daily simultaneously to glucogenic mixture administration. Both the glucogenic formulation and the water were administered at 0800 and 1900 h in the evening, by oral gavage using an esophageal feeding tube. This daily dosing schedule was set as close as possible to that adopted in previous experiments by our laboratories [twice daily, every 12 h (Berlinguer et al., 2012)]. From d 15 to d 21, i.e. throughout the treatment period, the sub-groups were kept indoors in separate pens. Indoor feeding consisted of 400 g/head/d of a commercial pelleted concentrate divided in two meals and individually fed at milkings, plus c.a. 900 g/head/d of dehydrated lucerne hay and 900 g/head/ d of chopped Italian ryegrass hay, which were administered in two equal meals in the morning and evening.

During the remaining days, i.e. before d 15 and after d 21, the ewes of both experimental subgroups were allowed to graze two 0.5 ha paddocks of mature (post-heading phase) Italian ryegrass (Lolium multiflorum Lam) pasture for 4 h/d (0700–1100 h). Paddock 1 was grazed by subgroups 1 of GLY and CTR ewes and paddock 2 by subgroups 2. During the grazing period, the ewes were supplemented with 400 g/head/d of commercial pelleted concentrate. The hay supplementation consisted of 1000 g/head/d of dehydrated lucerne hay before the flushing period and 900 g/head/d of chopped ryegrass hay plus 450 g/head/d of dehydrated lucerne after the flushing period. In this period, the increase of hay offer was aimed at compensating pasture defoliation of paddocks during the grazing period preceding the flushing treatment. The lucerne hay was reduced due to the simultaneous reduction of protein requirements, associated to the lowering milk yield. Dehydrated lucerne and grass hay were fed in separate troughs at grazing turning out.

### 2.2. Feedstuff composition and feed intake

Samples of the herbage on offer were collected at the beginning, at an intermediate date (before flushing treatment), and at the end of grazing period from both the paddocks by cutting five 0.5 $m^2$ quadrats per plot at 2 cm a.g.l. by an electric hand-driven shear. Supplement feed samples were collected weekly and pooled before further processing. All these samples were oven-dried at 65 °C and subsequently ground to pass a 1-mm screen to determine the content of dry matter (DM, oven drying at 100 °C overnight), ash (ID# 942.05), ether extract (EE, ID# 920.39) and crude protein (CP, N x 6.25, [ID# 988.05]) according to

AOAC (1990). Neutral detergent fiber on an ash-free basis (NDF), acid detergent fiber on an ash-free basis (ADF) and acid detergent lignin (ADL) were also determined (Van Soest et al., 1991). Net Energy ($NE_L$, Mcal/kg DM) and metabolic protein content of feedstuffs and diet were calculated using the equations published by Cannas et al. (2004). Diet formulation was done using Small Ruminant Nutrition System package. Mean data of forage mass on offer and herbage allowance are shown in Table 1, whereas data on herbage and feedstuff chemical composition are displayed in Table 2. Average $NE_L$ were 1.53 Mcal/kg DM, (concentrate), 1.19 Mcal/kg DM (dehydrated lucerne), 0.85 Mcal/kg DM (grass hay), and 2.70 Mcal/kg DM (glucogenic mixture). $NE_L$ of grazed herbage ranged between 1.0 and 0.7 Mcal/kg DM.

From d 15 to d 21, the concentrate intake was individually measured at the milking parlour by weighing the feed offered and corresponding orts at each meal. During the same period, group intake of hays was computed by weighing the forage offered to each subgroup and then subtracting the weight of the corresponding orts after each meal. Total group intake was then computed by summing up hays and concentrate supplement intake.

### 2.3. Animal measurements, samplings, and analyses

During the experimental period, the ewes were submitted to different measurements and their blood and milk were sampled. Fig. 1 shows the exact timing at which samplings and measurements were performed.

#### 2.3.1. Blood samplings for metabolite and hormone assays

Blood samples were collected at fasting (0800 h), right after the milking procedures. In addition, on d 18, the 3$^{rd}$ d of treatment administration, four consecutive samples were collected every 30 min, starting at fasting immediately before the morning administration of glucogenic mixture or water (0800, 0830, 0900 and 0930 h). At each sampling, from each ewe, two blood samples were collected, one using 3 mL vacuum collection tubes containing lithium heparin and mono-iodioacetate (Vacutainer Systems Europe; Becton Dickinson, MeylanCedex, France) for glucose assay, the other using 10 mL vacuum collection tubes containing EDTA K2 (Vacutainer Systems Europe; Becton Dickinson, MeylanCedex, France) for the remaining analyses. Immediately after recovery, blood samples were cooled at 4 °C, centrifuged at 1500 x g for 15 min. Plasma was removed and stored at −20 °C until assayed.

All plasma samples were measured in duplicate. Plasma osmolarity was measured using a freezing point osmometer (Osmomat 030, Gonotec, Berlin, Germany). Total proteins were measured in a multiple assay by a coloured method (BioSystems kit) where the proteins in the sample react with copper (II) ion in alkaline medium forming a stained complex measured spectrophotometrically at 545 nm. Intra- and inter-assay CV values were 1.8% and 0.9%, respectively.

**Table 1** - Forage mass at initial, mid, and final grazing day and herbage allowance in paddocks of Italian ryegrass at reproductive phase grazed by milked ewes during mating period.

| Item | N | Mean | s.d. |
|---|---|---|---|
| Paddock 1 | | | |
| Herbage mass first grazing day (t DM) | 5 | 3.5 | 0.6 |
| Herbage mass mid graazing day (t DM/ha) | 5 | 2.2 | 0.7 |
| Herbage mass last grazing day (t DM/ha) | 5 | 2.3 | 1.1 |
| Herbage allowance (kg DM/ewe/d) | | 2.6 | |
| Paddock 2 | | | |
| Herbage mass first grazing day (t DM) | 5 | 4.0 | 0.7 |
| Herbage mass mid grazing day (t DM/ha) | 5 | 2.3 | 0.6 |
| Herbage mass last grazing day (t DM/ha) | 5 | 2.6 | 0.7 |
| Herbage allowance (kg DM/ewe/d) | | 2.8 | |

**Table 2** - Feedstuff analyses.

| | | **DM %** | | **Ash % DM** | | **CP % DM** | | **EE % DM** | | **NDF % DM** | | **ADF % DM** | | **ADL % DM** | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Item** | N | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. | Mean | s.d. |
| **Concentrate** | 2 | 88.4 | 0.4 | 13.1 | 0.4 | 16.6 | 0.8 | 3.5 | 0.1 | 28.6 | 0.2 | 10.8 | 0.4 | 2.8 | 0.3 |
| **Ryegrass hay** | 3 | 84.9 | 0.4 | 8.5 | 0.5 | 7.6 | 2.2 | 1.9 | 0.2 | 59.9 | 3.6 | 36.0 | 2.3 | 3.9 | 0.6 |
| **Dehydrated lucerne** | 2 | 88.2 | 0.3 | 11.2 | 0.2 | 20.6 | 0.4 | 1.4 | 0.1 | 45.3 | 1.9 | 31.5 | 1.7 | 7.3 | 0.3 |
| **Herbage paddock 1** | | | | | | | | | | | | | | | |
| **First day of grazing** | 5 | 62.6 | 10.1 | 8.3 | 0.7 | 5.1 | 0.3 | 1.5 | 0.2 | 68.1 | 2.9 | 38.4 | 2.1 | 3.1 | 0.5 |
| **Mid-day of grazing** | 5 | 72.8 | 12.7 | 4.9 | 0.6 | 6.3 | 0.9 | 1.3 | 0.5 | 79.8 | 2.0 | 46.6 | 1.1 | 5.2 | 0.5 |
| **Last day of grazing** | 5 | 78.7 | 1.3 | 7.3 | 4.4 | 3.9 | 0.8 | 1.3 | 0.2 | 78.4 | 4.0 | 51.5 | 2.8 | 6.1 | 0.2 |
| **Herbage paddock 2** | | | | | | | | | | | | | | | |
| **First day of grazing** | 5 | 62.8 | 13.6 | 6.7 | 0.9 | 6.1 | 0.3 | 1.4 | 0.4 | 71.8 | 4.6 | 40.1 | 2.7 | 3.8 | 0.8 |
| **Mid-day of grazing** | 5 | 83.3 | 3.1 | 6.1 | 0.5 | 5.8 | 0.5 | 1.1 | 0.3 | 79.4 | 2.5 | 45.9 | 2.1 | 5.2 | 0.6 |
| **Last day of grazing** | 5 | 88.5 | 0.5 | 3.7 | 1.2 | 6.9 | 0.7 | 1.2 | 0.2 | 84.1 | 1.3 | 50.8 | 1.0 | 5.7 | 0.7 |

[a] Dry matter, [b] Crude protein, [c] Ether extract, [d] Neutral detergent fiber, [e] Acid detergent fiber, Acid detergent lignin.

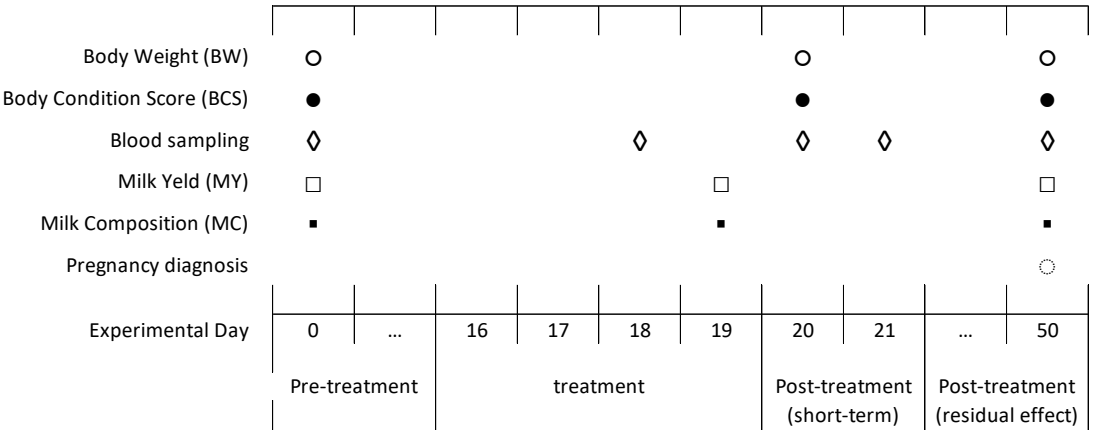


**Fig. 1.** Timing of measurements and sampling during the experimental period.

Glycerol concentration was measured in a single assay by a colorimetric method using a commercial Free Glycerol Assay Kit (Cell Biolabs, Inc, USA), with glycerol standards in the concentration range of 0 µM–400 µM. The kit measures free, endogenous glycerol by a coupled enzymatic reaction system. The glycerol is phosphorylated and oxidized, producing hydrogen peroxide which reacts with the kit's Colorimetric Probe (absorbance maxima of 570 nm). The analytical sensitive was 5 µM.

Glucose, NEFA and urea were measured using commercial kit and BS-200 Mindray clinical chemistry analyzer. Serum I Normal (Wako) and Serum II Abnormal (Wako) were used as multi control for each measured parameter. Glucose concentrations were determined in a single assay by liquid enzymatic colorimetric method (GOD - POD) (Real Time kit) with a glucose standard of 100 mg/dL for calibration. Intra-assay CV values was 1.1%. NEFA and urea concentrations were measured in multiple assays by enzymatic endpoint method (Diagnostic Systems kit), with a NEFA standard of 1 mmol/L and a urea standard of 50 mg/dL for calibration. NEFA intra- and inter-assay CV values were 1.07% and 0.98%, respectively. UREA intra- and inter-assay CV values were 1.7% and 1.6%, respectively.

Insulin concentration was measured in duplicate using a commercial Ovine Insulin ELISA Kit (Mercodia developing diagnostics, Germany) which is a solid-phase ELISA based on the direct sandwich technique. The kit is calibrated against an in-house reference preparation of ovine insulin, and it has been previously used for insulin determination in ovine plasma (Melendez et al., 2006; Mahjoubi et al., 2014). The mean ovine insulin concentrations of the six reference solutions were 0, 0.05, 0.15, 0.5, 1.5 and 3 µg/L. The

recovery upon addition was 94–114% (mean 103%). The analytical sensitivity was 0.025 μg/L and the intra- and inter-assay CV values were <7%.

#### 2.3.2. Body weight, body condition score, milk yield and composition

Body weight was measured before the morning meal using an electronic scale. Body condition score ranging from 1 (extremely thin) to 5 (obese) was estimated by two trained evaluators with an approximation of 0.25 BCS units (Russel et al., 1969). Their scores were averaged prior to data analysis.

Milk yield was measured by machine milking and weighting the production of each ewe in two consecutive milkings at 07.00 and at 15.00. Milk composition was assayed on composite samples for fat, protein, casein, and lactose using the Fourier-transformed infrared method (Milkoscan FT+, Foss Electric, Hillerød, Denmark) and milk urea concentration using an enzymatic colorimetric assay (Chem Spec 150; Bentley Instruments Inc., Chaska, MN, USA).

#### 2.3.3. Pregnancy scanning and reproductive performance

On Day 50, i.e. 20 days after the removal of the rams from the flock, pregnancy diagnosis was performed using trans-rectal ultrasonography (Aloka SSD 500, fitted to 82 mm prostate transducer UST-660-7.5, Aloka Co.). Pregnant sheep displayed enlargement of the uterine horns, embryo heartbeat was evidenced and, in more advanced stages of pregnancy, placentomes were seen. At parturition, the number of lambs born was recorded.

### 2.4. Statistical analyses

Circulating concentrations of metabolites and insulin measured on d 0, 18, 20, 21 and d 50 were analyzed by GLM with treatment as fixed effect using SAS (Version 8, SAS Institute Inc, Cary, NC, USA). Longitudinal data of plasma glycerol, glucose, insulin, NEFA, urea, osmolarity and total protein in the consecutive samples collected on d 18 (during treatment period) were analyzed by a mixed model for repeated measurements (PROC MIXED) in SAS with treatment, sampling hour and their first-order interactions as fixed effects, and sheep as random effect.

A mixed model for repeated measurements was also used to evaluate the effect of glucogenic treatment on the intake of concentrate during the flushing period, milk yield and milk composition before, during and after the treatment, with treatment, experimental day and their first-order interactions as fixed effects, and sheep as random effect. Live weight and BCS at the beginning, during, and after the glucogenic treatment period and their changes were analyzed by a mono-factorial GLM.

Finally, $\chi^2$-test was used to determine differences in reproductive performance between groups.

All results were expressed as mean ± SE and a probability of $P < 0.05$ was considered to be significant whereas trends where considered when probability ranged between $P = 0.05$ and $P = 0.1$.

## 3. Results

During the experimental period, temperatures were often above 30 °C. In particular, the treatment period and the subsequent mating days (days 14–28) were characterized by an average THI of 69.3, totalling 58 h of severe heat load (THI > 75).

### 3.1. Feed intake, body weight, and body condition

Concentrate intake significantly decreased in GLY compared to CTR group during the glucogenic mixture administration ($P < 0.001$; Fig. 2), while there was no difference in the intake before and after this period. Hay and total intake were similar between the two experimental groups. However, if total intake is calculated including the glucogenic mixture, GLY group showed a numerically higher energy intake and a lower CP/$NE_L$ ratio compared to CTR group (79 vs 139 g/Mcal, respectively; Table 3). No differences were observed in body weight and body condition between groups (Table 4).

### 3.2. Dynamics of glycerol, metabolites, and insulin in the bloodstream

The administration of the glucogenic mixture determined a sharp increase in glycerol circulating concentrations in treated ewes, with a maximum value 400-folds higher than in control ewes at 90 min post-dosing (Fig. 3, panel A). Despite this fast response, glycerol circulating concentrations did not differ between treated and control ewes if measured at fasting, during the nutritional treatment period, and in the two days after its end (Fig. 3, panel B).

The sharp increase in glycerol plasma levels consequent to its administration was followed by a parallel increase in glycaemia ($P < 0.001$) and insulinemia ($P < 0.01$; Fig. 4, panels A and B), and by a decrease in NEFA ($P < 0.001$) and urea circulating concentrations ($P < 0.05$; Fig. 4, panels C and D).

Glycaemia and insulinemia did not differ in treated and control ewes when measured at fasting (Fig. 5, panels A and B). The concentration of plasma NEFA and urea at fasting were significantly lower in treated than controls ewes on the 3$^{rd}$ day of glucogenic mixture administration ($P < 0.05$; Fig. 5, panel C and D).

### 3.3. Plasma osmolarity and total protein concentration

Plasma osmolarity increased significantly in GLY ewes compared to CTR ones, reaching higher values starting from 30 min after the glucogenic mixture administration ($P < 0.001$; Fig. 6, panel A). Plasma total protein concentration was significantly lower in GLY compared to CTR at 0 and 60 min from glucogenic mixture administration and as mean value (Fig. 6, panel B; $P < 0.05$).

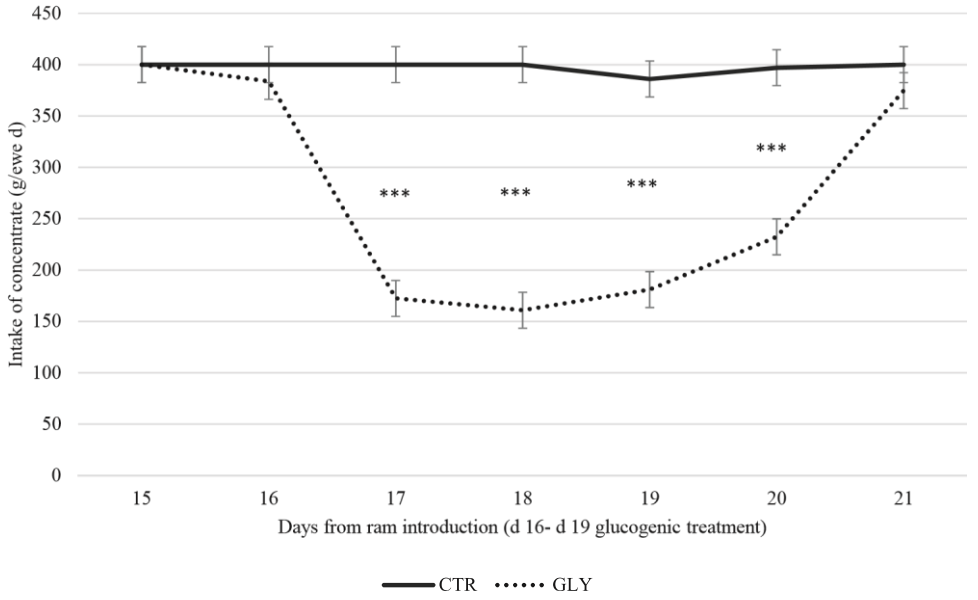


**Fig. 2.** Intake of concentrate in milked ewes orally dosed with either 400 mL/d of water (CTR n= 14) or 400mL/d of a glucogenic mixture (GLY n= 15). Concentrate was individually offered to all ewes at a level of 400g/d (356 g DM/d). Bars denote SE. *** Difference between groups at level of P < 0.001.

**Table 3** - Intake of feedstuffs (g DM) and proportion of concentrates in the diet of milked ewes either dosed with water (CTR) or with a glucogenic mixture (GLY). Concentrates were individually fed while forages were group fed in pens.

| | CTR | | | GLY | | | |
|---|---|---|---|---|---|---|---|
| Item | Intake g DM/d | CP[a] g/d | NEL[b] Mcal/d | Intake g DM/d | CP[a] g/d | NEL[b] Mcal/d | P < |
| Concentrate | 352 | 58 | 0.54 | 241 | 40 | 0.37 | 0.001 |
| Dehydrated lucerne | 719 | 148 | 0.86 | 711 | 146 | 0.85 | – |
| Ryegrass hay | 721 | 55 | 0.61 | 539 | 41 | 0.46 | – |
| Total intake of hay | 1440 | – | – | 1250 | – | – | – |
| Total feed intake | 1792 | 261 | 2.01 | 1491 | 227 | 1.67 | – |
| Glucogenic mixture | – | – | | 436 | – | 1.20 | – |
| Total intake[c] | 1791 | 261 | 2.01 | 1927 | 227 | 2.88 | – |
| Dietary proportion of concentrates[c] | 0.20 | – | – | 0.35 | – | – | – |
| Dietary proportion of glucogenic mixture | – | – | – | 0.23 | – | – | – |

[a] Crude Protein, [b] Net Energy for milk production, [c] Inclusive of glucogenic mixture.

**Table 4** - Body weight (BW) and body condition score (BCS) of milked ewes either dosed with water (CTR) or with a glucogenic mixture (GLY) before (d 0) and after the nutritional treatment (d 20 = one day after last administration; d 50 =30 days after last administration).

| | Day 0 | | | Day 20 | | | Day 50 | | |
|---|---|---|---|---|---|---|---|---|---|
| Item | CTR | GLY | P | CTR | GLY | P | CTR | GLY | P |
| BW | 40.9 ± 1.5 | 39.4 ± 1.3 | 0.43 | 43.0 ± 1.5 | 41.9 ± 1.3 | 0.59 | 42.0 ± 1.4 | 42.0 ± 1.2 | 0.99 |
| BCS | 2.8 ± 0.1 | 2.7 ± 0.1 | 0.31 | 2.7 ± 0.04 | 2.7 ± 0.01 | 0.76 | 2.7 ± 0.04 | 2.7 ± 0.01 | 0.65 |

### 3.4. Milk yield and composition

During the treatment (d 19), milk yield significantly decreased in GLY compared to CTR ewes (Fig. 7, panel A), together with its content in lactose (panel D), and urea (panel E). On the other hand, milk protein and casein percentages increased significantly in GLY compared to CTR ewes (Fig. 7, panel C and F). Milk fat percentage was not affected by the treatment (Fig. 7, panel B).

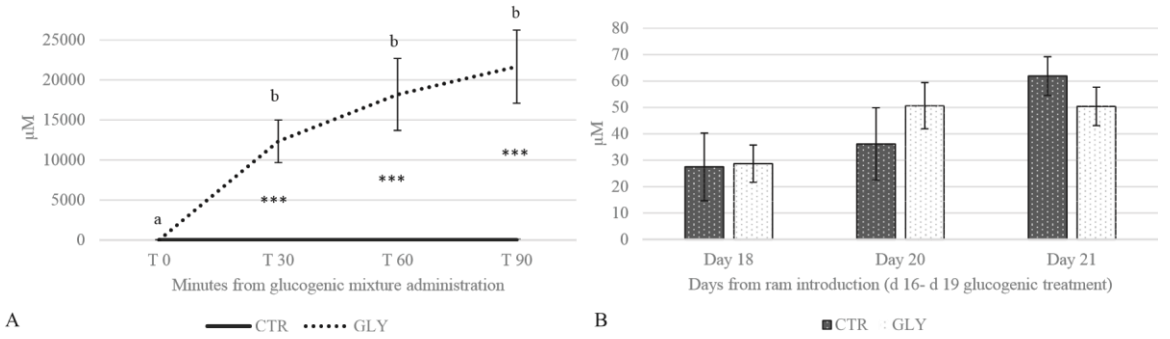


**Fig. 3.** Glycerol circulating concentrations - in ewes orally drenched with either 400 mL/d of water (CTR n =14) or 400 mL/d of a glucogenic mixture (GLY n =15) - during consecutive samplings performed every 30 min starting from the moment of their administration (panel A) and during different days of the experimental period at fasting (d 0 = vasectomized ram's introduction; d 16 - d 19 = nutritional treatment administration; panel B). Asterisks indicate significant differences between groups ($P < 0.001$). a, b indicates significant variations within GLY group.

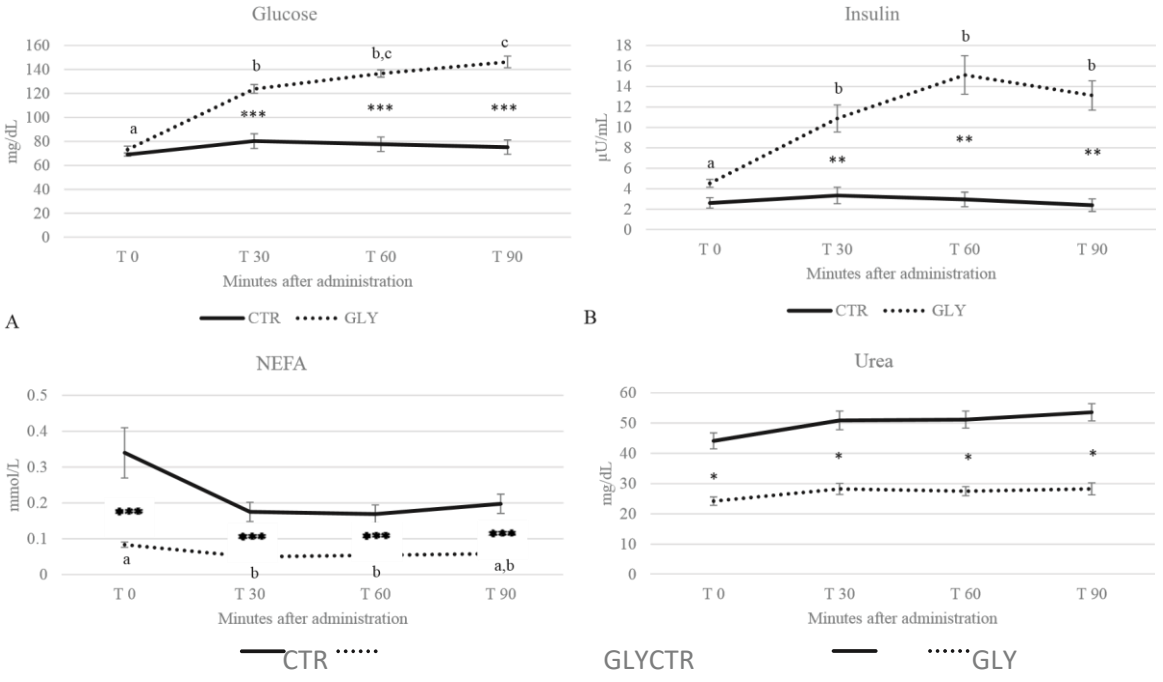


**Fig. 4.** Circulating concentrations of glucose, insulin, NEFA, and urea in ewes orally drenched either 400 mL/d of water (CTR n = 14) or 400 mL/d of a glucogenic mixture (GLY n= 15) during their 3rd day of administration in consecutive samples performed every 30 min. Asterisks indicate significant differences between groups (*P < 0.05; ** P < 0.01; *** P < 0.001). a, b, c indicates daily significant variations within GLY group.

### 3.5. Pregnancy scanning and ewe reproductive performance

No differences were recorded between treated and control ewe's reproductive performance (conception rate and prolificacy). In control group, 7 out of 14 ewes were diagnosed as pregnant at scanning (conception rate: 0.50) and gave birth to a total of 11 lambs (3 singleton plus 8 twins; prolificacy: 1.57). In treated group, 9 out of 15 ewes, were diagnosed as pregnant at scanning (conception rate: 0.60). One pregnant ewe from the GLY group died because of an acute pneumonia. Thus, 8 out of 11 ewes gave birth to a total of 11 lambs (5 singleton plus 6 twins; prolificacy: 1.38).

## 4. Discussion

Although a vast literature is available on the effects of feeding glycerol (Hippen et al., 2008; Silva et al., 2014; Khattab, 2015) and to a lesser extent propylene glycol to ruminants (Nielsen and Ingvartsen, 2004),

the effects of short-term drenching of their mixtures at high doses is much less investigated. Therefore, for sake of simplicity and clarity, the following discussion will primarily focus on literature relevant to the specific topic of this study (short-term high-dose drenching or feeding of glucogenic additives to ruminants).

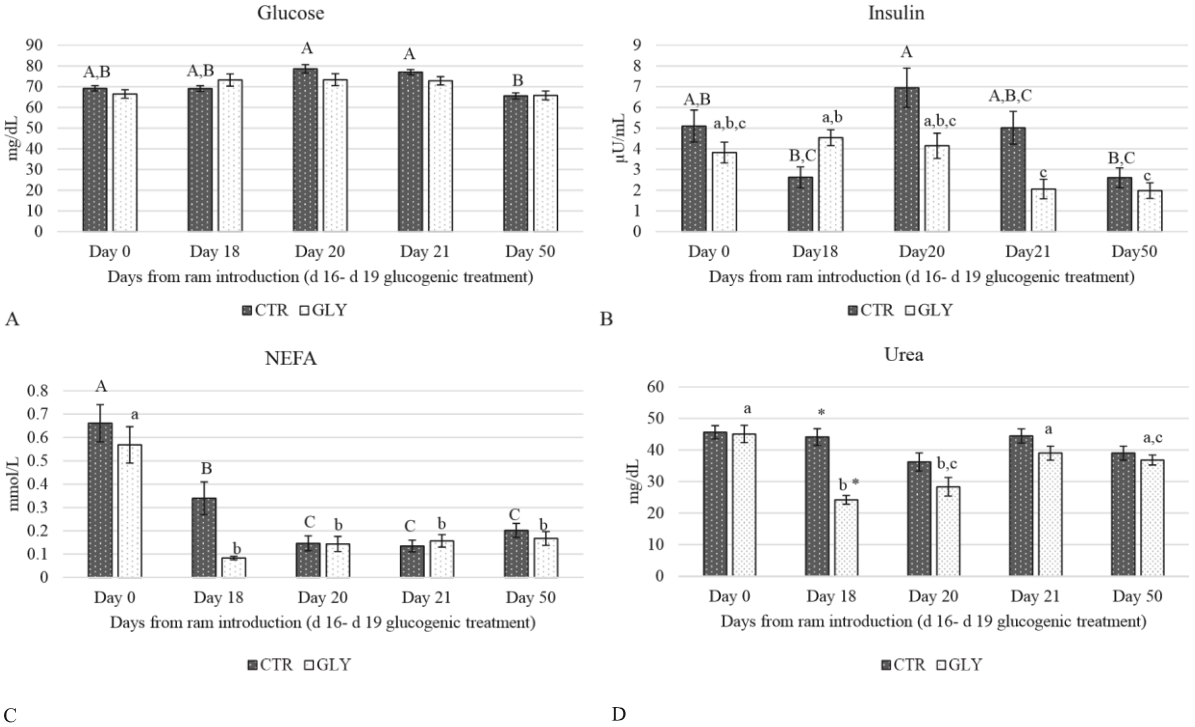


**Fig. 5**. Circulating concentrations of glucose, insulin, NEFA, and urea in ewes orally drenched either 400 mL/d of water (CTR n = 14) or 400 mL/d of a glucogenic mixture (GLY n =15) during different days of the experimental period (d 0 = vasectomized ram's introduction; d 16- d 19 = nutritional treatment administration). Asterisks indicate significant differences between groups ($P < 0.05$). a, b, c indicates daily significant variations within GLY group. A, B, C indicates daily significant variations within CTR group.

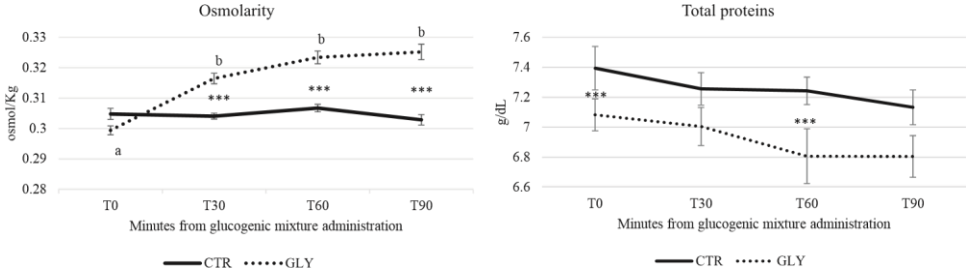


**Fig. 6.** Plasma osmolarity and total protein concentration in ewes orally drenched either 400 mL/d of water (CTR n =14) or 400 mL/d of a glucogenic mixture (GLY n =15) during their 3$^{rd}$ day of administration in consecutive samples performed every 30 min. Asterisks indicate significant differences between groups ($P < 0.001$). a, b, c indicates daily significant variations within GLY group.

### 4.1. Feed intake, body weight, and body condition

During treatment period, voluntary feed intake was lower in glucogenic-drenched than control ewes. A previous study in sheep reported no change in DMI in Merino ewes fed up to 12% DM crude glycerine (Meale et al., 2013), while others reported a linear decrease in lamb DMI when it replaced dry-rolled corn up to 30 or 45% DM (Gunn et al., 2010) or dry-rolled barley up to 21% DM (Avila-Stagno et al., 2013). A reduction in DMI was also observed in lactating cows when glycerol exceeded 10% of the diet DM (Donkin et al., 2009) or when cows were fed up to 30% crude glycerine (Ezequiel et al., 2015). In the latter study, the authors speculated that glycerine top dressing may have caused a decreased palatability of the diet. In the present study, the glucogenic mixture was administered by oral drenching and thus the post-ingestive effect can be evoked as the main factor underlying the decrease of feed intake (Provenza et al., 1996). Also, Rutter and Manns (1986) found a reduction of feed intake in ewes infused with glucose from d 7 to d 22 post-partum. It is not surprising that the effect of glucogenic drench on DMI was transient, since it was strictly related to the treatment. To the best of our knowledge, a transient reduction of concentrate intake was also found in cows fed crude glycerol at c.a. 5% DM dietary level in the first 6 days of treatment (Kass et al., 2013) or fed glycerol at 15% DM dietary level in the first 7 days of treatment (Donkin et al., 2009). Those cows were able to adapt to the glucogenic supply but the level of glucogenic in their diets was much lower than in the current study (23% on DM basis). An additional explanation of DMI drop can be related to propylene glycol. This can form sulfur-containing gases such as 1-propanethiol which could have passed an unpleasant onion-like flavour to the emitted or eructated air (Trabue et al., 2007). Thus, we cannot rule out a transient conditioned aversion towards the concentrate, fed immediately before the drenching.

Despite the effect of the treatment on feed intake, the short-term flushing did not impact on ewes' BW and BCS. This result confirms previous findings in sheep submitted to the same flushing protocol (Letelier et al., 2008; Porcu et al., 2017),.

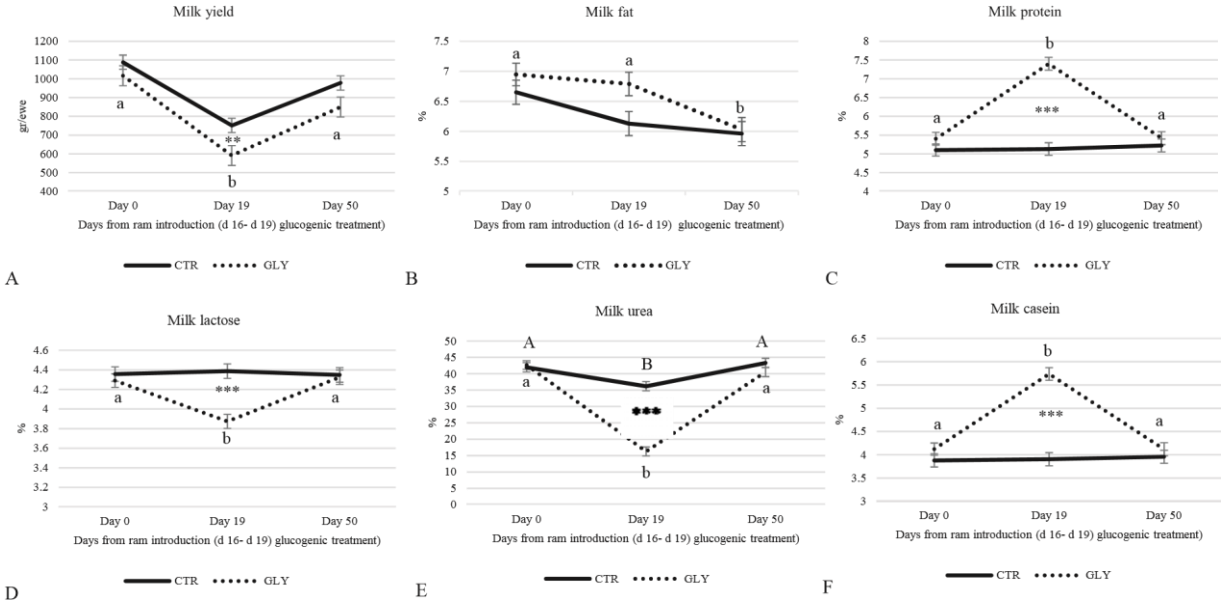


**Fig. 7.** Milk yield (panel A) and composition (panels B–F) in ewes orally drenched either 400 mL/d of water (CTR n =14) or 400 mL/d of a glucogenic mixture (GLY n =15) during different days of the experimental period (d 0 = vasectomized ram's introduction; d 16 - d 19 = nutritional treatment administration; d 50 = pregnancy diagnosis). Asterisks indicate significant differences between groups ($P < 0.001$). a, b indicates significant variations within GLY group. A, B indicates significant variations within CTR group.

### 4.2. Dynamics of glycerol, metabolites, and insulin in the bloodstream

After oral administration, a significant proportion of both glycerol and propylene glycol are absorbed by the rumen without fermentation through a passive diffusion and reach the circulation to serve directly as substrates in the liver for glucose synthesis (Ferraro et al., 2016). In this study, glucogenic mixture administration (23% DM) in dairy ewes caused a 400-folds increase in circulating glycerol concentration. Consequently, plasma osmolarity increased significantly and triggered water reabsorption from the extracellular fluids, as suggested by the decrease in plasma total protein concertation which indicates hemodilution. Glycerol clearance from the circulation was fast enough to allow the normalization of its level within 12 h form the oral drench, and we did not observe any residual effect of the treatment in the two days following its end. Both in GLY and CTR ewes glycerol plasma level at fasting were in fact similar, or even lower, than values reported for lactating ewes (Harmeyer and Schlumbohm, 2006).

Glycerol fast clearance from the bloodstream was due to its transformation to glucose in the liver. Glucose is the main source of energy for the ovary (Scaramuzzi et al., 2010), its effect on fertility being primarily related to its properties as a metabolic fuel (Dupont et al., 2014). At the ovarian level, it acts synergistically

with insulin in mediating the effects of acute changes in nutrient intake on follicular dynamics (Webb et al., 2004). Different glucose transporters, including the GLUT family, are expressed in the oocyte, the somatic cells of the follicle and in the early embryo, and the expression of some of them is controlled by steroids and insulin (Purcell and Moley, 2009). This system allows the follicle to regulate its growth and development, mainly by altering FSHinduced effects on the synthesis of estradiol by the granulosa cells, in accordance with the availability of glucose (Webb et al., 2004; Scaramuzzi et al., 2010). In addition, glucose is essential in determining the quality of the oocyte (Sutton-McDowall et al., 2010; Berlinguer et al., 2012). On the other hand, high circulating NEFA concentrations, which correlates with follicular fluid ones (Porcu et al., 2017), impair oocyte health and metabolism (Sutton-McDowall et al., 2016). In the same way, elevated plasma urea levels reduce ovine embryo viability and development in vivo and in vitro (Bishonga et al., 1996; McEvoy et al., 1997).

Thus, the administration of the glucogenic mixture, by causing a rise in circulating glucose and insulin levels and a decrease in NEFA and urea ones, may contribute in creating a suitable systemic metabolic milieu for the promotion of ovarian function in dairy ewes. These data confirm results reported in a previous study in which dry ewes were given the same nutritional treatment during an induced oestrus cycle (Porcu et al., 2017). However, NEFA and urea circulating levels were higher in the present study than in the previous one. These data confirm that during lactation lipolysis and proteolysis are increased to meet the energetic requirements of the mammary gland. Nevertheless, the glucogenic treatment significantly lowered NEFA and urea circulating levels, even if these effects were limited to the treatment period. This result agrees with previous studies aimed at assessing the effect of glycerol or glycerine supplementation in lactating cows (Carvalho et al., 2011; Lomander et al., 2012).

### 4.3. Milk yield and composition

In the present study milk yield was significantly reduced in GLY ewes when measured in the last day of treatment, and thereafter increased again to reach values similar to controls. This finding is in agreement with previous studies in cows reporting that the dietary inclusion of crude glycerine for long periods at high levels (21% DM) decreased milk yield in dairy cows fed corn silage-based diets (Paiva et al., 2016). However, other studies on dairy cows showed that milk yield did not change (Shin et al., 2012; Ezequiel et al., 2015) or even increased (Lomander et al., 2012) when feeding glycerol supplemented diets. These studies differ for type of glycerol fed, duration of supplementation, lactation stage and basal diet. To the best of our knowledge, this study is the first reporting the effects of glycerol and propylene glycol supplementation on milk yield and composition in dairy ewes.

The increase in milk protein and casein percentages observed in GLY as compared with CTR ewes suggests that protein uptake was probably sufficient for milk yield even if the dietary content of CP was lower in GLY (11.8% DM) than CTR ewes (14.6% DM).

A possible explanation of the decrease in milk yield in GLY ewes is the reduction of water availability needed for the dilution of the milk solid components. In GLY ewes, in fact, the osmotic effect of high circulating concentration of glycerol can have triggered water reabsorption from the extracellular fluids to the bloodstream. Water accounts for the 82.5% of milk volume in sheep (Pulina and Bencini, 2004), and an acute shorten in water availability to the mammary gland may possibly explain the decrease in milk yield observed during the administration of the glucogenic mixture in GLY ewes. However, further studies are needed to better evaluate this hypothesis.

The decrease in milk yield observed in GLY group could also be related to the decrease of its lactose content. It is well known that lactose, thanks to its osmoregulatory property, favours mammary uptake of water. Other authors reported that milk lactose concentration and milk lactose yield tended to decrease linearly with increasing inclusion of crude glycerine in dairy cows diet (Ezequiel et al., 2015). However, this reduction was not accompanied by a significant reduction in milk yield.

Regarding milk protein, previous studies in cows have shown increase of milk protein concentration in dairy cattle with administration of 3 kg/d of crude dietary glycerol (Harzia et al., 2013) or with a level of 120 g/kg DM of crude glycerin (Wilbert et al., 2013). In the current study, milk protein percentage increased during the treatment together with milk casein probably due to the higher intake of energy and lower MY in GLY ewes. In contrast, milk fat was not significantly enhanced by the treatment, possibly due to the decrease of acetate proportion in the rumen and lower mobilization of fatty acids from body depots in GLY than CTR ewes, as suggested by the lower level of plasma NEFA. The inclusion of glycerol in cow diet is expected to increase the molar proportion of propionate and butyrate at the expense of acetate (Silva et al., 2014).

Urea concentration in sheep milk is related both to the CP concentration in the diet (Cannas et al., 1998), and to the ratio of protein and energy intake (Molle et al., 2008). The CP concentration in the diet and the ratio of CP and $NE_L$ intake were lower in GLY than CTR ewes. Therefore, it can be assumed that both these factors probably concurred to the decrease of milk urea content observed in treated ewes.

### 4.4. Pregnancy scanning and ewe reproductive performance

Conception rate and prolificacy did not change between groups. Previous findings showed an increase in oocyte developmental competence in dry ewes given the same glucogenic treatment during an induced oestrus cycle (Berlinguer et al., 2012), most likely due the creation of a suitable environment for the final follicular growth, both at the systemic and at the follicular level (Porcu et al., 2017). Other authors showed

that dry ewes given a single oral glucogenic dosage (100 mL of the same mixture of the present study) immediately before introducing the rams in the flock had higher ovulation rates than controls, if primed with intravaginal sponges containing low medroxy-progesterone acetate (MPA) dose (10 mg). On the other hand, the opposite was found when sponges having high MPA dose (60 mg) were used (Iglesias et al., 1996). An increase in ovulation rate was found also in a previous study on Manchega ewes given the same treatment used in the present study and it was linked to an enhancement in the developmental competence of preovulatory follicles (Letelier et al., 2008). However, this response was not found in a previous study (Williams et al., 2001) adopting the same flushing protocol, but their ewes had high body condition score at mating in a way that flushing was probably unnecessary.

In the present study, the high external temperatures recorded in the mating days may have partially compromised mating success and reproductive performances, with particular reference to conception rate (Marai et al., 2007). Finally, the BCS and nutrition of CTR ewes were possibly good enough to express their genetic potential, at least in terms of prolificacy, as was the case of the study by (Williams et al., 2001).

## 5. Conclusion

In conclusion, the short-term administration of a high dose of glucogenic mixture to milked ewes at mating resulted in a temporary reduction of concentrate intake, milk yield and milk lactose content and an increase of milk protein and casein contents. Moreover, it resulted in a transient increase in blood volume and plasma concentration of glycerol, glucose, and insulin, with a decrease of plasma concentration of NEFA and urea. However, reproductive performances were not affected by the glucogenic mixture administration.

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