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Endocrine/metabolic status and reproductive performances in Sarda dairy ewes given different nutritional plans.

Docente Guida

Prof. Fiammetta Berlinguer

Il Coordinatore

Prof. Salvatore Naitana

Tesi di dottorato del

Dott. Porcu Cristian

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DOCTORAL DISSERTATION EX ANTE EVALUATION REPORT

PH. D. STUDENT: Cristian Porcu

TITLE: Endocrine/metabolic status and reproductive performances in Sarda dairy ewes given different nutritional plans

In Accordance with Article 16 of the Regulations for Doctoral Studies at the University of Sassari (approved by the Governing Council dated October12, 2012), Prof. Ana Josefa Soler Valls at the request of the Doctoral Program Coordinator of the University of Sassari ISSUES THE FOLLOWING REPORT ON THE DOCTORAL DISSERTATION CONTENT

Additional pages can be used, if necessary

1. Research lines in which the thesis is rooted:

The effect of the nutrition in the reproduction

2. Scientific, Technical, and Humanistic contributions of the doctoral dissertation:

The thesis contribute to better knowledge of the effect the glycaemia in the endocrine reproductive status. The results derived of this thesis will help to select the best treatment of nutrition for the conception period.

3. The doctoral dissertation compiles with the required quality standards:

YES ☒

NO ☐

Date: 02/14/2017

Signed: Ana J. Soler Valls

Departamento de
Producción Animal
y Ciencia de los Alimentos
Universidad Zaragoza

DOCTORAL DISSERTATION EX ANTE EVALUATION REPORT

PH. D. STUDENT: Cristian Porcu

TITLE: Endocrine/metabolic status and reproductive performances in Sarda dairy ewes given different nutritional plans

In Accordance with Article 16 of the Regulations for Doctoral Studies at the University of Sassari (approved by the Governing Council dated October12, 2012), Prof. José-Alfonso Abecia at the request of the Doctoral Program Coordinator of the University of Sassari ISSUES THE FOLLOWING REPORT ON THE DOCTORAL DISSERTATION CONTENT

Additional pages can be used, if necessary

1. Research lines in which the thesis is rooted:

Sheep reproduction, relationships between nutrition and reproduction,

2. Scientific, Technical, and Humanistic contributions of the doctoral dissertation:

The main scientific contributions are:
1. In Sarda ewes, a reduced time allocation in Italian ryegrass pasture during lactation, of less than 4 h/day, can have a negative effect on prolificacy. These results suggest that a restriction of time access to pasture during mid-lactation, can subsequently impair the reproductive performance of late-lactation sheep, even if submitted to both male effect and flushing with lupin.

2. A part-time grazing less than 4 hours, in Sarda sheep at mid lactation, can have negatives effects on reproduction.

3. A short-term administration of a glucogenic mixture significantly increased circulating concentration of glucose, insulin, and IGF-1, while reducing circulating concentration of NEFA and urea. These metabolic changes ultimately affected folicular fluid (FF) microenvironment. In particular, they brought about an increase in FF concentrations of glucose, without affecting FF insulin and IGF-1 concentrations, nor GSH content and total antioxidant capacity.

4. A short-term administration of a glucogenic mixture, creates a suitable micro-environment for the final follicular growth and thus for the conception period in the ewe.

5. Homeorhetic adaptation to lactation plays a central role in determining energy partitioning in mid lactation dairy ewes, and the metabolic status influences the response to the glucogenic treatment.

3. The doctoral dissertation compiles with the required quality standards:

YES ☒

NO ☐

Date: 14-2-2017

Signed:

Porcu Cristian. Endocrine/metabolic status and reproductive performances in Sarda dairy ewes given different nutritional plans. PhD thesis in Riproduzione, Produzione e Benessere Animale. Università degli Studi di Sassari.

TRAINEESHIP CERTIFICATE

Name of the trainee: Cristian PORCU

Name of the receiving organisation/enterprise: INRA UE FERLus

Sector of the receiving organisation/enterprise: 72.19
01.45

Address of the receiving organisation/enterprise *[street, city, country, phone, e-mail address]*, **website:**

INRA UE FERLUS, Les Verrines, CS 800006, 86600 Lusignan, 05.49.55.60.00

http://www.poitou-charentes.inra.fr/Le-centre-Les-recherches/Unites-du-centre/Ferlus

Start and end of the traineeship: from *17/02/15* till *13/05/15*

Traineeship title: Reproduction in goats

Detailed programme of the traineeship period including tasks carried out by the trainee:

Participation in different experimental studies run at INRA :

- Sexual precocity in doe kids : repeated measures and samples for AMH assays, weight, progesterone cyclicity
- Experimental inseminations on farms : pseudopregnancy diagnosis by ultrasonography, semen thawing and tracking of ejaculate/buck inseminated
- Buck semen preservation : conservation ability of fresh diluted semen, correlation between freezability and seminal plasma composition, assessment of different washing techniques
- Male behaviour during training and collection in AI center : behavioural assessment, timing and quality of semen in different collection conditions (dummy gender, dummy physiological status, collector,...)

Knowledge, skills (intellectual and practical) and competences acquired (learning outcomes achieved):

- Specific handling and blood sampling on young goats (from birth day till 6months), centrifugations, aliquoting plasma samples, traceability of samples AMH assays, transrectal ultrasound monitoring, fertility data analysis
- Buck semen collection, buck sexual behaviour recording, buck semen processing (washing, diluting, freezing, analysing with microscope and computer-assisted sperm analysis)
- On farm interventions : pseudopregnancy diagnosis by ultrasonography, semen thawing and tracking of ejaculate/buck inseminated, inseminations
- Improvement of French skills (oral and written)

Evaluation of the trainee:

Cristian integrated the team quickly and very easily.

He was in charge of blood sampling on young goats (weekly from birth) and plasma sampling for a study on sexual maturity with another intern.

He learnt really fast how to record buck behaviour and how to process semen for cryopreservation. We put him in charge of the recording and data compiling of a complete study on the effect of pheromones on semen collection parameters (4 bucks x 10 collections). Also he contributed in the lab to different experiments on the "washing" of seminal plasma and on "freezability" assessment of buck semen.

The technical team taught him how to detect pseudopregnancy through ultrasonography, which is a specific phenomenon in goats. He had the opportunity to take part in detections and inseminations on farm. He was very efficient and helpful with ultrasonography, he was also of great help preparing semen for AI.

He made great progress in French. He readily made a presentation of his PhD thesis in French for the local team and fellow intern students.

Date: 20/05/2015

Name and signature of the responsible person at the receiving organisation/enterprise:

Alice FATET, ingénieur de recherche en reproduction des petits ruminants
INRA UE FERLUS

François GASTAL, directeur d'unité
INRA UE FERLUS

INRA
UE Fourrages Environnement Ruminants
Les Verrines - CS 80006
86600 LUSIGNAN

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SUMMARY

Numerous studies link nutrition with reproduction, and have demonstrated the diet deficiencies exert a negative effect on reproductive performances. Since in the typical breeding system in the Mediterranean area, mature ewes are mated when in their mid to late lactation, ewe energy requirements are still high and farmers have to supplement diet to minimize possible detrimental effect of diet deficiencies on reproductive performance. Currently a common strategy to improve the reproductive performance of sheep is to use short, targeted feeding regimes. However, the response to these nutritional treatments has been shown to vary significantly between commercial flocks. One cause often overlooked is represented by the carry-over effects of nutrition on reproductive performance since the metabolic consequences of dietary supplementation vary according to the nutritional history of the animals. Thus, the first part of this dissertation aimed at studying the carry-over effects of a background grazing during lactation on ewe's reproductive performances. Two experiments were designed to evaluate whether different time access to pasture during mid-lactation may exert a residual effect on ewe's body weight, body condition, milk yield at mating, and reproductive performance in ewes submitted to a flushing with lupin grains. These experiments demonstrated that ewes with severely restricted access to pasture during mid-lactation seems unable to adequately respond to a short-term flushing, with detrimental effect on reproduction.

Focus feeding supplementations used to improve reproductive performance are often based on glucogenic treatment since glucose is one of the key nutrients having an effect on the ovary. Thus, the second study of the present thesis aimed at assessing the effects of a short-term administration of a glucogenic mixture on metabolism and reproductive performances. The study was branched in two experiments: the first one was designed to assess the effects of a short-term administration of a glucogenic mixture in non-lactating Sarda ewes on plasmatic and intrafollicular concentrations of metabolites and hormones, while the second one was performed in lactating Sarda ewes to evaluate its effects on metabolism, milk production and reproductive performances.

Results obtained showed that short-term administration of a glucogenic mixture significantly affected circulating concentration of metabolites and hormones in dry ewes, leading to an increase in glucose and insulin concentrations and a decrease in NEFA and urea ones. This changes, partially reflected in follicular fluid, affected follicular microenvironment, as evaluated 12 h after the end of the nutritional treatment, suggesting that short-term administration creates a suitable micro-environment for the final follicular growth and thus for the conception period in the ewe. . A reduced clearance of NEFA was observed within the follicle, thus underlying that the negative effects on follicular growth related with high NEFA concentrations can persist even after the re-establishment of a positive energy balance.

These findings were only partly confirmed when the mixture was administered in lactating ewes, because homeorhetic adaptation during lactation lead to a different

response, with higher circulating glucose values and lower circulating insulin values if compared to dry ewes. Moreover, the glycerol based mixture lead to a 400-folds increase in circulating glycerol concentration, affecting plasma osmolarity and total protein concentration, and likely blood volume. Milk yield was significantly reduced in treated ewes, probably as a consequence of the decrease in its lactose content, or altered metabolizable protein availability. A shorten in water availability needed for the dilution of the milk solid components, due to the increased plasma osmolarity that triggered water reabsorption from the extracellular fluids to the bloodstream, may also explain the decrease in milk yield observed during the treatment. Moreover, milk protein content increased whereas milk fat was not affected. As for reproductive performances, the glucogenic treatment applied numerically increased ewe's fertility, but the difference was not statistically significant, and overall reproductive performances did not differ between the two experimental groups.

A severe time restriction to pasture in ewes in mid lactation can directly affect nutrient intake and milk performance and then exert a detrimental residual effect on the response to glucogenic treatments. The ovary is able to take advantage of the transient hyperglycaemia, and the consequent hormones-related releasing, but NEFA clearance in the ovary is less efficient than at the systemic level, and thus the negative effect of high FF NEFA concentration can persist even after the re-establishment of a positive energy balance. This could possibly explain while, under some circumstances, short-term flushing treatments based on lupins or glucogenic mixtures failed to

improve reproductive performance of sheep submitted to long-term systemic underfeeding.

1. INTRODUCTION

1. INTRODUCTION

In the Mediterranean countries, sheep production represents important economic, environmental, and sociological issues. European dairy sheep production is mainly located in the Mediterranean basin (52% of the total dairy sheep stock in Europe is raised in Italy and Greece). The typical farming system is semi-extensive or semi-intensive, which consists of grazing natural pastures or forage crops associated with farming techniques typical of traditional intensive systems (e.g. mechanical milking and feed supplementation during almost the entire lactation). The breeding system implies one lambing per year, with the mating season starting in late spring for mature ewes and in early autumn for ewe-lambs (two tooth sheep) [1]. After lamb's weaning, the ewes are milked twice a day from December to June and once a day until they dry off in July [2]. Primiparous ewes milking period usually starts in March and ends in July.

Mediterranean regions are characterised by seasonality in food availability, with wet winters and springs and dry summers. Herbage availability is strongly affected by rainfall distribution and air temperature. The herbage-growing season starts in autumn and early winter, especially in the mountains, where the herbage availability is often low but its quality is high [3]. Flocks are spread all over the region and their size are related to the soil and climatic conditions. On the lowlands, a high percentage of cultivable land permits rich forage production associated with a high stocking rate and generally high milk production per hectare [4, 5]. On the other hand, in the hill and

mountain areas, the production is based on the utilisation of natural pasture with a low stocking rate, between two and 12 ewes per hectare [6].

In these semi-extensive conditions, ewes may face nutritional deficiencies and adverse weather conditions [7], and weight loss is common during late spring and autumn. Semi-extensive system, in fact, have much more risk of nutritional deficiencies due to lack of pasture in critical seasons [8]. In some cases, ewes reared extensively had to increase the time of grazing from the normal 8 hours to a maximum of 13 hours, to make up for the shortage pasture which would otherwise cause weight loss [8, 9].

Since in the typical breeding system in the Mediterranean area the mating season starts in late spring for mature ewes and in early autumn for young ewes [1], farmers have to supplement ewe's diet during these periods to minimize possible detrimental effect of diet deficiencies on reproductive performance. In addition, mature ewes are often bred during mid to late-lactation, when their energy requirements are still high. Therefore, to improve reproductive outcomes ewes usually receive a flushing supplementation around the time of mating.

However, the response to feeding supplements has been shown to vary significantly between commercial flocks. The causes of this high variability may include genetics, nutritional plans applied, duration of supplementation, and ewe's physiological status. In addition, one cause often overlooked is represented by the carry-over effects of nutrition on reproductive performance because the metabolic consequences of dietary supplementation vary according to the nutritional history of the animals. This concept is a cornerstone of behavioural ecology, considering that many animals *finance*

reproduction from energy stores (predominantly fat) gained in the months prior to the breeding season (*capital*-based breeding strategy), rather than using energy gained concurrently during reproduction (*income*-based breeding strategy) [10, 11]. As a general rule among capital breeders, individuals with larger fat stores upon initiation of reproduction tend to have the greatest reproductive success [12], with fitness penalties incurred by those individuals who initiate reproduction with lower/insufficient energy stores [13, 14]. In fact, the proportion of ewes that displays oestrus following ram introduction in spring seems to reflect live weight changes over the previous winter rather than the live weight at the time the rams are introduced [15]. It takes approximately 6 months from when follicles first commence growing to when one or more of these undergo final maturation and ovulate [16]. Imposing nutritional handicaps at distinct stages of folliculogenesis have been shown to influence ovulation rate in the ewe. Restricting feed intake 6 months prior to ovulation, when those follicles destined to ovulate first commence growing, reduced ovulation rate [17]. The carry-over effects of nutrition on reproductive performance may be particularly evident in the Mediterranean farming system, considering that dairy ewes are usually mated during mid-lactation, and their energy requirements around mating can be fairly high, often thrice their energy maintenance level. For these reasons, the study of nutrition carry-over effect as drivers of differences in dairy ewe's reproductive performance may help improving the outcome of flushing treatment applied in preparation to mating.

Currently a common strategy to improve the reproductive performance of sheep is to use short, targeted feeding regimes, "focussed feeding" [18]. Focus feeding is already being used to boost sperm production, increase ovulation rate, and improve offspring survival. In particular, focus feeding is a reliable strategy to improve ovulation rate and the costs can be minimised by feeding supplements for periods as short as 4 days, as long as the supplement is offered only during the late luteal phase [19-21]. Studies report that supplementary feeding such as lupin grain (Lupin angustifolius) during mating can increase ovulation rate and reproductive performances [22, 23]. The effect of lupin grain supplementation, is likely to be associated with metabolic changes in response the increase in nutrient supply [24]. There is some evidence that a shorter period of increased metabolic status could stimulate the follicular growth and ovulation rate in sheep and goats [25, 26]. Energy yielding substrates can increase the number of larger follicles [27, 28] and decrease follicular atresia [29-31]. High-energy short-term feeding of ewes have been also associated with progestin-sponge, resulting in increased folliculogenesis [32]. Moreover, Vinoles et al (2005)[33] found an increase in glucose and metabolic hormones when short-term nutritional supplementation are administered during the luteal phase of the estrous cycle.

Focus feeding is often based on glucogenic treatment, considering that glucose is one of the key nutrients having an effect on the ovary [24, 32]. Glucose has distinct roles in follicular function: first as a nutrient to generate ATP, and second as a signalling molecule to stimulate folliculogenesis when nutritional conditions are favorable to reproduction [34]. The role of glucose is also essential in determining the quality of the

oocyte [35]. In a previous study on dairy sheep, our research group showed that short-term flushing with a glucogenic mixture based on glycerol and propylene glycol improves oocyte quality, evaluated by the kinetics of their *in vitro* development and by the production of blastocysts [27]. In another study, the same nutritional treatment increased the ovulation rate [36]. Glucogenic precursors such as propylene glycol and glycerol and their mixture have been used in veterinary practice to increase blood glucose and to reduce nutritional problems in dairy cows during the peripartum [37-39]. It has been reported that feeding glycerol as a top dress [37, 40] or supplied in water [39] to transition dairy cows resulted in a positive energy status with higher concentrations of serum glucose and lower concentrations of plasma non-esterified fatty acids (NEFA)[38]. Glucose can be metabolized within the follicle by the pentose phosphate pathway to provide precursors for the synthesis of purine nucleotides and NADPH which are in turn used in various biosynthetic pathways, including those related with the antioxidant defense. Hence glucose follicular fluid (FF) concentration can also influence follicular oxidative status [41].

Increasing glucose concentration in blood, leads to a rise in insulin blood concentration. In ruminants, as other mammals, insulin have a central role on metabolism regulation, and plasma levels are increased after feeding [42, 43]. Insulin's effects on ovarian cells are positive, stimulating granulosa cell proliferation and the production of progesterone and enhancing luteal cell steroidogenesis. The follicles have glucose transporters, and the increase in the availability of glucose and metabolic hormones should increase the energy available to the follicles, and thus their ability to

grow [44-46]. In fact, when nutritional treatments stimulate insulin-glucose system, granulosa and theca cells increase the expression of GLUT-1 and GLUT-4 proteins [46] and glucose uptake by the ovary [47].

In addition to the glucose-insulin system, another hormone playing a key role in the energy status and function of the ovary is the insulin-like growth factor 1 (IGF-1). Insulin-like growth factor 1 and gonadotropins are synergistic for growth and differentiation of the follicle [48, 49].The IGF system (receptors, ligands, and binding proteins) is expressed within granulosa and theca cells [48-50].

Intra-follicular actions of the glucose-insulin and IGF systems have been demonstrated to be nutritionally regulated [34, 41, 51], both using infusion of glucose and lupin grain feeding [32].

However, in sheep, the actual effect of a short-term administration of a glucogenic mixture on the follicular glucose-insulin system still need to be fully investigated. Moreover, most of the studies are carried on in dry sheep, and the effect of these nutritional treatment in lactating ewes are often overlooked.

Starting from these premises, two main studies were designed. The first study (STUDY 1) aimed at investigating the carry-over effects of a background grazing during lactation on ewe's reproductive performances. This study includes 2 experiments which assessed the residual effect of restricted time access to pasture in dairy sheep on their response to the ram effect and flushing in terms of ovarian activity resumption, fertility, and prolificacy. These experiments differed by the types of

pasture offered. In experiment 1 (E 1.1) a grass-based pasture rich in fiber and carbohydrates but low in protein was grazed, while in experiment 2 (E 1.2) a leguminous pasture rich in protein was grazed. The purpose was to evaluate the residual effects of grazing two of the most spread forage crops in Mediterranean basin.

The second study (STUDY 2) aimed at assessing the effects of a short-term administration of a glucogenic mixture, in both dry and lactating Sarda ewes, on their metabolism and reproductive performances. Also this study includes 2 experiments. The first experiment (E 2.1) aimed at assessing the effect of a short-term administration of a glucogenic mixture in non-lactating Sarda ewes on plasmatic and intrafollicular concentrations of metabolites and hormones which play a key role in follicular maturation and quality.

The second experiment (E 2.2) was performed in lactating Sarda ewes. The same nutritional treatment (glucogenic administration) as in E 3 was applied to evaluate its effects on ewe's productive (milk) and reproductive performances.

2. LITERATURE REVIEW

2. LITERATURE REVIEW

2.1. Glucose-Insulin and IGF System

Sheep can face different physiological states where energetic needs are high, such as foetal growth, lactation and pubertal growth, and failure to meet nutrient demand can compromise reproduction.

The metabolic system of the follicle is nutritionally regulated in the ewe and the infusion of glucose and lupin grain feeding [32] have been shown to increase reproductive performance acting directly at the follicular level on the insulin-glucose and the IGF systems [34, 41, 51].

2.1.1. Glucose and insulin

In adult sheep, the direct uptake of glucose from the diet is usually limited, due the poor absorption from the small intestine (especially if fed with a roughage diet). To meet glucose requirement the main source is the liver *via* gluconeogenesis [52](Weekes, 1991), based on the metabolism of rumen-derived volatile fatty acids (mainly propionic acid) and amino acids. With gluconeogenesis, sheep are able to keep their basal glucose level relatively stable [53].

Overall, whatever the source, the intake of glucose or glucose precursors can markedly impact on milk production in ruminants. In dairy sheep, milk yield is affected by dietary non-fiber carbohydrates (NFC), such as starch, pectin, galactans, and simple sugars.

During the first part of lactation, diets with high NFC concentration based on starch generally give much higher milk yield than medium-low NFC diets [54-56]. However, during mid to late lactation, feeding sheep with diet rich in starch did not improve significantly milk yield or even slightly decreased it. Molle et al. [57] found, in mid-lactating dairy ewes grazing Italian ryegrass in afternoon rather than in morning hours, an increase in the intake of herbage water soluble carbohydrates (mainly sugars), enhancing milk yield and body condition score.

Anyway, during lactation, glucose requirements in the mammary tissue increase dramatically, considering that it is the main precursor of milk lactose, and it is also involved in milk protein and lipid metabolism.

The homeostasis of glucose is regulated by insulin. Feeding sheep with concentrates or feeding with grains led to an increase of glucose and consequently of insulin blood concentration. In ruminants, as other mammals, insulin have a central role on metabolism regulation, and plasma levels are increased after feeding [42, 43] by pancreas secretion [58]. The ruminant digestion, seems to modify the physiological insulin secretion, with less importance of stimulating removal of an exogenous glucose load [52]. Many papers have reported that short-term nutritional supplementation increased the plasma concentrations of insulin in ewes [24, 29, 33, 46, 59], and cows [60-62]. Elevated insulin concentration in plasma diminished hepatic uptake of glucose precursor [63] and depressed hepatic glucose production [64].

Studies showed that feeding with lupin have a direct effect on glycaemia and then on insulinemia. In addition, infusion with glucose increase plasma concentrations of

glucose and insulin reaching higher values than the normal physiological range for glucose leading to a quicker increase in insulin concentrations as lupin feeding [24, 32], probably related to a different impact on metabolic homeostasis.

In sheep, ovarian follicles are sensitive to nutritional signal and the manipulation of the diet can increase folliculogenesis. Studies report that glucose infused for 3 or 5 days stimulated folliculogenesis, increasing the numbers of large follicles without any effect on the number of small and medium sized follicles [24, 32]. Large follicles are increased also by Lupin in a lesser extent than glucose-infusion.

Glucose is the principal source of energy of the ovary and it is well described the positive effects on fertility are related to its properties as a metabolic fuel [65].

Glucose have a direct effect on the ovary [24, 32] and can be metabolized within the follicle by the pentose phosphate pathway to provide precursors for the synthesis of purine nucleotides and NADPH which are in turn used in various biosynthetic pathways, including those related with the antioxidant defense.

Glucose and insulin have distinct roles in follicular function. The roles of glucose are essential in determining the quality of the oocyte [35], acting as a nutrient to generate ATP, and as s signalling molecule that stimulate folliculogenesis when nutritional conditions are favorable to reproduction [34]. Insulin, on ovarian cells, stimulate granulosa cell proliferation and the production of progesterone and enhancing luteal cell steroidogenesis. It works as signal mediating the effects of acute changes in nutrient intake on follicle dynamics [41, 66]. Moreover, insulin had specific effects on

granulosa and theca cell function in the ewe [67] and with glucose acted together to influence directly ovarian function in situ [68]. The follicles have glucose transporters, and the increase in the availability of glucose and metabolic hormones should increase the energy available to the follicles, and thus their ability to grow [44-46]. In fact, when nutritional treatments stimulate insulin-glucose system, granulosa and theca cells increase the expression of GLUT-1 and GLUT-4 proteins [46] and glucose uptake by the ovary [47].

2.1.2. IGF-1

In addition to the glucose-insulin system, another hormone playing a key role in the energy status and function of the ovary is the insulin-like growth factor 1 (IGF-1). IGF-1 is a peptide related to insulin, and its secretion is influenced by both glucose and insulin. It is an extremely potent stimulator of follicular proliferation and follicular steroid secretion in most species and its action is synergic with gonadotropins for growth and differentiation of the follicle [48, 49].

IGF-I is a hormone that modulates the maturation of the dominant follicle during the first follicular wave postpartum [69], and circulating IGF-I in ovulatory cows at the first follicular wave postpartum is higher than that in anovulatory cows, regardless of parity. In fact, systemic IGF-I concentrations in the first 2–3 weeks of lactation have been associated with increased probability of ovulation of the first postpartum dominant follicle [70].

The IGF system (receptors, ligands, and binding proteins) is expressed within granulosa and theca cells [48-50]. In the bovine granulosa cells, IGF-I induced the upregulation of steroidogenic and apoptotic regulatory genes via the activation of phosphatidylinositol-dependent kinase/AKT [71]. Both insulin and IGF-I enhance follicle cell function in vitro in several species including cattle [72, 73]. In addition, in vitro research has shown that the addition of IGF-I to the culture medium improved the development of caprine preantral follicles [74]. Moreover, dose-dependent stimulatory effects of IGF-1 on the proliferation and steroid synthesis of bovine granulosa cells and theca cells were found; these effects can be exerted via their direct stimulatory actions on the follicle as well as by increased local responsiveness of the follicle to FSH and LH [49].

2.2. NEFA

The evolutionary modification of the stomach in ruminants allowed the use of microbial fermentation in the rumen to digest cellulose and to use non protein nitrogen [75, 76].

In ruminant, most of the carbohydrates contained in the diet, instead of being absorbed, are fermented into volatile fatty acids, which are readily absorbable for energy production [76]. Fatty acids are the most common and important component of all classes of lipids. They are rarely present in the form of free fatty acids but more frequently incorporated with ester or amide bond in the various lipid classes (mainly triglycerides, phospholipids, cholesterol esters, sphingolipids, etc.). Rumen

fermentation of poly- and monosaccharaides results in the formation of pyruvate, which is an intermediate substrate for the production of short-chain fatty acids, primarily acetate, propionate and butyrate. Propionate is primarily used for hepatic gluconeogenesis, whereas acetate is activated to form acetyl-CoA, the main energy-providing substrate.

NEFA are the primary source of fatty acid metabolized from the liver, and the uptake depend on the blood concentration, which increase with negative energy balance (NEB). Changes in body reserves in ruminants are related to different periods during the year. Excess of nutrients are stored when feed is abundant, while the mobilization happens under exigent situation such as when the dam has to nourish the offspring during cold and dry season or in presence of scarcity in feed availability [77]. The stress of late gestation and early lactation increases NEFA supply to the liver, where they cause deposition of fat. To prevent the accumulation of fat, liver increase ketogenesis and oxidation, but in the peripartum period it is not sufficient to prevent it [78].

High producing dairy cows are exposed to the risk of NEB and various metabolic disorders. The metabolic patterns in maternal tissues of well fed, late-pregnant ruminants, are characterized by increased hepatic gluconeogenesis but reduced glucose utilization in peripheral tissues, unchanged or decreased peripheral utilization of acetate, and moderately increased mobilization of NEFA from adipose tissue, associated with similar increases in peripheral utilization of NEFA and their derivatives [79]. Insufficient glucose supply can lead cow metabolism to develop NEB and as consequences of it, the animal can experience fatty liver and fatty liver-related

metabolic and infectious health disorders. It has been established that high concentrations of NEFA have cytotoxic effect on different type of cells, such as Leydig cells [80], nerve growth factor differentiated cells [81], hepatocytes [82], and pancreatic b-cells with consequent impairment of insulin secretion, considered as an important factor in the pathogenesis of diabetes [83].

High-yielding dairy cows reproductive performance has declined over the past few decades [84] leading to a prolonged interval to first ovulation [85], and delayed oestrus have been related to NEB [86, 87]. For this reason, reproductive performances of high producing cows depend on the occurrence of a negative energy balance and concurrent metabolic changes [88, 89].

When NEB occurs, body fat is mobilized, reflected trough an increased plasma NEFA concentration [90-92], with a negative impact on fertility [88, 93]. In cow, the energy loss during milk production results in NEB that cannot be compensated by the energy intake [94-96] and seems to impair the fertility of these cows [95-98]. This stress leads to a massive body fat mobilization with an increase of free fatty acid plasma (fatty acid complexed to albumin) and follicular fluid concentration [91, 98-102], and impairs fertility by affecting oocyte quality due to transport of fatty acid into the oocyte [101, 103].

NEFA uptake occurs at ovarian level, and there is a strong positive correlation between the concentration of NEFA in plasma and the follicular fluid. NEFA have a negative effect on fertility [88, 93], possible related to the harmful effects of NEFA on either granulosa cells or the oocyte [99, 104].

NEB negative effect on follicular growth and development can be exerted through different mechanisms, like reduced LH-pulsatility and lower IGF-1 concentrations [86], with NEFA elevated concentrations [105]. These concentrations are closely reflected in the follicular fluid of dominant follicles [99]. In fact, in cattle, the presence of NEFA in follicular fluid of large follicles is highly correlated with blood concentration [106], leading to an increase of NEFA concentration on ovarian cells affecting follicular growth and development.

In cows NEFA concentrations are reflected in the follicular fluid of the pre-ovulatory ovarian follicle [100], directly affect the granulosa cell viability and steroidogenesis [107, 108] as well as the developmental capacity of the oocyte [100, 107, 109]. This effect on reproductive cells have been showed also in human granulosa cell, where Palmitic Acid (PA) and Stearic Acid (SA), suppress granulosa cell survival [110]. Have been reported that elevated NEFA concentrations during oocyte maturation have negative consequences for the resulting preimplantation embryo, measured 8 days later at the blastocyst stage.

In addition, the fatty acid composition of high quality and low quality oocytes differs [111], and in porcine, cow, and sheep oocytes fatty acid composition, mirror the composition of free fatty acid present in blood and follicular fluid. [100, 112, 113]. In addition, the fatty acid composition seems to be related to those of the adipose tissue that they are liberated [105], suggesting that the environment can influences oocytes development competence. Usually, in somatic cells, fatty acid are esterified into

triacylglycerols (TAG) and cholesterol-esters and stored as neutral lipids in lipid droplets.

Some studies found that increased NEFA concentration have been related with low blood progesterone concentrations and a decrease in CL weight [114, 115], while other report only lower CL weights with no lower plasma progesterone concentrations [116].

2.3. Urea

The fate of the urea originating from catabolism of amino acids and hepatic detoxification of ammonia can enter the gastrointestinal tract for subsequent hydrolysis by bacterial urease to ammonia. Ammonia is a source of nitrogen for microbial synthesis of proteins. It can be absorbed in the intestine for anabolic purpose.

The process of urea recycling is more evident in ruminant then non-ruminant animal [117] and about 30–98% of urea produced by the liver can enter the gastrointestinal tract, leading ruminants to survive when protein supplies are insufficient or inadequate [118, 119].

In a state of inferior nutrition, glucose is insufficient for energy expenditure. Energy deficiency, lead to mobilization of body protein stores. If the negative energy balance increases, then the catabolism of body tissue also increases, resulting in degradation not only the body fat but also the body protein and therefore urea blood levels are then enhanced. Moreover, poor-quality protein or excessive nitrogen in the diet can

increase blood urea concentrations through excessive deamination of amino acids and result in increased urea concentration in plasma and milk.

Caldeira et al. (2007) [120] indicated that ewes in poor nutritional condition have lower blood concentration of glucose, insulin, and higher NEFA and blood urea nitrogen (BUN). When in a balanced status, glucose and insulin are at intermediate levels, and BUN is low. [121] also reported that plasma glucose declined and BUN levels increased when BCS of cows was reduced.

On the other hand, in sheep production system, the ration of high producing ewes during early lactation requires protein supplementation, because the rumen microbial protein does not meet the animal's net requirements of amino acids [122].

Increasing crude protein in the diet of dairy cows and sheep is associated with increase of plasma and milk urea nitrogen (PUN; MUN,) [88] and may also have detrimental effects on the oocyte or embryo development. In cow [123, 124] and sheep [125, 126], high peripheral concentrations of nitrogenous compounds, related to the diets, have been associated with impaired reproductive performance. Elevated PUN or MUN was reported to be associated with a reduction in the conception rate in lactating dairy cows [124] and dairy heifers [127]. High PUN concentrations were associated with elevated ammonia and urea nitrogen concentration in follicular fluid of preovulatory follicle, as well as in the uterine fluid during the luteal phase of the estrous cycle [128]. Changes in uterine environment have direct effect on oocyte and subsequent effect on fertility in cow [129]. Rhoads et al. (2006) [130] demonstrated that high PUN concentrations detrimentally affected oocytes or embryos of lactating cows before Day

7 of pregnancy. Feeding ewes diets with excess rumen degradable proteins lead to elevated urea and ammonia in the plasma and in utero [125]. Moreover, PUN have been related with a decreased embryo viability in superovulated lactating cows, resulting in higher pregnancy rate related to embryos from donor cows with a moderate PUN than high-PUN. Moreover, urea feeding without an adaptation period was associated with a decrease in embryos recovered from superovulated dairy cows [131].

Elevated plasma urea nitrogen and ammonia did not affect LH surge before ovulation but they reduced the embryo viability and development in vivo and in vitro. In addition, feeding excess degradable protein to ewes during estrous cycle and the first 5 days after breeding delayed embryo transport and accelerated embryo development through oviduct [132].

2.4. Nutrition in lactation

Mediterranean region is characterised by seasonality in food availability, influenced by wet winters and dry springs and summers. Sarda lactating ewes are mainly fed native pastures or forage crops, and receive as supplements commercial concentrates, cereal grains, hay or silages, at levels depending on herbage availability and ewe physiological status. Under grazing conditions, sward height, an index of sward structure and herbage availability, shows a strong relationship with animal intake and nutrition [133-135].

After the weaning of lambs, ewes are usually milked twice daily until late spring or early summer. During this period, pasture is usually turning to a reproductive phase and grazed herbage is featured by a raising content of fiber (high fiber NDF-40-60 % DM), and a declining content of crude protein (14-8% DM). This decay of pasture nutritive value is usually mirrored by the lowering of milk yield [136], with a decrease in herbage availability and quality, limiting intake and digestibility [137, 138].

Nutrition during middle lactation, besides affecting immediately milk yield, can exert a residual effect on reproductive performance.

Dairy ewes are usually mated during lactation, so their energy requirements around mating can be fairly high, ranging between 130 and 200% of maintenance, and if previous nutrition had been poor, body condition may not reach the optimum score for reproduction [139]. The proportion of ewes that displays oestrus following rams introduction in spring seems to reflect live weight changes over the previous winter rather than the live weight at the time the rams were introduced (Oldham, Lindsay, and Martin 1990). In the same period (May/July), both herbage availability and quality from annual Mediterranean pasture decrease quickly, limiting intake and digestibility. In these circumstances, body condition may not reach the optimum score for reproduction particularly if previous nutrition had been poor (Molle et al. 1995).

Ovulation rate is associated with the absolute level of body weight and is closely related to adiposity; as adiposity increases so does ovulation rate (Morley et al. 1978; McNeilly, Jonassen, and Rhind 1987; Vinoles et al. 2002). Considering that Sarda adult sheep is usually mated in anoestrous season, during lactation, the reproductive

success can be modulated by factors related to the animals, such as body weight (BW), body condition (BCS), lactation stage and productive level (Kafi and McGowan 1997).

It takes approximately 6 months from when follicles first commence growing to when one or more of these undergo final maturation and ovulate [16]. Imposing nutritional handicaps at distinct stages of folliculogenesis have been shown to influence ovulation rate in the ewe. Restricting feed intake 6 months prior to ovulation, when those follicles destined to ovulate first commence growing, reduced ovulation rate [17]. That, has major implications for the reproductive performance of sheep [140]. A too restricted part-time grazing of Italian ryegrass during mid-lactation (before mating period) has been proven to lead to low herbage and energy intake [141]. As described by Molle [142] the effect of different part-time grazing to pasture of berseem clover, a flock exposed to 2 hours/day of restricted time allocation, displayed a lower energy intake than flocks that grazed 4 and 6 hours/day, resulting in lower milk yield during mid-lactation period and a trend to lower recovery in BW and BCS.

Severe malnutrition can significantly extend the length of the seasonal anestrous period (Knight Tw 1983). Therefore, an inadequate nutrition plane in lactating ewes can have a negative influence on ovarian activity resumption and ewe fertility (Shevah et al. 1975), inhibiting hypothalamic GnRH release, and thus leads to reduced secretion of pituitary LH and eventually to anovulation and anestrus (Scaramuzzi and Martin 2008). It follow that metabolic challenges, such as food deprivation or restriction, inhibit GnRH secretion into the pituitary portal circulation [143]. Metabolic signals are permissive for the neuroendocrine events that control ovulatory cycles and fertility. In

fact, among adult ewes, undernutrition may alter the resumption of ovary cyclicity and the expression of sexual behaviour [144, 145]. This can depend to a specific behavioural response or to a reduction in GnRH pulse frequency that prevents the cascade of events leading to ovulation: these same events are necessary for the induction of behavioural oestrus [146].

It comes to itself that long-terms nutrition plan implemented during lactation can impact on reproduction (Molle et al. 1995), and improvement of ovulation rate and reproductive performances through nutritional supplementation can be achieved by increasing live weight and body condition (Scaramuzzi et al. 2006).

3. STUDY 1

3. STUDY 1

3.1. Introduction

Seasonal pattern of reproduction in ruminants is usually managed to ensure that births occur at the optimal time of the year, usually spring, which allows the new born to grow under favourable conditions of weather (mild temperature) and food availability (plenty of high quality herbage for their dams) [147]. Ewe's reproductive performance in terms of fertility, prolificacy, and fecundity are affected by genetics and by a variety of environmental factors including current and previous nutrition, photoperiod, socio-sexual factors, weather pattern (mainly temperature and relative humidity), and stress [148].

Mediterranean region is characterised by seasonality in food availability (influenced by wet winters and dry springs and summers), in a way that sheep can experience weight loss in autumn, i.e. the natural mating season. Therefore, Mediterranean breeds are adapted to conceive out-of-season (May-June), being their response to photoperiod modulated by socio-sexual (male effect or permanent contact ewes - rams) and other environmental factors, such as nutrition [149].

The sudden introduction of males can induce sufficiently synchronized ovulations to allow strategic feed supplementation in females that are reproductively quiescent because, out of season, or lactating [150, 151]. This event, called "ram effect", during late anestrus and after at least 8 weeks of separation, can lead to ovulation and heat synchronization [152, 153]. Ewe's response to male introduction, can be modulated by

several factors related to the animals, such as body weight (BW), body condition (BC), lactation stage, and productive level [154].

On the other hand, environmental factors such as changes in feed availability can also have an impact on reproduction. This effect can be exerted either by short-term, pre-mating flushing [20, 155] or by long-term high nutrition plans, usually implemented during lactation [156]. Both strategies are aimed at improving the ewe status at mating. The effect of nutrition on ovulation rate is more evident when the nutritional treatments is implemented during the transition between anestrus period (seasonal or lactational) and breeding season [157, 158]. The sudden raise of the nutrition plan during this period is often named as flushing.

Short-term effect of nutrition is associated with a short-term boost in nutrient intake that can stimulate folliculogenesis by increasing the number of small and medium-sized follicles [29, 32]. This can increase ovulation rate [24, 159] without a corresponding change in body weight [51] and this effect is probably associated with increased insulin-stimulated uptake of glucose by the follicle [51]. Numerous studies have demonstrated that the ovulation rate in ewes can be increased by supplementary feeding of lupin grain, glucose or other energy-yielding substrates [24, 33, 159-161]. In fact, a short-term feeding of lupin grain increased plasma concentrations of both glucose and insulin, with an increase number of follicles with no detectable change in live weight [162].

However, the proportion of ewes that displays oestrus following ram introduction in spring seems to reflect live weight changes over the previous winter rather than the

live weight at the time the rams were introduced [15]. Moreover, an improvement of ovulation rate and reproductive performances can be achieved through a high level of supplementations which usually result in an increase of BW and BC [34]. Body condition and, to some extent, body weight are closely related to adiposity and, as adiposity increases so does ovulation rate up to a threshold which is set by the genotype [163-165].

This influence is more evident on ovulation rate than ovarian cyclicity, sexual activity and fertility, although severe malnutrition can significantly extend the length of seasonal anestrous period [166]. An inadequate nutrition plane in lactating ewes can have a negative influence on ovarian activity resumption and ewe fertility. Insufficient supply of energy in the diet, or unmet demand of energy such as in early-lactating ruminants, inhibits hypothalamic GnRH release, and thus leads to reduced secretion of pituitary LH and eventually to anovulation and anestrus [148].

Nutrition during mid-lactation, besides affecting immediately milk yield, can exert a residual effect on reproductive performance. Sarda dairy ewes are usually mated during lactation, so their energy requirements around mating can be fairly high, often thrice energy maintenance level. In the same period (May/July), both herbage availability and quality from annual Mediterranean pasture decrease quickly, limiting intake and diet digestibility. In these circumstances, body condition may not reach the optimum score for reproduction, particularly if previous nutrition had been poor [156]. Forage-based diets are conventionally used for small ruminant feeding for both growing and fattening periods in many parts of the world and pasture is the main

feeding source for sheep raised in Sardinia. The grazing flock is generally fed on pastures characterised mainly by annual grass species that have to meet animals feed requirements. For this reason, in many Mediterranean pastoral areas, annual forage grasses are often cultivated to be grazed during winter and spring to complement the production of natural pasture [167].

Starting from these premises, two experiments were designed to evaluate whether different time access to pasture during mid-lactation may exert a residual effect on ewe's body weight, body condition, milk yield at mating, and reproductive performance. These experiments differed by the type of pasture offered, named as background pastures hereunder. In experiment 1 (E 1.1) a grass-based pasture of Italian ryegrass (*Lolium multiflorum*, Lam) was grazed. This pasture was featured by relatively high fiber but low protein contents [167], while in experiment 2 (E 1.2) a high quality leguminous-based pasture of berseem clover (*Trifolium alexandrinum* L) was grazed. It was featured by high concentrations of energy and crude protein and relatively low content of fibre [168]. These experiments are part of a wider study conducted by Agris Sardegna, in northwestern Sardinia, from February 2014 to November 2015. Each experiment was branched into two parts. The first part was run from February to end of April (named as background period) and was aimed at assessing the direct effects of time access to pasture restricted to 2 h/d, 4 h/d or 6 h/d on feeding behavior, intake and performance of dairy ewes rotationally grazing the above forage crops in spring [57, 169]. In contrast, the second part, described in this thesis, was aimed at evaluating the carry over effects of the above treatments on the

reproduction performance of the same animals. The reproductive performances were evaluated in term of behavioral estrus resumption when ewes were exposed to male effect and in terms of fertility, prolificacy, lambing concentration and twin rates after being submitted to a lupin-based flushing treatment.

3.2. Materials and Methods

3.2.1. Experiment site and duration

Both experiments were conducted at the Bonassai research station of Agris, in northwestern Sardinia (40°N, 8°E, 32 m a.s.l.). The climate is Mediterranean with a long-term (1995–2013) average annual rainfall of 568 mm. The experimental period, described in this manuscript, ranged from February to the end of the lambing season (November) in both E 1.1 and E 1.2, which were run in 2013 and 2014, respectively.

3.2.2. Experimental design

The animal protocol and the implemented procedures were in accordance with the ethical guidelines in force at Agris and the University of Sassari, in compliance with the European Union Directive 86/609/EC and the recommendation of the Commission of the European Communities 2007/526/EC. The research and technical personnel had been previously trained to properly handle the animals during measurement procedures. Before being subjected to the experimental procedures, the animals were familiarized with the equipment and routine described below.

The pre-reproductive nutritional treatments consisted in three different part-time grazing periods: 2-h access to pasture (2 h/day), approximately from 0800 to 1000 Central European Time (CET); 4-h access to pasture (4 h/day), approximately from 0800 to 1200 CET; and 6-h access to pasture (6 h/day), approximately from 0800 to 1400 CET. At the end of the part-time grazing periods, the ewes were subjected to ram effect and a five-day flushing with lupin grain.

3.2.3. Animals

In both experiments E 1.1 and E 1.2, at the end of February thirty-six mature Sarda ewes in mid lactation, previously treated against gastro-intestinal parasites, were selected from the farm flock, homogeneous for age and lactation stage. The ewes were weighted (mean ± s.d., 42.5 ± 4.0 kg, in E 1.1 and 41.8 ± 4.1 kg in E 1.2), body condition scored (2.42 ± 0.14in E 1.1 and 2.39 ± 0.12, in E 1.2) and their milk yield was measured (1449 ± 206 g/day/head, in E 1.1 and 1952 ± 127 g/day/head, in E 1.2). Thereafter, the ewes were managed as follows: they were machine milked twice daily at 7:00 and 15:00 and from late February to the second week of March, adapted to graze all together for 4 h/day spare plots of the forage species under focus (Italian ryegrass, in 2013, berseem clover in 2014), receiving commercial concentrates (700 g/day/ewe split into three meals) and ryegrass hay as supplements. In both experiments, after 2 adaptation weeks, in mid-March, the ewes were divided into three groups (G2h; G4h; G6h), balanced for age and for the pre-experimental measurements. The groups had access to three plots of Italian ryegrass (E 1.1) or berseem clover (E 1.2), of equal

dimension, divided into subplots which were rotationally grazed for a restricted daily time: G2h (n=12), 2 hours/day, from 08.00 to 10.00; G4h (n=12), 4 hours/day, from 08.00 to 12.00; G6h (n=12), 6 hours/day, from 08.00 to 14.00. The ewes were carted to and from the plots being housed overnight. Indoor feeding consisted of individually fed commercial concentrates (400 g/day/ewe) split into two meals at milkings and ryegrass-based hay (700 g/day/ewe). In E 1.1, after 2 weeks, because of the lowering quality of Italian ryegrass pasture, 300 g of lupin grain, was offered to all groups. Lupin was fed to the groups at pasture turnout. In E 1.2, due to the high protein content of berseem clover, lupin was replaced with whole maize grain, group fed at pasture turnout at the same daily dose than the lupin (300 g/day/ewe).

After approximately fifty days of experiment, on April 30 in both experiments, the part-time grazing period (background period) was discontinued, and all ewes were managed as a flock, being allocated to a pasture of Italian ryegrass at heading stage for 6 hours/day (07:30-13:30) with and indoor supplementation of 400 g/day/ewe of concentrate split in two meals at milkings and 700 g/day/ewe of ryegrass hay overnight.

On May 1 (identified as day 0 in both E 1.1 and E 1.2 for experimental purpose) to induce the ram effect, four adult, entire and not shorn rams were introduced in the flock (Male/Female ratio = 1/9). In both experiments from day 16 to day 20, the ewes were submitted to a five days flushing, replacing 400 g/day of concentrate with 400 g/day of lupin seed. On day 17, heat detection started two times a day (07:00, 15:00),

using crayons on the rams, and checking the mark on the ewes at the milking parlor. Heats and mating were detected for two more reproductive cycles.

Lambing period were distributed from October to November. The following reproductive data were collected: estrus behavior resumption at male introduction, fertility and prolificacy, both related to the first heat and to the breeding season, lambing rate and twinning.

In E 1.1, during pregnancy, five ewes died after been exposed to Blue Tongue disease (2 ewes from G2h, 2 from G4h and 1 from G6h). In E 1.2 one ewe died during pregnancy [167][167][167][167][167]due to abortion and therefore its record was discarded from data analysis.

3.2.4. Measurements

In both experiments body weight, body condition score and milk yield were determined in all the ewes on Day 0 before ram introduction (the day after background treatment discontinuing), on Day 13, (three days before flushing start) and on Day 29. The ewes were weighted using an electronic scale before the morning meal. Body condition score [170] ranging from 1 (extremely thin) to 5 (obese) was estimated by two trained evaluators with an approximation of 0.25 BCS units. Their scores were averaged prior to data analysis. Milk yield was measured by weighting the production of each ewe in two consecutive milkings.

3.2.5. Statistical analyses

Body weight, BC and milk yield on each measurement date were analysed by a mono-factorial GLM. χ^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.

3.3. Results

3.3.1. Experiment 1 (E 1.1)

No significant differences between groups were found in terms of live weight (Figure 1), body condition (Figure 2) and milk yield (Figure 3) on Day 0, Day 13, and Day 29. As expected, milk yield dropped from Day 0 to Day 29, with no difference between treatment groups (Figure 3).

After rams' introduction, heat behavior was detected in 10 out of 12 ewes coming from G2h group, 9 out of 12 from G4h and 9 out of 12 from G6h with no significant differences between groups (Figure 4a). A trend in fertility related to the first heat was found, with 3 out of 10 ewes from G2h, 7 out of 10 from G4h and 7 out of 11 from G6h (p=0,07 between G2h and G6h) becoming pregnant at the first heat after ram's introduction and giving birth earlier. No differences were found in fertility related to the breeding season (three reproductive cycles with a total of 7 out of 10 from G2h, 8 out of 10 from G4h and 8 out of 11 from G6h (Figure 4a) giving birth.

Prolificacy was higher in G6h group compared to G2h one ($p<0.05$), both if related to the first heat (3/3 in G2h - 1 lamb/ewe, 11/7 in G4h - 1,6 lamb/ewe, 13/7 in G6h - 1,9 lamb/ewe; $p<0.05$ between G2h and G6h) and if related to the breeding season (10/7 in G2h - 1,4 lamb/ewe, 12/8 in G4h - 1,5 lamb/ewe, 15/8 in G6h - 1,9 lamb/ewe; $p<0.05$ between G2h and G6h – Figure 4b).

A positive trend was found increasing the time access to pasture from 2 to 6 hours/d for lambing concentration (3/7 in G2h, 7/8 in G4h, 7/8 in G6h, $p<0.07$ between G2h and G4h/G6h) and twin rate (3/7 in G2h, in 4/8 G4h and 7/8 in G6h $p<0,07$ between G2h and G6h – Figure 5).

Figures

Figure 1 - Body weight (BW) of late-lactating ewes at mating submitted to the residual effect of time restricted allocation to a ryegrass-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).

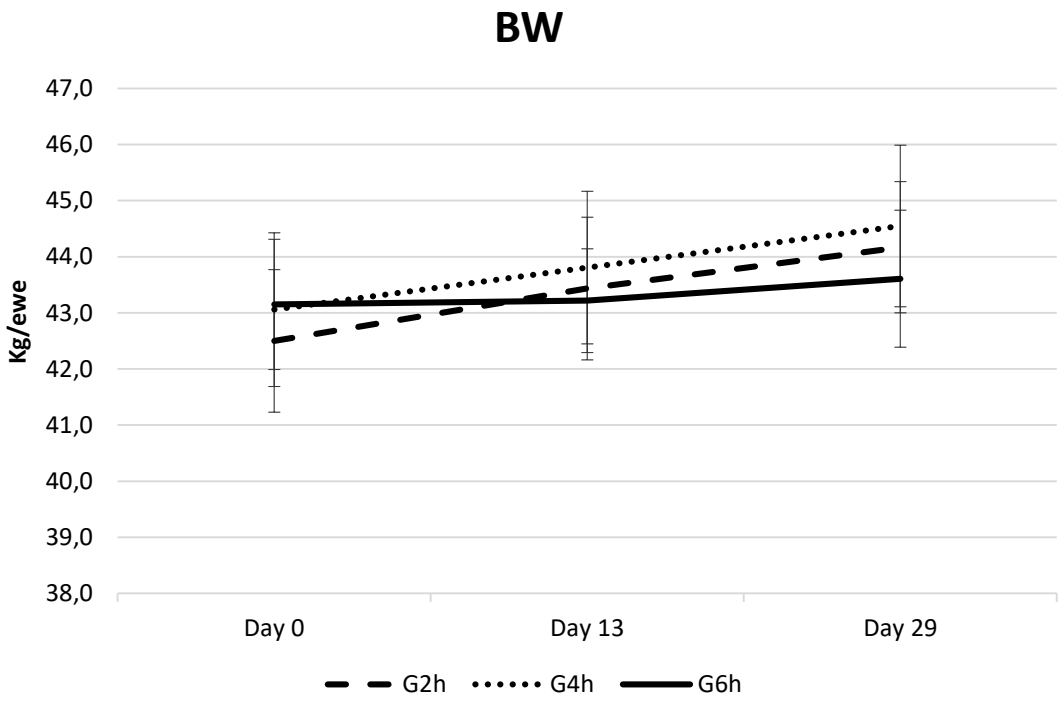


Figure 2 - Body condition score (BCS) of late-lactating ewes at mating submitted to the residual effect of time restricted allocation to a ryegrass-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).

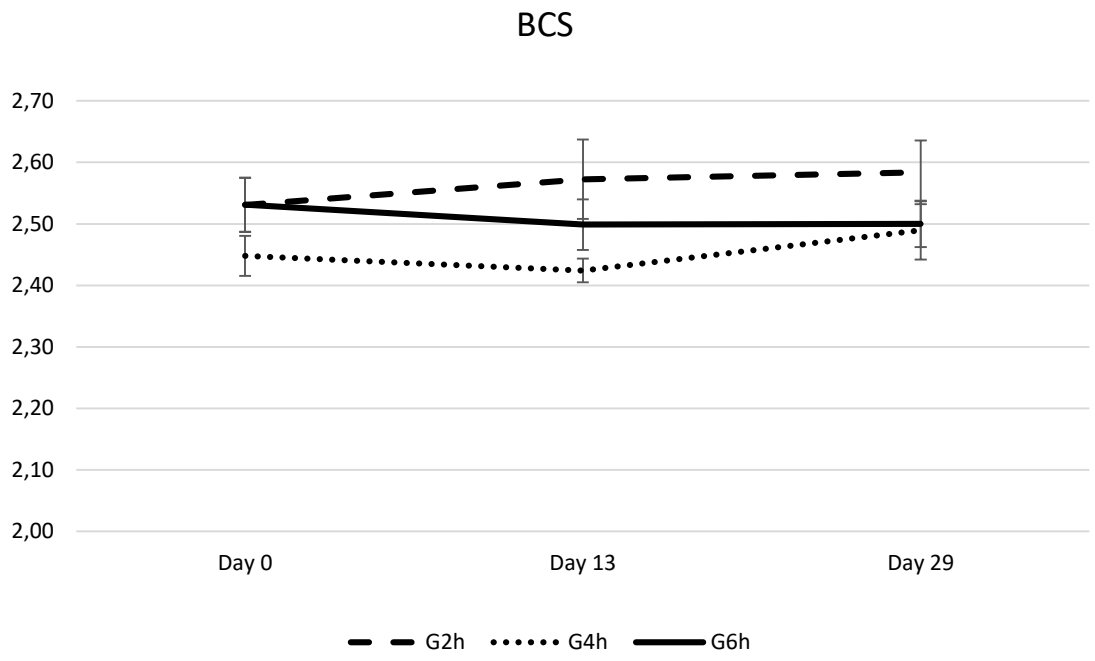


Figure 3 - Milk yield of late-lactating ewes at mating submitted to the residual effect of time restricted allocation to a ryegrass-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). Letters a, b indicate significant differences between Day 0 and Day 29 in all the groups ($p<0.01$).

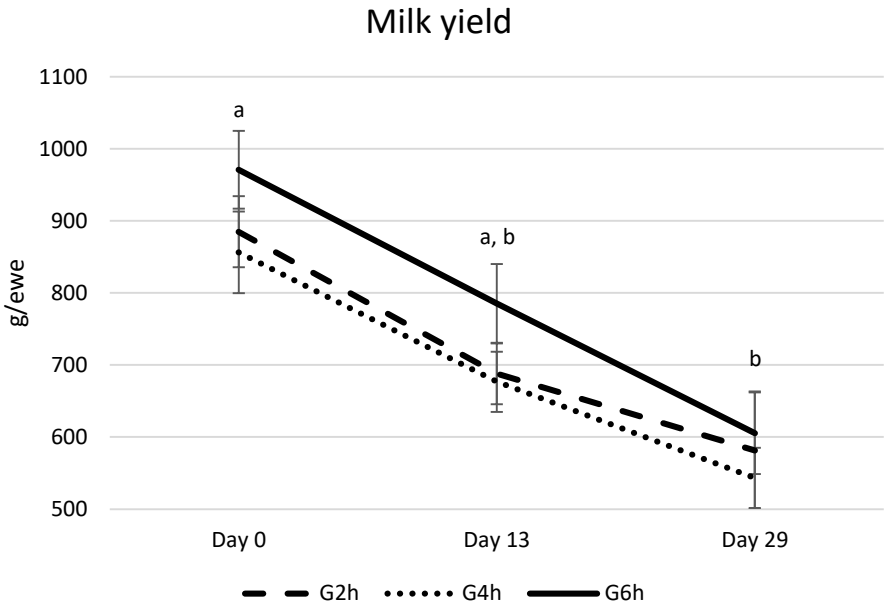
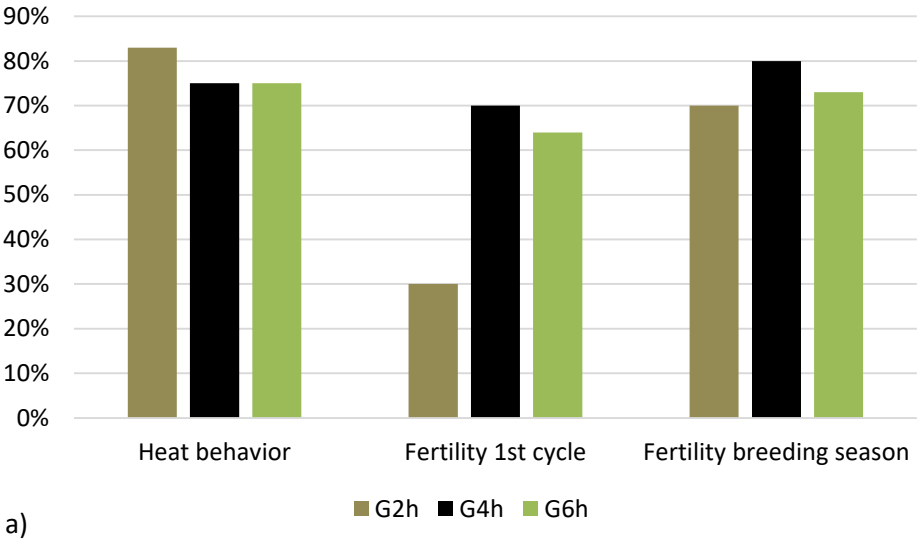
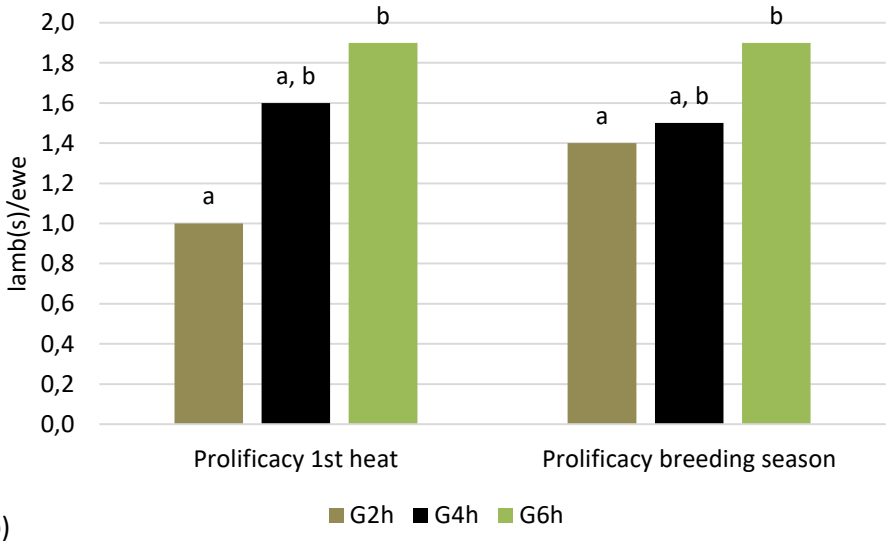


Figure 4 - Panel a) Heat behavior, fertility at the first heat (p=0.07 between G2h and G6h), and related the breeding season; Panel b) prolificacy related to the first heat (P<0.05 between G2h and G6h) and to the breeding season (P<0.05 between G2h and G6h) in late-lactating ewes submitted to the residual effect of time restricted allocation to a ryegrass-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).

a, b indicate significant variations within groups.

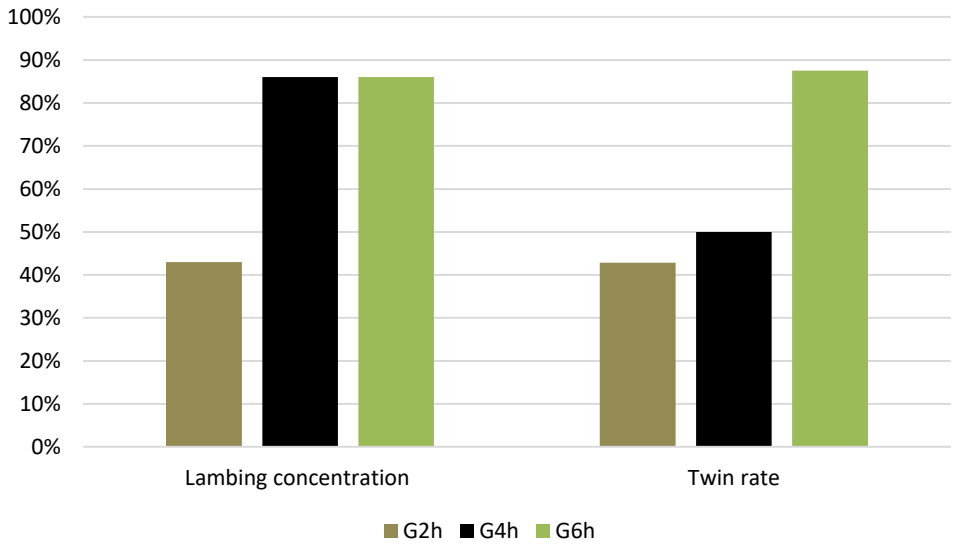


a)



b)

Figure 5 - Lambing concentration (p<0.07 between G2h and G4h/G6h) and twin rates (p<0.07 between G2h and G4h/G6h) in late-lactating ewes submitted to the residual effect of time restricted allocation to a ryegrass-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).



3.3.2. Experiment 2 (E 1.2)

When reproductive experiment began, at the end of April, the sheep sourced from the grazing background treatment with access to pasture restricted to 2h/d (G2h) showed lower BW ($P<0.06$ as compared with G4h/d and $P<0.11$ as compared with G6h – Figure 6) and BCS ($P=0.14$ as compared with G6h – Figure 7). During pre-flushing period and afterwards, until early pregnancy (Day 55), G2h group showed a progressive recovery in BW as compared to the others groups, with significant differences between groups only in the pre-flushing period (Day 0 – Day 13: 1.3 vs. – 0.3 and -0.4 kg per ewe in G2h, G4h and G6h, respectively $P<0.05$, – Figure 6). In a similar way, BCS showed a recovery trend, numerically higher in G2h than in the other groups ($P>0.15$ between groups – Figure 7).

Milk yield differed between treatments only on the first measurement date (day 0), with higher yields in the G4h than G2h treatment ($P < 0.05$), the G6h performing as intermediate (Figure 8)

After male introduction, during the first reproductive cycle, heat behavior was detected in 6 out of 12 ewes coming from G2h group, 10 out of 12 from G4h and 11 out of 11 from G6h with significant differences between G2h and G6h ($P<0.05$ between G2h-G6h – Figure 9/a). No differences between groups in terms of fertility were found. Fertility related to the first heat was 4 out of 6 ewes inG2h, 8 out of 10 in G4h and 9 out of 11 in G6h with no differences between groups (Figure 9/a) and fertility related to the breeding season (three reproductive cycles) was 10 out of 12 from G2h, 10 out of 12 from G4h and 11 out of 11 from G6h (Figure 9/a).

A trend in prolificacy related to the first heat was found with 5 lambs/4 ewes lambed in G2h (1.25 lamb/ewe), 11/8 in G4h (1.38 lamb/ewe) and 13/9 in G6h (1.44 lamb/ewe; p=0.063 between G2h and G6h– Figure 9/b). Prolificacy related to the breeding season was 13/10 (1.30 lamb/ewe) in G2h, 14/10 (1.40 lamb/ewe) in G4h and 17/11 (1.55 lamb/ewe) in G6h (Figure 9/b). Lambing concentration was 4/10 in G2h, 8/10 in G4h, 9/11 in G6h ($p<0.05$ between G2h-G6h – Figure 10). Twin rates were 3/10 in G2h, in 4/10 G4h and 6/11 in G6h (p=0.51 – Figure 10).

Figures

Figure 6 – E2 Body weight of late-lactating ewes at mating and in early pregnancy submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). All ewes were submitted to ram effect and flushing with lupin seed.

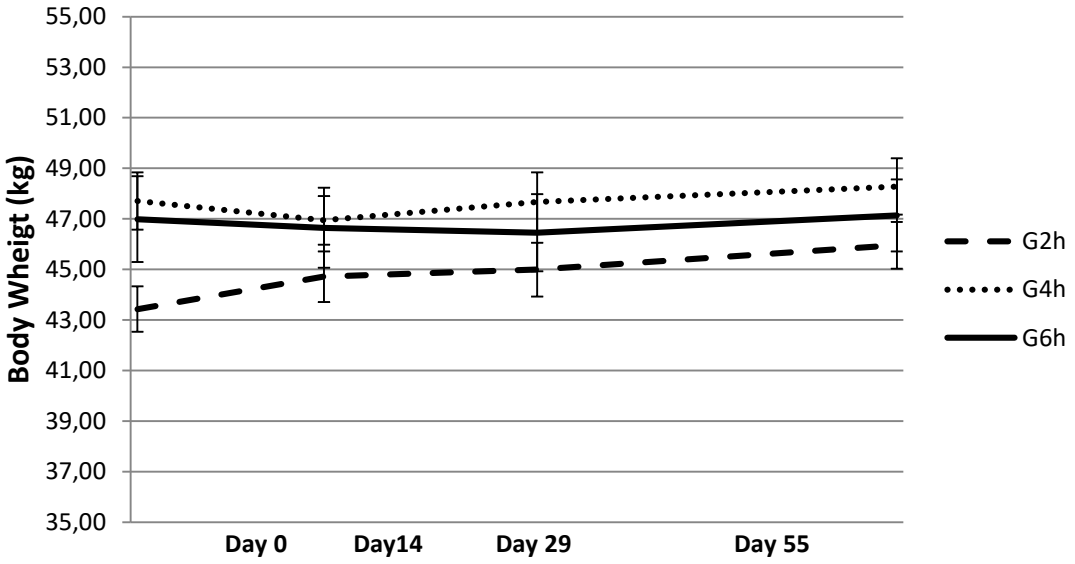


Figure 7. E2 Body condition score of late-lactating ewes at mating and in early pregnancy submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). All ewes were submitted to ram effect and flushing with lupin seed.

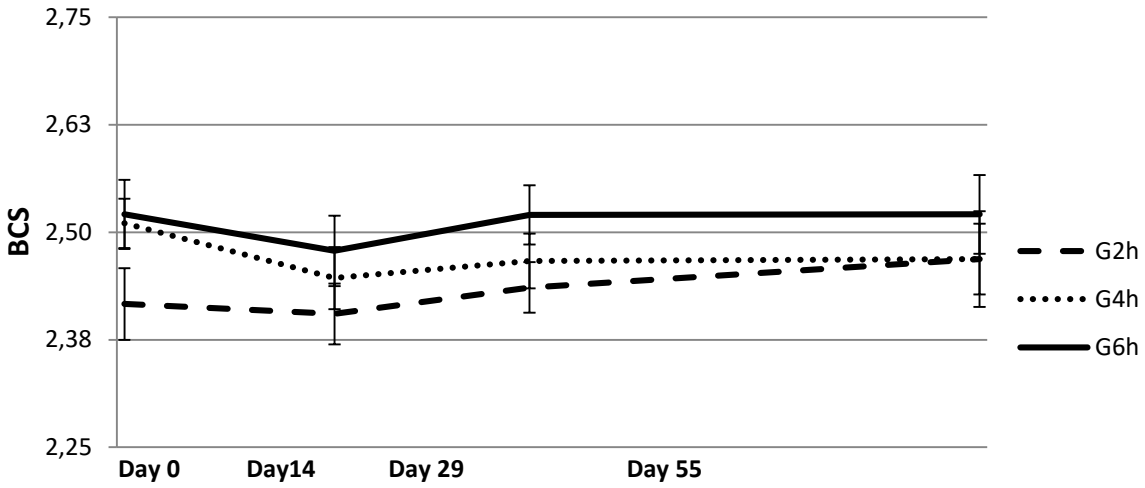


Figure 8. Milk yield determined on Day 0 before ram introduction (the day after background treatment discontinuing), on Day 13, (three days before flushing start) and on Day 29 in ewes submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).

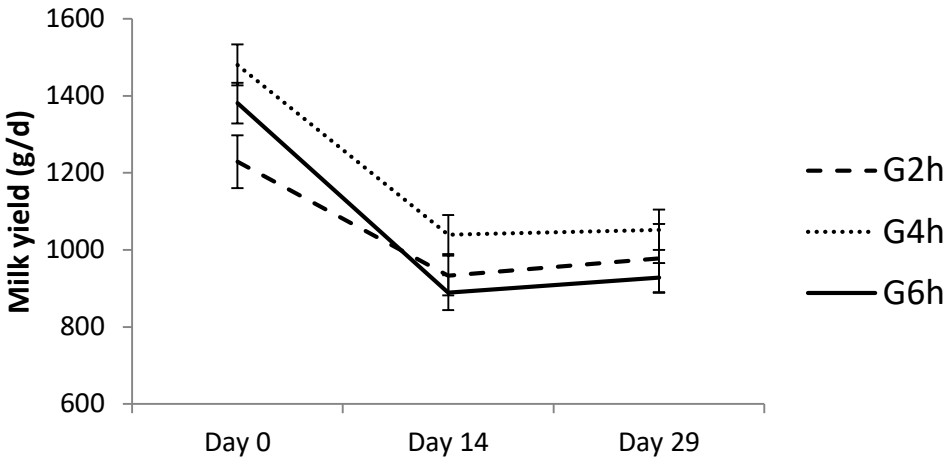


Figure 9 - Panel a) Heat behavior (p<0,05 between G2h and G6h), fertility at the first heat, and related the breeding season; Panel b) prolificacy related to the first heat (P<0.06 between G2h and G6h) and to the breeding season in late-lactating ewes submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h).

a, b indicate significant variations within groups.

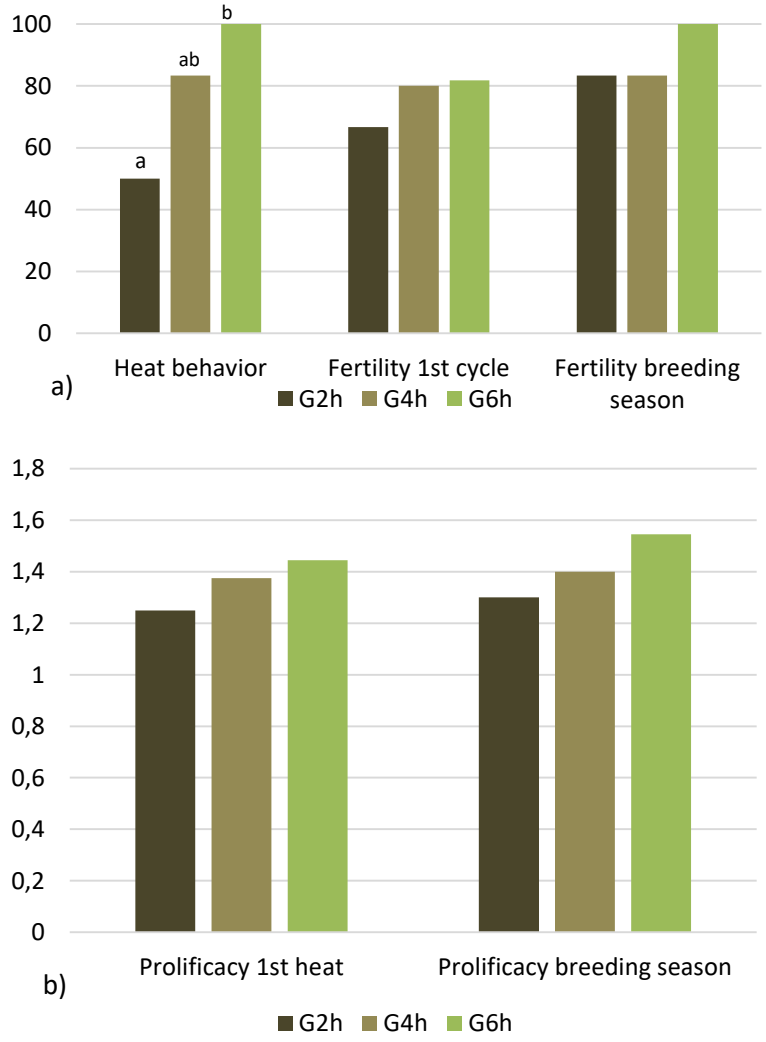
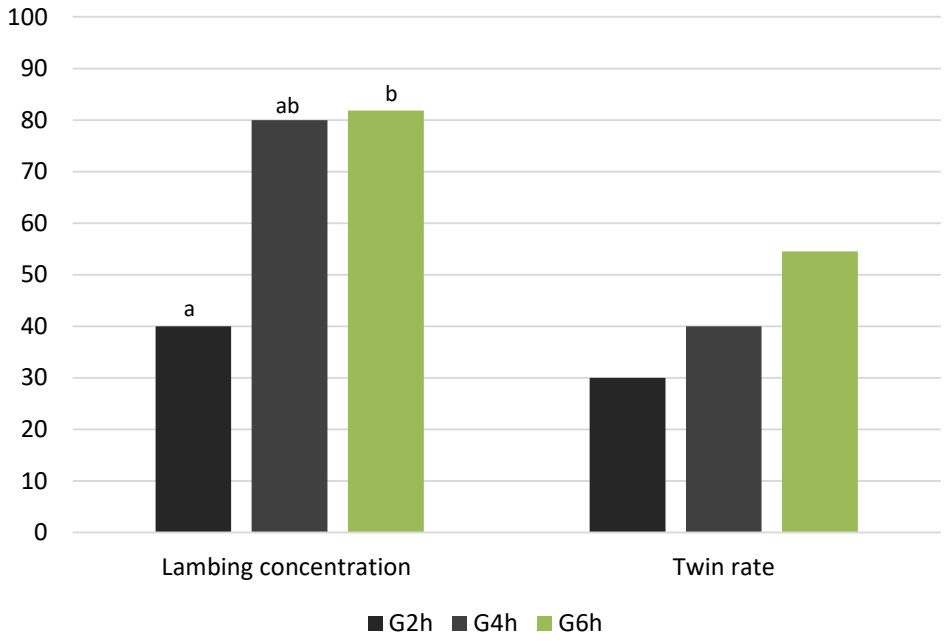


Figure 10. E2 - Lambing concentration ($p<0,05$ between G2h and G6h) and twin rates in late-lactating ewes at mating and in early pregnancy submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). All ewes were submitted to ram effect and flushing with lupin seed.

a, b indicate significant variations within groups.



3.4. Discussion

3.4.1. Experiment 1 (E 1.1)

Different time access to Italian ryegrass pasture in dairy sheep at mid-lactation stage did not affect behavioural oestrus resumption in the anoestrus period. In fact estrus onset were detected in the large part of the ewes in all the groups, according with expected consequence of rams effect [153]. Despite this, a trend towards a lower fertility related to the first heat was found for G2h as compared to G6h group. In the same way, lamb concentration and twin rates, also showed a negative trend for G2h. The metabolic consequences of dietary supplementation in sheep can depend on the energy balance condition. In fact, despite different studies indicate that supplementation with lupin grain for 4–6 days before ovulation increases ovulation rate [21, 171], in this experiment, the success of flushing with lupin grain, in terms of ovulation rates and then prolificacy, seems to be related to the nutrition management during lactation, as showed by the significant differences on prolificacy related to the first heat between G2h and G6h groups. It takes approximately 6 months from when follicles first commence growing to when one or more of these undergo final maturation and ovulate [16]. Restricting feed intake 6 months prior to ovulation, when those follicles destined to ovulate first commence growing, reduced ovulation rate [17]. That, has major implications for the reproductive performance of sheep [140]. The adoption of flushing before and during the mating period should result in a significant increase in ovulation rates [155], decrease of follicular atresia [30] and higher incidences of twin births [172]. A too restricted part-time grazing of Italian

ryegrass during mid-lactation (before mating period) has been proven to lead to low herbage and energy intake [141]. This underfeeding can have overridden the flushing treatment effect, explaining the negative trends of reproductive performance showed by G2h group as compared with the other background treatment groups, particularly in terms of prolificacy. In fact, energy intake is a key limiting factor of sheep production in animals raised at pasture, hence insufficient energy resulted in lowered reproductive performance [30]. Metabolic challenges, such as food deprivation or restriction, inhibit GnRH secretion into the pituitary portal circulation [143]. Metabolic signals are permissive for the neuroendocrine events that control ovulatory cycles and fertility. Nutritional deprivation of the female, whether as a result of an insufficient supply of energy in the diet, or a reduced availability because of excessive demands (processes such as lactation), inhibits hypothalamic GnRH release and thus leads to reduced secretion of pituitary LH and eventually to anovulation and anoestrus [148].

To conclude, these results overall suggest that a restriction of time access to pasture of Italian ryegrass during mid-lactation can subsequently impair the reproductive performance of late-lactation ewes, even if submitted to both male effect and flushing with lupin grains. In particular, a time allocation of 2h/d during mid-lactation can result in negative carry over effects on Sarda ewe reproductive performance, namely a trend to lower lambing concentration and lower prolificacy as compared with the 6 h/d treatment group.

3.4.2. Experiment 2 (E 1.2)

As described by Molle [142], during part-time grazing phase (background treatment period), when ewes were exposed to the direct effects of the restricted allocation time to pasture of berseem clover, Gh2 displayed a lower energy intake than Gh4 and Gh6, which resulted in lower milk yield during mid-lactation period (March to end of April) and a trend to lower recovery in BW and BCS. An inadequate nutrition due to a severe time restriction to pasture in ewes in mid lactation, besides directly impacting on nutrient intake and milk performance, can exert detrimental residual effects on the response to male effect and flushing with lupin. This had already been showed in the previous experiment (E 1.1) carried out on Sarda ewes in mid-lactation part-time grazing Italian ryegrass for 2 h/d. In that case, G2h group exhibited a significant reduction of prolificacy related to first heat and to the breeding season and a negative trend on lamb concentration and twin rates [173]. Also in this experiment (E 1.2), the access to pasture of berseem clover for a duration of 6 h/d during mid-lactation had a positive impact on subsequent reproductive performance as compared with the shortest time access (2 h/d). The residual effect was evidenced by a prompter resumption of behavioral estrus, followed by a higher concentration of lambing and a trend towards higher prolificacy as consequence of the expected acute effect by supplying nutritional inputs for 5 days [20, 174]. In fact, among adult ewes, undernutrition may alter the resumption of ovary cyclicity and the expression of sexual behaviour [144, 145]. This can depend to a specific behavioural response or to a reduction in GnRH pulse frequency that prevents the cascade of events leading to

ovulation: these same events are necessary for the induction of behavioural oestrus [146]. In this experiment, after ram introduction, during the first reproductive cycle, heat behavior was detected in 6 out of 12 ewes coming from G2h group and 11 out of 11 from G6h ($p<0.05$ between G2h-G6h). It seems that the proportion of ewes continuing to ovulate and displaying oestrus following ram introduction in spring appears to reflect BW changes over the previous winter rather than the BW at the time the rams were introduced [21].

Despite the trend found in the previous experiment, fertility was not affected by the treatment. On the other hand, prolificacy related to the first heat showed a positive trend in G6h respect G2h groups, while prolificacy related to the whole breeding season showed a recovery in G2h.

These results overall confirm those of the previous experiment, suggesting that a restriction of time access to pasture during mid-lactation, besides constraining intake and productive performance in mid lactation, can subsequently impair the reproductive performance of late-lactation sheep, even if submitted to both male effect and flushing with lupin. This happens despite a late recovery of body weight and condition score during pre-flushing period and afterwards until early pregnancy, in the ewes submitted to the most severe restriction of time at pasture during mid-lactation..

It is noteworthy that in this experiment milk yield was particularly high during mating, therefore the energy demand of the ewes was particularly high, which can explain why in E 1.2 even cycle resumption was negatively affected by the feeding restriction during mid lactation. In other words in E 1.2 more than in E 1.1, the lactation anoestrus

contributed to constrain the expression of estrus behaviour after submission to male effect, particularly in the G2h group.

3.5. Conclusion

The first experiment underlined that background grazing during lactation can exert carry-over effects on ewe's reproductive performances, modulating the response to a short-term flushing and to the male effect, even if mating is prepared by a flushing treatment. The variability of the response at focused nutritional treatments appear to find its cause on historical nutritional plans applied previously to the mating season, and possibly related to ewe's physiological status. Ewes mated in late anoestrus with severely restricted access to pasture during mid lactation are unable to adequately respond to a short-term flushing and male effects. Although different studies indicate that supplementation with lupin grain for 4–6 days before ovulation increases ovulation rate [21, 171], in Study 1, the success of flushing with lupin grain, in terms of ovulation rates and then prolificacy, seems to be related to nutrition management during lactation. A severe time restriction to pasture in ewes in mid lactation can directly affect nutrient intake and milk performance and then exert a detrimental residual effect on the response to male effect.

Sheep that grazed for 2 h/d in Italian ryegrass, compared with sheep that grazed for 6 h/d, exhibited a significant reduction of prolificacy related to first heat and to the breeding season and a negative trend on lamb concentration and twin rates. The same restriction on berseem clover, showed a residual effect evidenced by a lower

resumption of behavioral estrus, followed by a negative concentration of lambing and a negative trend in prolificacy.

4. STUDY 2

4. STUDY 2

4.1. Introduction

Farmed animal feed prices and availability have led researches to look for alternatives to use. Byroducts and co-products from different agriculture industries have been considered, such as distilleries and biodiesel production plants [175]. In the case of biodiesel production, vegetable oils, produced from oilseed crops, are subjected to a transesterification process, using methanol or ethanol in the presence of a catalyst. The major by-product of this industry is glycerine and the rapid expansion of biodiesel production has led to increase its availability, creating a surplus [176]. As matter of fact, the glycerine refining market produced about 2 billion kg of refined glycerine worldwide [177]. This increase in availability drove prices downward and contributed to glycerine excess, which may be used for other purposes, such as animal feeding [178, 179].

Glycerine, also known as glycerin and glycerol or as propane-1,2,3-triol, 1,2,3-propanetriol, 1,2,3-trihydroxypropane, glyceritol, and glycyl alcohol, is a colourless, odourless, hygroscopic, and sweet-tasting viscous liquid. It is a sugar alcohol with a high solubility index in water. There are a wide range of applications for glycerol in the food, pharmaceutical, and cosmetic industries. Glycerol has been used in the food industry with the property to enhance the water-holding capacity [180]. The attribute of being sweet tasting so it is well accepted by animals [181]. Thus, glycerol has been used in dairy cows during early lactation [40, 182, 183] or mid-lactation [178, 184] to

ensure energy balance during the transition period as an additional glucose precursor [182], because of its gluconeogenic properties [178].

Different studies have examined administration strategies: top-dressed or mixed into the total mixed ration [40, 182, 183], within the concentrate ration [184, 185] or by oral gavage using an esophageal feeding tube [27].

In general, glucogenic substrates are glucose precursor which improve energy balance [185, 186]. In ruminants, consumed glycerol can have different fates: fermented in the rumen, absorbed across the rumen epithelium or escape the rumen by outflow through the reticulo-omasal orifice. Glycerol escaping the rumen may be absorbed from the intestine likewise in monogastric species [187]. Omazic [188] found that approximately 25% of the glycerol entering the rumen was fermented, while approximately 45% was absorbed from the rumen, very likely by passive diffusion via the cellular pathway. The remaining 30% of the glycerol escaped through the reticulo-omasal orifice to be absorbed by the small intestine. Those results indicated that approximately 75% of the glycerol escaped rumen fermentation to reach the circulation and to become an available gluconeogenic substrate.

The direct uptake of glucose from the diet is usually limited as its main source is the liver *via* gluconeogenesis based on the metabolism of rumen-derived volatile fatty acids (mainly propionic acid) and amino acids. The administration of glycerol can increase total volatile fatty acids, because of the rumen microbiome metabolism [189], reducing the acetate to propionate ratio [190]. In fact, the proportion of propionate (which is a glucose precursor) generally increases at the expense of acetate when diets

are supplemented with glycerol [191-193]. Absorbed glycerol is channelled by the liver to triose phosphate and then further to glucose via gluconeogenesis, or it is catabolised via glycolysis [194, 195].

Feeding strategies had implemented the use of glycerol as primary feed ingredient to replace corn grains in rations in dairy cows [178]. In dairy cows, up to 15% of the total of dry matter (DM) can be replaced by high purity glycerine without causing negative effects on feed intake, milk production or milk composition [178]. Other studies have been focussed on feed lot diets offered to beef cattle with glycerol provided for short time and high doses [196-198]. Compound such as propylene glycol and glycerol and their mixture have been used to increase blood glucose and to reduce nutritional problems in dairy cows during the peripartum (transition) phase [37-39]. It has been reported that feeding glycerol as a top dress [37, 40] or supplied in water [39] to transition dairy cows resulted in a positive energy status with higher concentrations of serum glucose and lower concentrations of plasma non-esterified fatty acids (NEFA) [38].

Glucogenic precursors have been used in veterinary practice not only to increase energy balance but also to optimize reproductive performance. As matter of fact, glucose is one of the key nutrients having an effect on the ovary [24, 32], since it has distinct roles in follicular function: first as a nutrient to generate ATP, and second as a signalling molecule to stimulate folliculogenesis when nutritional conditions are favourable to reproduction [34]. The role of glucose is also essential in determining the quality of the oocyte [35]. That was showed in a previous experiment on non-lactating

dairy sheep, were our research group found that short-term flushing with a glucogenic mixture based on glycerol and propylene glycol improves oocyte quality, evaluated by the kinetics of their *in vitro* development and by the production of blastocysts [27]. In another study, the same nutritional treatment increased the ovulation rate [36].

Starting from these premises, two experiments were designed to evaluate the effect of a short-term administration of a glucogenic mixture:

- **Experiment 1 (E 2.1)** - was performed in dry ewes and aimed at assessing the effect of a short-term administration of a glucogenic mixture on increasing the plasmatic and intrafollicular concentrations of metabolites and hormones having an effect on the follicle;
- **Experiment 2 (E 2.2)** - aimed at investigating whether the glucogenic mixture administration in lactating dairy ewes could create the same metabolic and hormonal milieu observed in dry ewes, and at evaluating the effects of glucogenic treatment on food intake, milk yield, milk composition and reproductive performances.

4.2. Experiment 1 (E 2.1)

4.2.1. Aim

The ovary uses glucose as its principal source of energy and its well described positive effects on fertility have been related to its properties as a metabolic fuel [65]. Glucose can be metabolized within the follicle by the pentose phosphate pathway to provide precursors for the synthesis of purine nucleotides and NADPH, which are in turn used in various biosynthetic pathways, including those related with the antioxidant defence. Hence glucose follicular fluid (FF) concentration can also influence follicular oxidative status [41].

The physiological link between energy intake and folliculogenesis most probably involves several metabolic hormones and growth factors including insulin and insulin-like growth factor 1 (IGF-1). 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 [199]. IGF-1, which is a hormone playing a key role in the energy status and function of the ovary, and gonadotropins are synergistic for growth and differentiation of the follicle [48, 49]. The IGF system (receptors, ligands, and binding proteins) is expressed within granulosa and theca cells [48-50].

This experiment aimed at assessing the effect of a short-term administration of a glucogenic mixture on increasing the plasmatic and intrafollicular concentrations of metabolites and hormones which play a key role in follicular maturation and quality. In

particular, we measured plasma levels of glucose, insulin, IGF-1, NEFA, and urea during the nutritional treatment and their corresponding levels in the FF as measured 12 h after the last administration of the glucogenic mixture. At the same time, the oxidative status of the FF was also investigated.

Finally, to assess possible changes in IGF-I bioavailability in the FF, the concentration of pregnancy-associated plasma protein A (PAPP-A), a protease regulating the levels of free IGF-1 in dominant follicles [200, 201] were determined in the FF.

This information is pivotal to better explain the effect of a rise in glycemia on the endocrine and metabolic milieu of sheep at mating and to set the basis for the formulation of short-term flushing treatment able to create the best conditions for the conception period.

4.2.2. Materials and methods

The experiment was run at the experimental facilities of the Department of Veterinary Medicine at the University of Sassari, Italy (40°43'40.33"N, 8°33'1.33"E). The experimental procedures with animals were approved by the Animal Care and Use Committee of the University of Sassari.

The ewes were penned outdoor with access to a sheltered area. These facilities meet the requirements of the European Union for Scientific Procedure Establishments. The ewes were group fed a maintenance ration at a level of 46 g of dry matter per kg of metabolic weight ($BW^{0.75}$) consisting of hay and concentrate fed twice daily. The

experiment was run during October 2014, within the natural breeding season (late August – late December) described for this breed at this latitude.

4.2.2.1. Animals and treatments

Twenty Sarda ewes, 4-5 years old, were used. In brief (Figure 1), synchronization was induced in all the animals with the insertion of one intravaginal progestagen-impregnated sponge (45 mg fluorogestone acetate, FGA, Chronogest; Intervet International, Boxmeer, The Netherlands) which remained *in situ* for 6 d. On the day of sponge withdrawal (day 0), the ewes received 125 µg of a prostaglandin analogue (cloprostenol, Estrumate™, Essex Animal Health, Friesoythe, Germany) by i.m. injection. At the same time, ewe live weight (42.3 ± 0.9 kg) was determined. On day 0 the ewes were divided in two experimental groups at random. From day 7 to day 10 after sponge withdrawal, one group (GLU: n=10; weight 42.2 ± 1.3 kg;) received, orally twice daily at 8.00 a.m and at 19.00 p.m., 200 mL of a glucogenic mixture, as previously described [36]. The glucogenic formulation contained 70% glycerol and 20% propylene glycol (both from Sigma Chemical Co., St. Louis, MO, USA) and 10% water. The control animals (WAT: n=10; weight 42.3 ± 1.3 kg;) received 200 mL of water twice daily simultaneously to treatment administration. Both the glucogenic formulation and the water were administered by oral gavage using an esophageal feeding tube.

From day 8 to 10, follicular development was stimulated in all the ewes by the administration of 175 IU of FSH (Folltropin; Bioniche Animal Health, Bio 98, Milano, Italy) given every 12 h in six equal doses.

At day 11, 12 h after the last FSH administration, the ewes were weighed and then conducted to the slaughterhouse where they were sacrificed. After having collected the ovaries, follicles and corpora lutea on their surface were counted and FF from follicles ≥ 4 mm was aspirated with a 2.5 mL syringe fitted with a 22-gauge needle.

4.2.2.2. Blood and FF sampling

Plasma concentrations of glucose, NEFA, urea, insulin, IGF-1 and progesterone (P4) were determined from samples drawn from jugular vein at 8.00 a.m from day 6 to day 11. Glucose, insulin, NEFA and urea plasma concentrations were also determined in three further samples collected every 30 min starting from the time of morning administration of glucogenic mixture (8.30, 9.00 and 9.30 a.m.) from day 7 to day 10. At each time point and 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 and centrifuged at 1500g for 15 min. Plasma was removed and stored at -20°C until assayed.

Follicular fluid concentrations of metabolites (glucose, NEFA and urea), hormones (insulin and IGF-1) and pregnancy-associated plasma protein A (PAPP-A) were determined on day 11. At the same time, to evaluate FF oxidative status, glutathione (GSH) and Trolox equivalent antioxidant capacity (TEAC) were assayed. Given the small

volume recovered from a single follicle, to allow the determination of the above mentioned metabolites and hormones, the FF collected from the ovaries of a single ewe was immediately pooled and stored in Eppendorf tubes at -20°C until assayed.

4.2.2.3. Determination of metabolite concentrations in plasma and follicular liquid samples

Both FF and plasma samples were measured in duplicate.

Glucose, NEFA and urea were measured using commercial kit and BS-200 Mindray clinical chemistry analyzer. We used Serum I Normal (Wako) and Serum II Abnormal (Wako) 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 were 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.

4.2.2.4. Hormone analyses

All assays were ELISA assays and were performed using the Personal Lab Adaltis (Adaltissrl, Rome, Italy), which is a tool that performs automated ELISA protocols.

Progesterone concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany), which is a solid-phase ELISA, based on the principle of competitive binding. All kit reagents, controls and stored samples to be analysed were thawed and warmed to 25°C at the beginning of the test. The analytical sensitivity was 0.045 ng/mL and the intra- and inter-assay CV values were <10%.

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 [202, 203]. 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%.

IGF-1 concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany) which is a solid-phase ELISA based on the principle of competitive binding. The IGF1 assay kit used in the present experiment has been previously used in goats [204] and sheep [205]. The analytical sensitivity was 9.75 ng/mL and the intra- and inter-assay CV values were <8%. Before being assayed,

IGF-1 was extracted following the assay procedure of the ELISA kit. Briefly, 50 µL of sample and standard solution were added to 50 µL of 0.2 N HCL. After 30 min incubation, the sample and standard solutions were neutralized with 10 µL neutralization buffer and immediately assayed. Efficiency was calculated as recovery percentage. Samples were spiked by adding IGF-1 solution with known concentrations in a 1:1 ratio. The recovery (%) was calculated by multiplying the ratio of the measurements and the expected values with 100. The recovery upon addition was 86-126% (mean 102%).

Pregnancy-associated plasma protein A concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany) which is a solid-phase ELISA based on the sandwich principle. The analytical sensitivity was 0.133 µg/mL and CV values were <7%. Our PAPP-A concentrations are in accordance with those already reported in literature using RIA Kits [206, 207].

4.2.2.5. Oxidative status determination in FF

4.2.2.5.1. Glutathione

GSH was measured after derivatization with N-ethylmaleimide (NEM) as described by Moore et al. [208]. A liquid chromatographic system with triple quadrupole mass spectrometry detection (LC-MS/MS) Varian 310-MS (Varian, Palo Alto, CA, USA) was used. GSH was quantified in follicular fluid using thiosalicylic acid (TSA) as an internal standard (IS) (Table 1). Chromatographic separation was achieved on a LUNA C18 column (5 µm, 100 × 2.1 mm Phenomenex, Bologna, Italy) fitted with a Phenomenex

C18 security guard cartridge (4 × 2.0 mm ID). A linear gradient with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed as follows: 1 min at 5% B; in 0.5 min solvent B was increased from 5 to 97%, and in another 1.5 min it was decreased to 95%. Then, in 0.5 min solvent B was decreased from 95% to 5% and remained constant for 2.5 min. The total run time was 6 min. The electrospray interface operated in positive mode with the following conditions: capillary voltage, 5000 V; drying gas temperature, 200 °C; nebulizer gas pressure, 50 psi; electron multiplier voltage, 1795 V. The collision gas used was argon with a pressure of 2mTorr. The transitions related to free GSH were also monitored in order to verify the complete derivatization of GSH with NEM, and none was detected. Individual parameters for GSH-NEM, IS and GSH are listed in table 1. The values of GSH in the samples were calculated using a standard curve built in 15% methanol (range from 10 to 5000 ng/mL) and expressed as µM[208, 209]. GSH content was normalized for protein concentration. Total proteins in FF 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.

4.2.2.5.2. Trolox equivalent antioxidant capacity (TEAC)

Plasma TEAC was determined as described by Re et al. [210] and modified by Lewinska et al. [211]. Briefly, a fresh solution was prepared by dissolving 19.5 mg 2,20-azinobis 3- ethylbenzthiazoline -6-sulphonic acid (ABTS) and 3.3 mg potassium persulphate in 7 mL of 0.1 mol/L phosphate buffer, pH 7.4. This solution was stored in the dark for 12

hours for completing the reaction. ABTS solution was diluted in 0.1 mol/L phosphate buffer, pH 7.4, to give an absorbance reading at 734 nm of 1.0. The absorbance of the mixture was measured twice in a spectrophotometer (ThermoElecrom Corporation Genesys 10 UV, Madison, Wisconsin, USA), at 734 nm, 3 min after mixing a sample with the ABTS solution. The extent of ABTS bleaching (decrease in absorbance) is proportional to the activity of antioxidants in a given sample. The antioxidant capacity was expressed as TEAC, that is the concentration of trolox producing the same effect as the sample studied. The values of TEAC in the samples were expressed as mM Trolox equivalent normalized for volume of FF and were calculated using trolox for a standard curve.

4.2.2.6. Statistical analyses

Live weight at the beginning and end of the glucogenic-treatment period and their changes were analysed by a mono-factorial ANOVA.

Circulating and intra-follicular concentrations of analysed metabolites and hormones on day 6 and day 11 were analysed by GLM with treatment as fixed effect. Longitudinal data of plasma glucose, insulin, NEFA and urea in the samples collected from day 7 to day 10 (during treatment period) were analysed by a mixed model for repeated measurements (PROC MIXED in SAS Version 8, SAS Institute Inc., Cary, NC, USA) with treatment, sampling day, sampling hour and their first-order interactions as fixed effects and sheep as random effect. In addition, to compare the composition of blood plasma and FF on the last sampling date (day 11) a GLM model was used with

treatment, source of sampling and their interaction as fixed effects. Finally, on the same sample, the relationship between the concentration of metabolites and hormones in blood and FF was evaluated by correlation analysis using Pearson's correlation coefficients.

All results were expressed as mean ± SEM and a probability of $P < 0.05$ was considered to be significant.

4.2.3. Results

No differences were observed in live weight between groups ($P = 0.98$) at the beginning (day 0) and the end (day 11) of the nutritional treatment (live weight, 42.1 ± 1.3 vs 42.8 ± 1.3 kg in WAT and GLU groups, respectively).

No difference between groups was observed in ovarian follicular population, as evaluated one day after the end of the nutritional treatment (day 11; Figure 2).

4.2.3.1. Effect of short-term glucogenic treatment on the concentrations of plasma metabolites and hormones

Before the beginning of the treatment (day 0), circulating concentrations of analysed metabolites and hormones were within the physiological ranges [212] in all the ewes and showed no differences between GLU and WAT group (table 2).

Progesterone plasma levels rose in both experimental groups during the experimental period ($P < 0.0001$, Figure 3), with no differences ($P = 0.184$) between groups (GLU 3.6 ± 0.4 ng/mL; WAT 4.5 ± 0.4 ng/mL).

Short-term administration of the glucogenic mixture had an immediate effect on glycaemia (Figure 4A) and insulinemia (Figure 4B). Glucose rose significantly in GLU group, being higher than the control both during the 4 d of glucogenic mixture administration ($P < 0.0001$; GLU 79.3 ± 1.9 mg/dL; WAT 64.1 ± 1.9 mg/dL) and in the day after the end of the treatment (day 11: $P < 0.05$; GLU 98.9 ± 13.1 mg/dL; WAT 69.3 ± 3.3 mg/dL). This fast response became evident from 60 min after the administration of the glucogenic mixture in the GLU group. In the same group, as a consequence of the high glucose plasma concentration, a parallel increase in insulinemia was detected from the second day of nutritional treatment. As for glycaemia, insulin concentrations were significantly higher in GLU than in WAT group both during the nutritional treatment (GLU 0.82 ± 0.07µg/L; WAT 0.13 ± 0.07µg/L; $P < 0.0001$), and in the day after its end (day 11: $P < 0.05$; GLU 0.4 ± 0.09 µg/L; WAT 0.1 ± 0.01 µg/L).

The circulating concentration of IGF-1 (Figure 5) was significantly higher in GLU compared to WAT group (90.7 ± 2.8 ng/mL vs 77.3 ± 2.8; $P < 0.01$). The rise in IGF-1 circulating concentration was slower compared to what observed for insulin, being detectable from the 3rd d of nutritional treatment and reaching its highest values on the day after its end.

The glucogenic mixture administration caused a sharp drop in NEFA plasma levels in the GLU group (Figure 4C) detectable soon after 60 min from the first glucogenic dose.

Subsequently, NEFA mean circulating concentrations remained stably lower in GLU than in WAT group both during the treatment period (GLU 0.04 ± 0.02 mmol/L; WAT 0.20 ± 0.02 mmol/L; $P < 0.0001$), and after its end (day 11; GLU 0.02 ± 0.01mmol/L, WAT 0.40 ± 0.08mmol/L, $P < 0.001$).

The same pattern was observed in the concentration of urea in blood plasma (Figure 4D). The nutritional treatment caused a significant drop in urea concentration in GLU group as compared to the WAT one. This effect was found during the treatment period (GLU 15.7 ± 0.84 mg/dL; WAT 22.2 ± 0.84 mg/dL; $P < 0.0001$) and after its end (day 11; GLU 12.4 ± 1.7mg/dL; WAT 29.7 ± 3.1 mg/dL; $P < 0.0001$). In this case, however, the response was delayed and became evident starting from the 2nd day of the treatment.

4.2.3.2. Effect of short-term glucogenic treatment on the concentrations of metabolites and hormones in the follicular fluid

The glucogenic supply significantly affected FF concentrations of glucose, insulin, IGF-1, NEFA and urea, as evaluated 12 h after the end of the nutritional treatment (day 11). The observed changes in FF composition only partly mirrored those observed in the plasma. In particular, in the GLU group the glucogenic mixture administration resulted in higher intra-follicular glucose concentrations as compared to those in WAT one ($P < 0.0001$; Figure 6A). However, in both groups FF glucose concentrations were significantly lower as compared to the corresponding circulating levels ($P < 0.01$).

Moreover, the higher intra-follicular glucose concentration found in the GLU group compared to WAT one was not accompanied by any increase in intra-follicular insulin

concentration. On the contrary, while in WAT group no difference was observed between circulating and intra-follicular insulin concentrations, in GLU group intra-follicular insulin concentrations (Figure 6B) were significantly lower than circulating ones ($P < 0.0001$). In other words, as confirmed by the significant interaction found between treatment and sample source (blood or FF) for insulin ($P < 0.01$), circulating and FF insulin levels varied differently in GLU and WAT group. Likewise, the higher circulating IGF-1 concentrations (Figure 6C) in GLU group was not accompanied by significantly higher intra-follicular concentrations in GLU than WAT ewes. However, in both groups, plasma levels of IGF-1 were higher than intra-follicular ones ($P < 0.01$).

Changes in circulating concentrations of NEFA (Figure 6D) and urea (Figure 6E) were mirrored by changes in their follicular fluid concentrations. In particular, in the GLU group a drop in mean FF NEFA (GLU 0.217 ± 0.027 mmol/L; WAT 0.360 ± 0.034 mmol/L; $P < 0.0001$) and urea (GLU 11.52 ± 1.94 mg/dL; WAT 26.96 ± 2.82 mg/dL; $P < 0.0001$) levels were observed. However, FF NEFA concentrations were higher than circulating ones only in GLU group ($P < 0.05$) with a significant interaction between group and sample source ($P < 0.05$).

No significant difference between groups was found in FF concentrations of PAPP-A, GSH, and in its total anti-oxidant capacity (Figure 7).

Correlations between plasma and intra-follicular concentrations of metabolites and hormones are shown in table 3. Presented results show that the increase in circulating glucose concentration is accompanied by a parallel increase in its follicular concentration, in circulating insulin, and in circulating and follicular IGF-1. In addition,

insulin is negatively correlated with circulating NEFA and urea, and positively correlated with IGF-1 concentrations both in the plasma and in the follicle. NEFA and urea show a strong positive correlation both in their plasmatic and follicular concentrations.

Tables

Table 1 - Liquid chromatography–tandem mass spectrometry parameters.

	RT (min)$_a$	Capillary(V)	MRM transitions (*m/z*)$_b$	CE (eV)$_c$
GSH	2.3	50	308.3→ 179.1	-10
			308.3→ 161.9	-14
GSH-NEM	3.1	32	433.4→ 304.0	-11.5
			433.4→ 286.9	-16.5
IS	3.4	30	280.4→ 262	-7.5
			280.4→ 163.3	-20

$_a$RT retention time, $_b$MRM multiple reaction monitoring, $_c$CE collision energy

Table 2 - Circulating concentration of glucose, insulin, IGF-1, NEFA and urea in Sarda ewes before starting a 4-days nutritional treatment with a glycogenic mixture (day 0). No significant differences were found between groups. Values are expressed as means ± S.E.

	Control group (n=10)	Treated group (n=10)
Glucose (mg/dL)	50.8±2.7	51.9 ± 2.28
Insulin (µg/L)	0.095±0.008	0.08±0.02
IGF-1 (ng/mL)	82.6 ±4.1	74.1 ±3.8
NEFA (mmol/L)	0.26±0.04	0.28±0.03
Urea (mg/dL)	26.1±2.9	29.3 ± 3.8

Table 3 - Pearson correlation coefficients between plasma and follicular concentrations of glucose, insulin, NEFA, IGF-1, and urea as evaluated 12 hrs after the end of a 4-days glycogenic mixtures administration in treated (n=10) and control ewes (n=10). Corresponding *P*-values are shown within parenthesis.

		Glucose		Insulin		NEFA		IGF-1		Urea	
		Plasma	Follicle	Plasma	Follicle	Plasma	Follicle	Plasma	Follicle	Plasma	Follicle
Glucose	Plasma		0.8151	0.7815	- 0.0918	-0.3028	-0.143	0.7032	0.7458	-0.3096	-0.1589
			(0.0001)	(0.0001)	(0.7171)	(0.2219)	(0.5713)	(0.0011)	(0.0004)	(0.2112)	(0.5288)
	Follicle	0.8151		0.7139	-0.4655	-0.3753	-0.231	0.4962	0.5519	-0.4113	-0.3856
		(0.0001)		(0.0009)	(0.0515)	(0.1249)	(0.3563)	(0.0362)	(0.0176)	(0.0899)	(0.114)
Insulin	Plasma	0.7815	0.7139		-0.0546	-0.5353	-0.3583	0.5853	0.6294	-0.5486	-0.4171
		(0.0001)	(0.0009)		(0.8297)	(0.0221)	(0.1443)	(0.0107)	(0.0051)	(0.0184)	(0.085)
	Follicle	-0.0918	-0.4655	-0.0546		0.0765	0.1451	0.2006	0.0225	0.2692	0.3622
		(0.7171)	(0.0515)	(0.8297)		(0.7627)	(0.5657)	(0.4247)	(0.9294)	(0.28)	(0.1397)
NEFA	Plasma	-0.302 8	-0.3753	-0.5353	0.0765		0.6991	-0.2984	-0.217	0.8122	0.7876
		(0.2219)	(0.1249)	(0.0221)	(0.7627)		(0.0012)	(0.2291)	(0.387)	(0)	(0.0001)
	Follicle	-0.143	-0.231	-0.3583	0.1451	0.6991		-0.2226	-0.0501	0.6699	0.6777
		(0.5713)	(0.3563)	(0.1443)	(0.5657)	(0.0012)		(0.3746)	(0.8435)	(0.0024)	(0.002)

IGF-1	Plasma	0.7032	0.4962	0.5853	0.2006	-0.2984	-0.2226		0.4414	-0.2059	-0.1062
		(0.0011)	(0.0362)	(0.0107)	(0.4247)	(0.2291)	(0.3746)		(0.0667)	(0.4124)	(0.675)
	Follicle	0.7458	0.5519	0.6294	0.0225	-0.217	-0.0501	0.4414		-0.4119	-0.2525
		(0.0004)	(0.0176)	(0.0051)	(0.9294)	(0.387)	(0.8435)	(0.0667)		(0.0895)	(0.312)
Urea	Plasma	-0.3096	-0.4113	-0.5486	0.2692	0.8122	0.6699	-0.2059	-0.4119		0.9454
		(0.2112)	(0.0899)	(0.0184)	(0.28)	(0.0001)	(0.0024)	(0.4124)	(0.0895)		(0.0001)
	Follicle	-0.1589	-0.3856	-0.4171	0.3622	0.7876	0.6777	-0.1062	-0.2525	0.9454	
		(0.5288)	(0.114)	(0.085)	(0.1397)	(0.0001)	(0.002)	(0.675)	(0.312)	(0.0001)	

Figures

Figure 1 - Experimental protocol

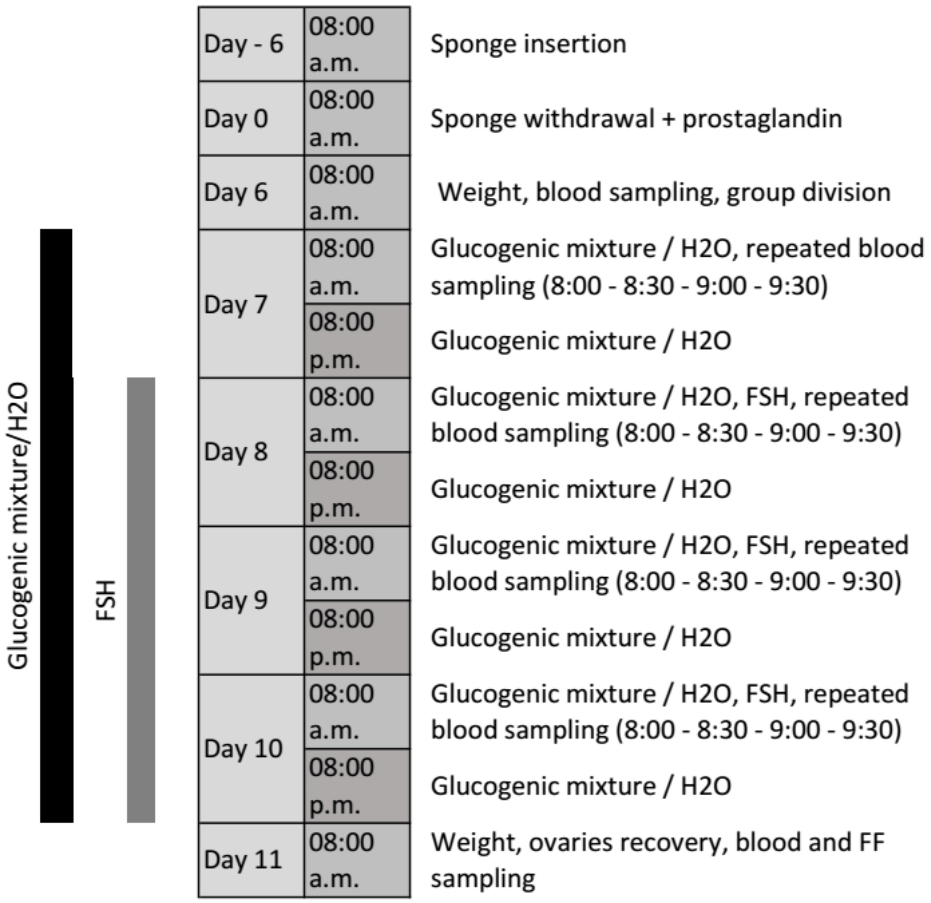


Figure 2 - Ovarian follicular and corpora lutea (CL) population in ewes orally drenched with a glucogenic mixture (GLU n=10) or with water (WAT n=10), 12 h after the end of a 4-d administration. Follicles are either expressed as total count (tot) or subdivided by size categories (2-3 mm; 4-5 mm; >6mm).

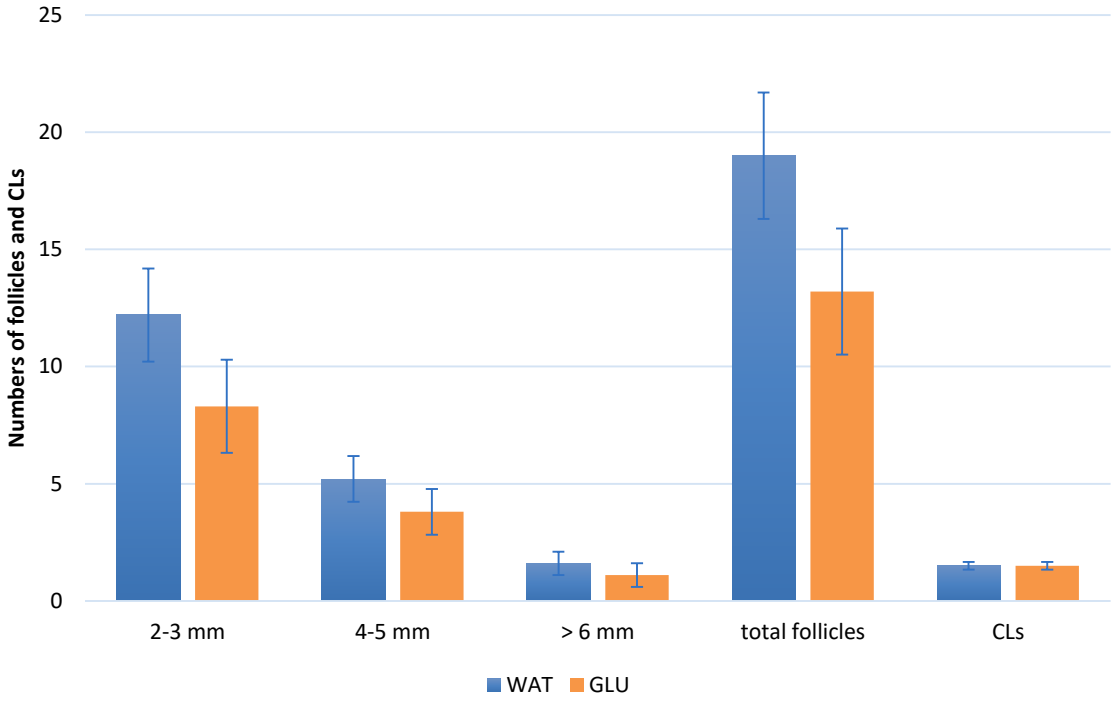


Figure 3 - Progesterone mean circulating concentrations in ewes orally drenched with a glucogenic mixture (GLU n=10) or with water (WAT n=10) during the experimental period (day 6 – day 11; day 0 = sponge withdrawal); a,b,c indicate significant variations within GLU group; A,B,C indicate significant variation within WAT group ($P < 0.0001$).

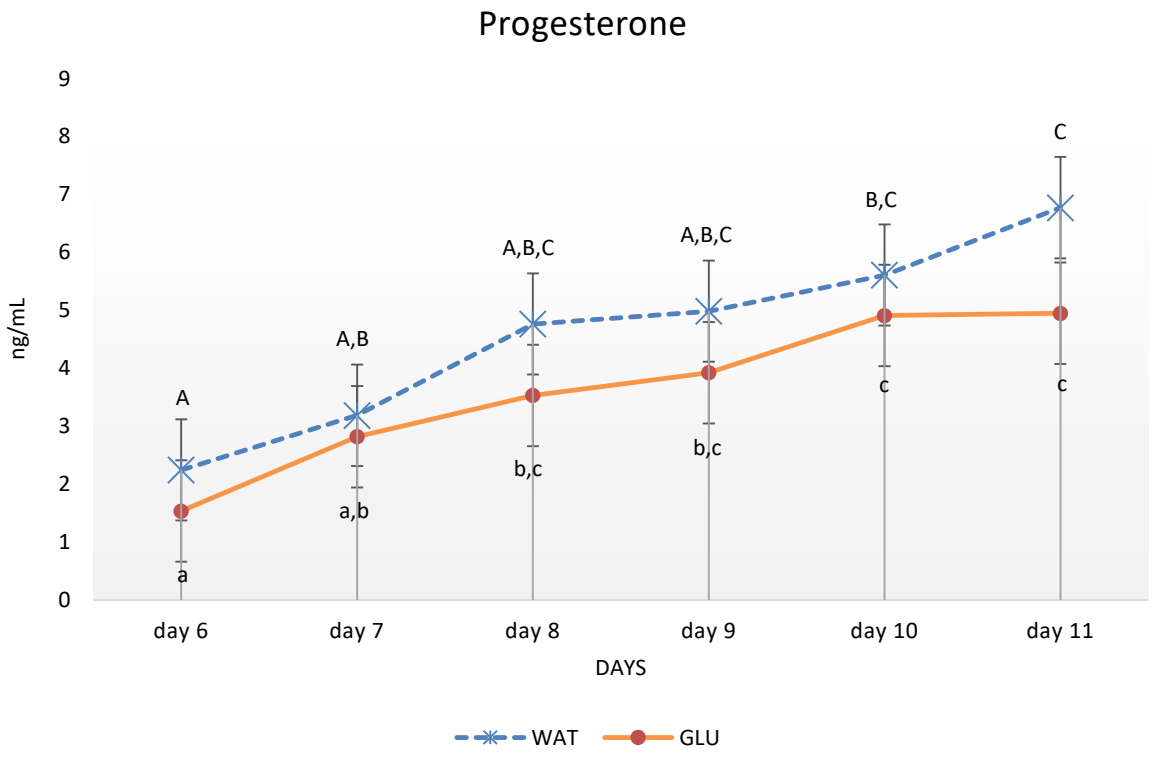
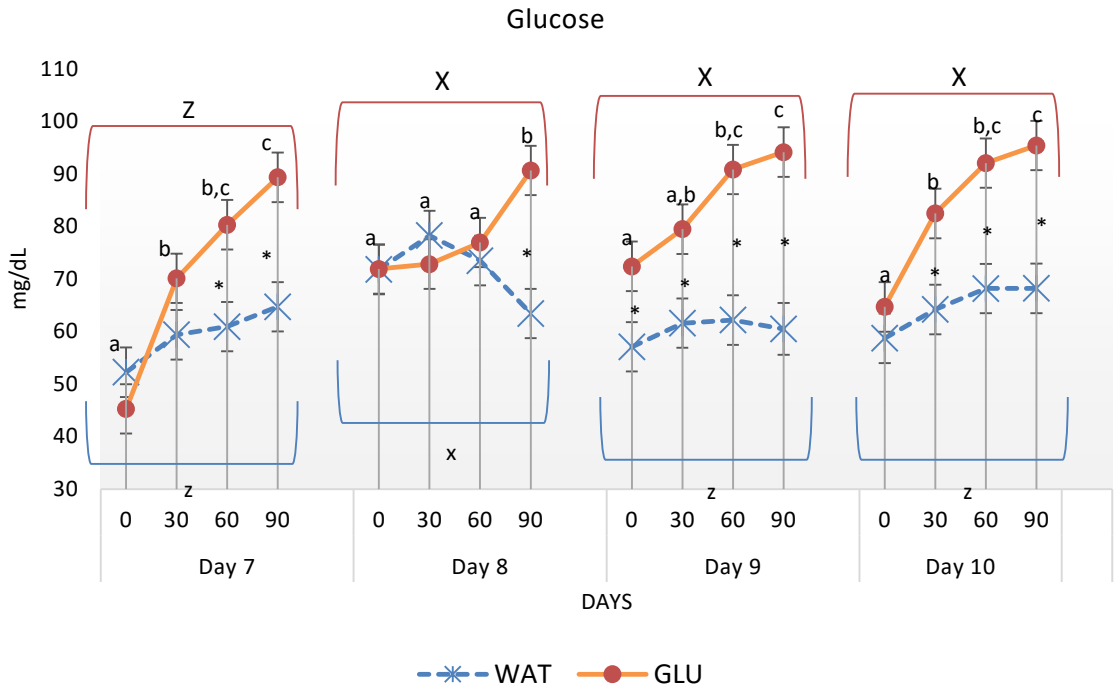
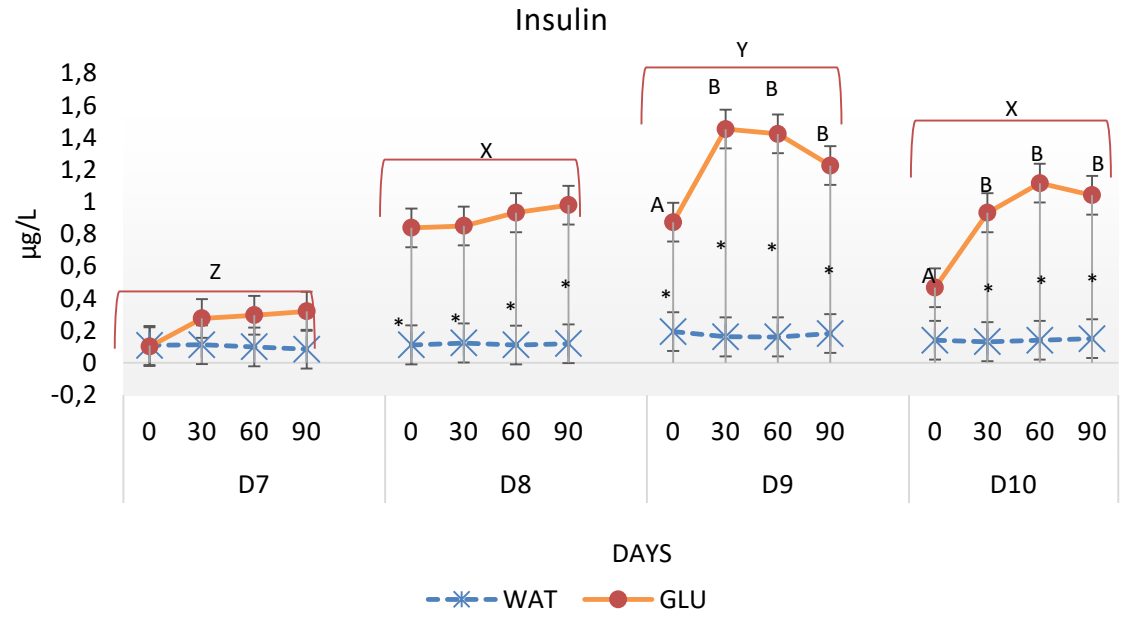
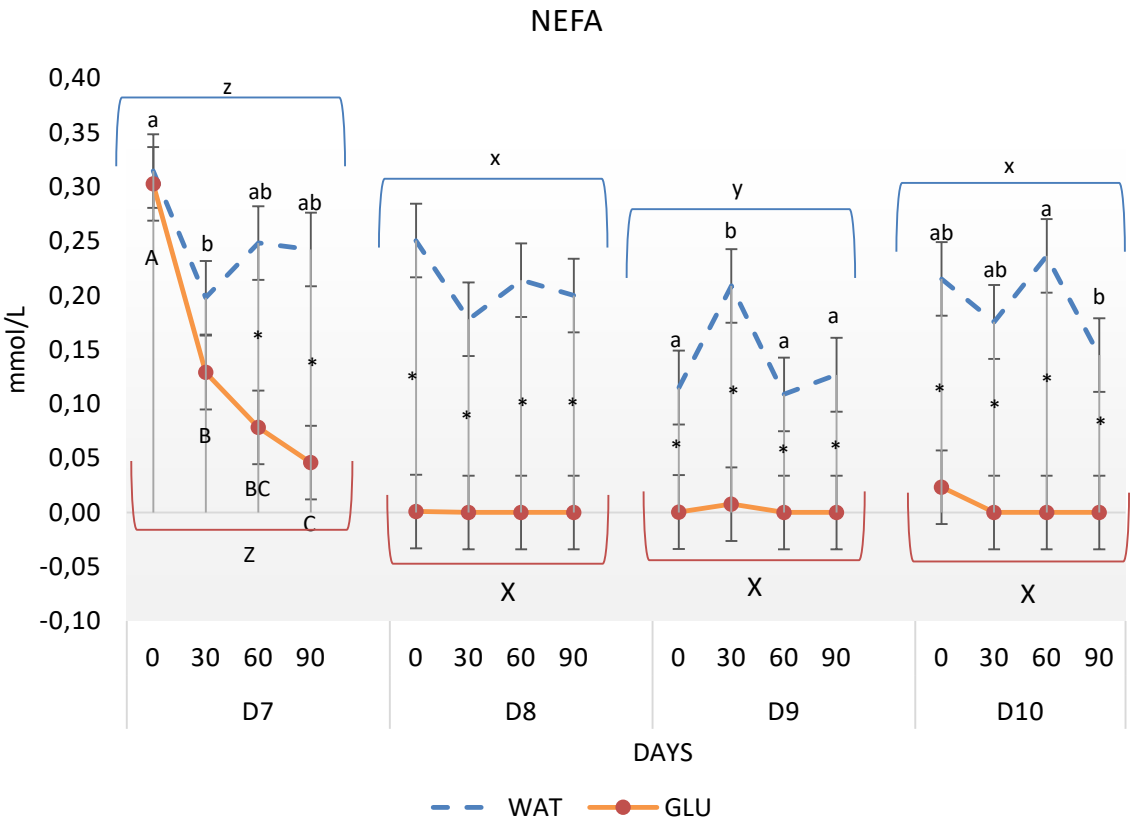


Figure 4 - Circulating concentrations of glucose, insulin, NEFA and urea in ewes orally drenched with a glucogenic mixture (GLU n=10) or with water (WAT n=10) during treatment period (D7 – D10; D0 = sponge withdrawal). Asterisks indicate significant differences between groups ($P < 0.0001$). a, b, c indicates daily significant variations within GLU group. A, B, C indicates daily significant variations within WAT group. Z, X, Y indicates significant variations within GLU group on daily mean levels. z, x, y indicates significant variations within WAT group on daily mean levels.




Insulin
µg/L
1,8
1,6
1,4
1,2
1
0,8
0,6
0,4
0,2
0
-0,2
Z
X
Y
X
A
B
0 30 60 90
D7
D8
D9
D10
DAYS
WAT
GLU


NEFA
mmol/L
0,40
0,35
0,30
0,25
0,20
0,15
0,10
0,05
0,00
-0,05
-0,10
z
x
y
x
a
b
ab
A
B
BC
C
Z
X
0 30 60 90
D7
D8
D9
D10
DAYS
WAT
GLU

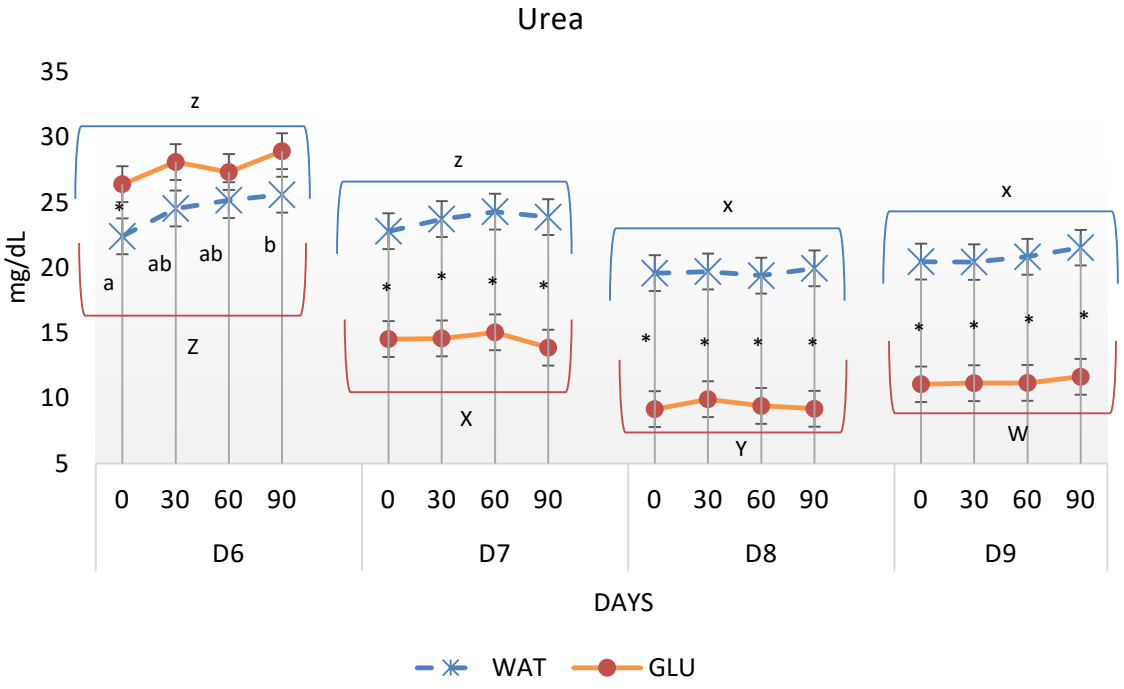

Urea
35
30
25
20
15
10
5
mg/dL
z
z
x
x
a
ab
ab
b
Z
X
Y
W
0 30 60 90
0 30 60 90
0 30 60 90
0 30 60 90
D6
D7
D8
D9
DAYS
WAT
GLU

Figure 5 - Mean circulating concentrations of IGF-1 in ewes orally drenched with a glucogenic mixture (GLU n=10) or with water (WAT n=10) during the experimental period (day 6 – day 11; day 0 = sponge withdrawal); *indicate significant differences between groups ($P < 0.01$). a,b,c indicate significant variations within GLU group ($P < 0.01$).

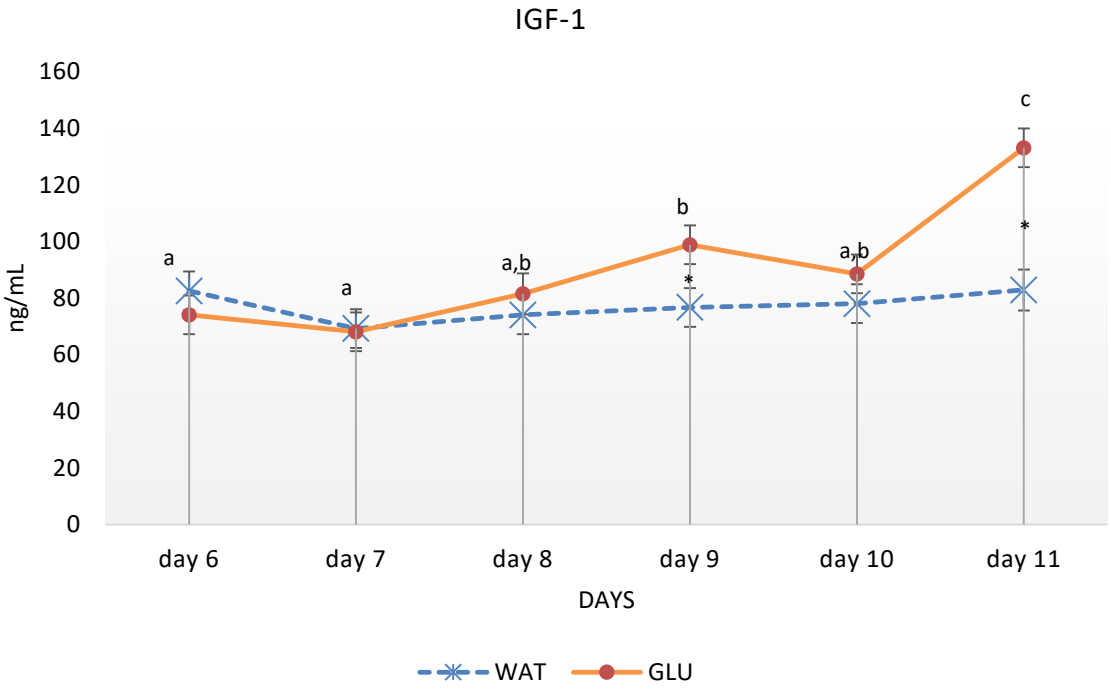


Figure 6 - Glucose, insulin, IGF-1, NEFA and urea concentrations in the plasma and follicular fluid collected form Sarda ewes 12 h after the end of a 4 d oral drenching with a glucogenic mixture (GLU n=10) or with water (WAT n=10). Follicular fluid was collected from follicles ≥ 4 mm in diameter. Asterisks indicate significant differences between groups in plasma ($P < 0.0001$) and FF concentrations ($P < 0.0001$). Different letters indicate statistical differences between plasma and follicular concentrations within the same group: upper case letters: WAT group; lower case letters: GLU group. Asterisks indicate statistical differences between WAT and GLU group in plasma (single asterisk) and in follicular concentrations (double asterisk).

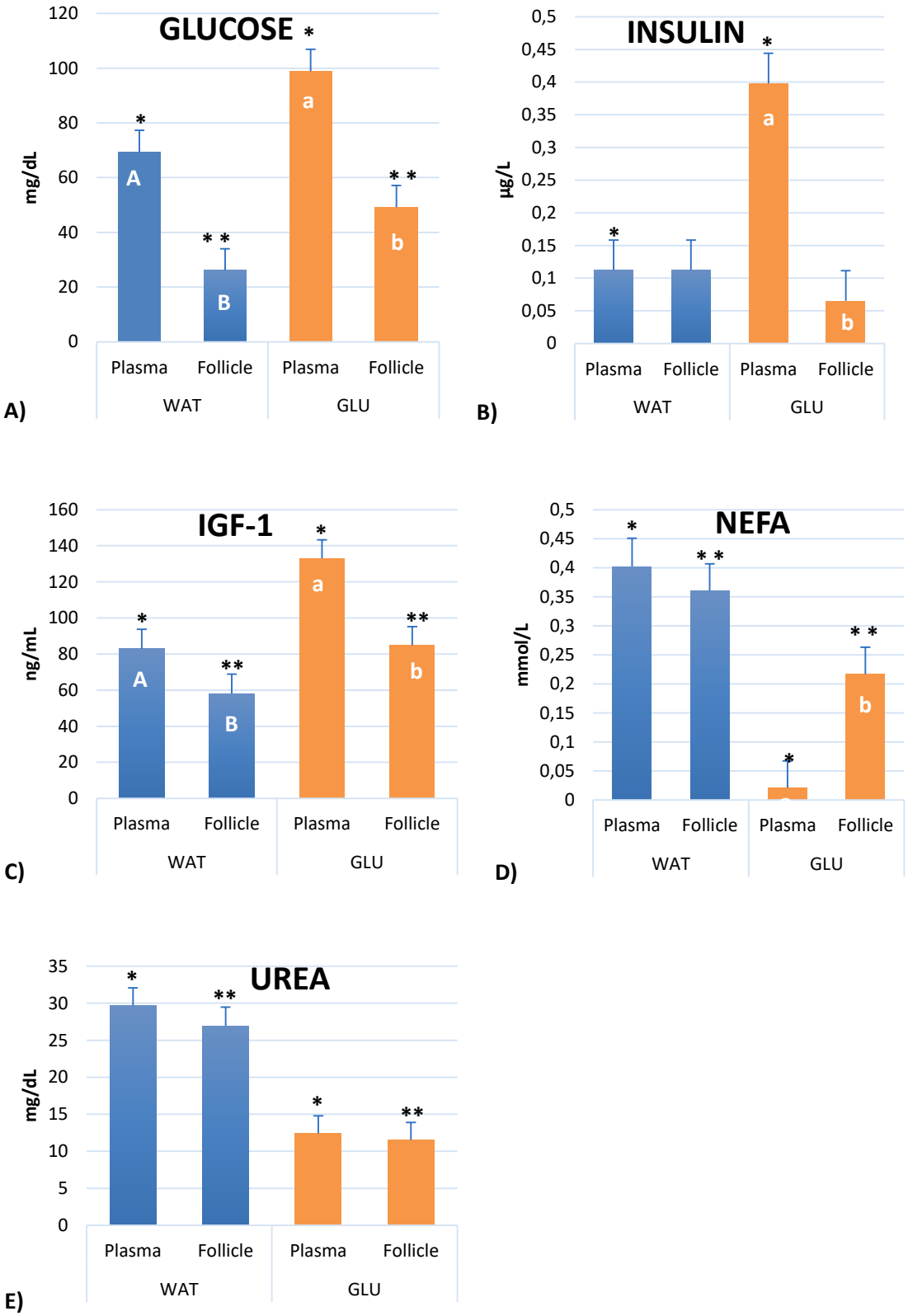
GLUCOSE: GLM follicle vs plasma $P < 0.01$; WAT vs GLU $P < 0.0001$; treatment x source of sample $p>0.05$

NEFA: GLM follicle vs plasma $p>0.05$; WAT vs GLU $P < 0.0001$; treatment x source of sample $P < 0.05$

UREA: GLM follicle vs plasma $p>0.05$; WAT vs GLU $P < 0.0001$; treatment x source of sample $p>0.05$

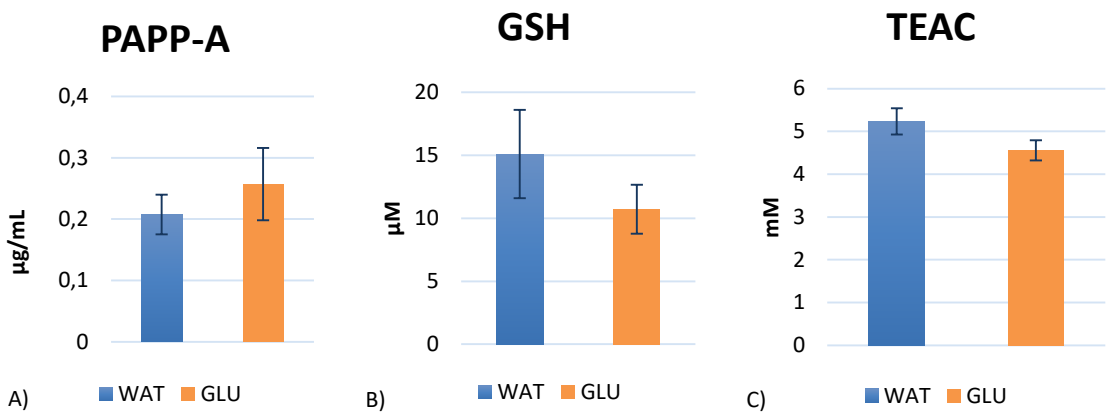
INSULIN: GLM follicle vs plasma $P < 0.0001$; WAT vs GLU $P < 0.01$; treatment x source of sample $P < 0.01$

IGF-1: GLM follicle vs plasma $P < 0.01$; WAT vs GLU $P < 0.001$; treatment x source of sample $p>0.05$



Porcu Cristian. Endocrine/metabolic status and reproductive performances in Sarda dairy ewes given different nutritional plans. PhD thesis in Riproduzione, Produzione e Benessere Animale. Università degli Studi di Sassari.

Figure 7 - Concentrations of PAPP-A (panel A) and GSH (panel B), and trolox equivalent antioxidant capacity (TEAC; panel C) in follicular fluid collected from Sarda ewes 12 h after the end of a 4 d oral drenching with a glucogenic mixture (GLU n=10) or with water (WAT n=10).



4.2.4. Discussion

As expected, the short-term flushing did not impact on ewes' live weight. This was already found by others [36], in sheep submitted to the same flushing protocol.

The rise in circulating progesterone confirmed that ovulations were efficiently synchronized, and ewes were at the beginning of the luteal phase during the nutritional treatment. Despite this, the effect of short-term flushing on follicle dynamics was below expectation. The lack of difference between GLU and WAT groups in the number of ovarian follicles and CL as response to exogenous FSH administration partially disagree with previous findings of our laboratory [27], and others [36] who found at least at some stage an increase of the number of 2-3 mm follicles at day 8 [27] or CL [36]. This could be partially explained by the overriding effect of FSH.

In contrast with the lack of effect on follicular dynamics, the short-term flushing with a glycerol-propylene glycol mixture caused significant changes in the plasma and follicular fluid milieu. These changes were detectable from day 1 of administration and lasted up to the day after its end. The main impacts were on the glucose-insulin-IGF-1 system homeostasis with a positive trend of all the above levels, whereas NEFA and urea levels dropped in both the plasma and the follicular fluid.

The use of glycerol and propylene glycol as glucogenic precursors is based on *in vitro* and *in vivo* studies showing that the concentration of ruminal propionic acid increases when

forage is supplemented with these organic compounds [192, 213-215]. 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 [213, 216].

In the present experiment, 4 days of glucogenic mixture administration had an immediate effect on glycaemia and insulin plasma levels. Circulating concentration of glucose rose sharply in GLU group, reaching levels similar to those obtained in ewes by continuous glucose infusion at 10 mM/h for 72 h [217]. Consequently, in the same group, insulin circulating levels increased significantly, as showed by several studies in which nutritional treatments lupin grain [29, 32]; a mixture of soya meal and maize [33]; steamed corn flakes [218]; a mixture of glycerol and propylene glycol [36] increased the concentrations of glucose and insulin in jugular venous plasma in ewes.

Although a previous study reported that IGF-I itself does not respond to short-term nutritional supplementation [32], in the present experiment mean IGF-1 plasma levels were significantly higher in GLU than WAT group. The rise in IGF-1 plasma levels was slower than glucose and insulin's one, since it was detected from the 3rd day of the nutritional treatment and reached its peak after its end (day 11).

The follicle has a functional insulin-glucose-IGF-1 system [51] which is affected by short-term nutritional treatments, and it is clear that components of this metabolic system are nutritionally regulated in the follicle [24, 36].

The higher circulating glucose concentrations found in energy-supplemented ewes compared to controls are likely to exert a positive effect on ovarian function, especially because they were accompanied by a rise in glucose FF concentrations. It seems that the effect of glucose on fertility is primarily related to its properties as a metabolic fuel [41], being glucose the main source of energy for the ovary [51, 219]. 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 [199]. This system allows the follicle to regulate its growth and development, mainly by altering FSH-induced effects on the synthesis of oestradiol by the granulosa cells, in accordance with the availability of glucose [51, 66]. In addition, glucose is essential in determining the quality of the oocyte [35]. Our research group had already showed that in sheep glucogenic treatments improve oocyte quality, evaluated by the kinetics of their *in vitro* development and by the production of blastocysts [27]. Present findings suggest that those results were likely linked to the increased intra-follicular concentration of glucose, which was driven by the rise in its circulating concentrations.

Insulin is another key signal mediating the effects of acute changes in nutrient intake on follicle dynamics [66]. Insulin and glucose seems to act synergistically at the ovarian level, and it is likely that the effects of short-term nutrition may be mediated by direct ovarian actions of insulin and glucose [68]. *In vitro* research has shown dose-dependent stimulatory effects of insulin on the proliferation and steroid synthesis of the bovine

granulosa and theca cells; these effects can be exerted via their direct stimulatory actions on the follicle as well as by increased local responsiveness of the follicle to FSH and LH [220]. An increase in insulin concentrations over a short lapse of time has been shown to exert a positive effect on the growth of small follicles prior to superovulatory treatment and a beneficial influence on subsequent embryonic development [221, 222]. Thus, the rise in circulating insulin levels, triggered by glucose's rise, may contribute in creating a suitable systemic metabolic milieu for the promotion of ovarian function. However, no difference was found in insulin FF concentrations between the two experimental groups. Insulin likely reaches FF from the circulation by transudation, but, as observed in the present experiment, FF insulin concentrations do not correlate with plasma insulin [223]. Even if insulin plays an essential role in the final stage of follicular development, abnormal concentrations of the hormone can lead to follicular dysfunction, resulting in excessive atresia or the formation of follicular cysts [224]. Therefore, we can speculate that the follicle can buffer a rise in insulin circulating concentration to prevent its potential harmful effects at the local level.

IGF-1 is a potent stimulator of both follicle growth and oestradiol secretion [34], and the ovary is a site of IGF-I gene expression and reception [48]. In the present experiment, the significant rise in IGF-1 circulating concentration observed in energy-supplemented ewes was not accompanied, as for insulin, by a consequent rise in its FF concentrations.

According to the somatomedin hypothesis, nutritionally induced changes in liver IGF-1 secretion have a direct effect on the ovary through the endocrine actions of IGF-I, and thanks to its contribution to FF IGF-1 [225, 226]. At this regard, Scaramuzzi et al. [227] presented compelling evidence for an endocrine effect of IGF-I on ovine follicular development. They infused an IGF-I analog into an autotransplanted ovary and demonstrated increased estradiol secretion after PGF2α-induced luteolysis. Endocrine IGF-I secretion is controlled by somatotropin, and by the nutritional status (energy and protein intake relative to requirements) [226]. The nutritional treatment applied in the present experiment stimulated endocrine IGF-1 secretion, probably with accumulative effect that determined the insurgence of the highest values at the end of the treatment period. However, in the present experiment we determined whole IGF-1 concentrations, which comprise both biologically active and inactive (bond to the IGFBP) IGF-I.

To assess possible changes in IGF-1 biological activity at the ovarian level consequent to the nutritional treatment applied, we determined intra-follicular PAPP-A concentrations, the main IGFBP protease regulating the levels of free IGF1 in bovine dominant follicle [200, 201]. However, no significant differences were observed between the two experimental groups.

The nutritional treatment applied also caused a significant drop in NEFA and urea circulating concentrations, which remained stably lower than in WAT group. Supplying meal or water with glycerol to transition dairy cow resulted in a positive energy status

with higher concentrations of serum glucose and lower concentrations of plasma NEFA [37, 39, 40]. Lien et al. [228] showed that animal treated with propylene glycol had lower levels of blood urea nitrogen respect to the control group. In ewes, supplementation of soluble sugar reduced urea levels in serum and FF [229], and short-term high energy feeding diets lowered serum urea nitrogen levels while increasing the numbers of large follicles [230].

In the present experiment, glucogenic mixture administration caused a drop in NEFA and urea concentrations not only in the plasma but also in the follicular fluid. Although Rabiee et al. [104] report that there was no significant uptake of NEFAs by the bovine ovary, others have reported that in cattle increased concentrations of NEFAs in blood were reflected in the FF microenvironment [100, 106]. Elevated concentrations of plasma NEFA frequently observed in the FF of the dominant follicle in dairy cows were associated with reduced *in vitro* developmental competence of the oocyte [100, 107, 109]. In addition, high NEFAs levels can compromise the viability of the bovine granulose cells [108]. Therefore, diets or metabolic states that favour high concentrations of NEFAs should be avoided during the cycle of conception and the early post-conception period [41]. The present experiment while confirming the negative relationship between plasma concentrations of NEFA from one hand and insulin and glucose to the other, also showed that the drop in FF NEFA concentrations found in energy-supplemented ewes was slower than the one observed in the plasma. In other words, NEFA clearance in the ovary is less

efficient than at the systemic level, and thus the negative effect of high FF NEFA concentration can persist even after the re-establishment of a positive energy balance. This could possibly explain while, under some circumstances, short-term flushing treatments based on lupins or glucogenic mixtures failed to improve reproductive performance of sheep submitted to long-term systemic underfeeding [161].

In the same way, high levels of urea in blood have been associated with lower fertility due to a changed uterine environment and poor embryo viability [125, 127, 231]. *In vitro* studies showed that urea can impair meiosis and thereby reduce the percentage of oocytes fertilized and of embryos that develop [232]. Elevated plasma urea levels reduced ovine embryo viability and development *in vivo* and *in vitro* [125, 233]. Circulating urea concentrations are reflected in FF and may affect the quality of both the oocyte and the granulosa cells [106, 234]. Therefore, the observed drop in NEFA and urea concentration and the systemic and follicular level can contribute to the creation of a suitable environment for follicular growth and for the acquisition of oocyte developmental competence.

The survival of follicles subjected to gonadotropin stimulation is also related to an increase in the expression of antioxidant enzymes since inadequate protection against ROS constitutes a trigger for follicular atresia [235]. Apoptosis of granulosa cells during follicular atresia is indeed preceded by oxidative stress, partly due to a drop in the antioxidant GSH. Within the follicle, glucose metabolism, *via* the pentose phosphate

pathway, provides precursors for the synthesis of NADPH and thus for GSH regeneration. Results obtained in the present experiment showed no difference in FF GSH concentrations and total antioxidant capacity between GLU and WAT group, and no correlation was found between glucose FF concentration and its antioxidant defenses. Previous studies in *in vitro* cultured somatic cells showed that high glucose concentrations (27.5 and 11 mM) in culture media decrease intracellular GSH by impairing cystine uptake capability [236]. However, this effect was not observed at lower glucose concentrations (5.5 mM), similar to those found in FF in the present experiment. Further studies are needed to better address the relationship between glucose FF concentration and follicular antioxidant defenses.

4.3. Experiment 2 (E 2.2)

4.3.1. Aim

Lactating ewes are mainly fed native pastures or forage crops, and receive as supplements commercial concentrates, cereal grains, hay or silages, at levels depending on herbage availability and ewe physiological status. Considering that animals are bred during the anoestrus period when they are in mid to late lactation, 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. During this period, pasture is usually turning to a reproductive phase and grazed herbage

is featured by a raising content of fiber (high fiber NDF-40-60 % DM), and a declining content of crude protein (14-8% DM). This decay of pasture nutritive value is usually mirrored by the lowering of milk yield [136].

To cope with these adverse nutritional conditions and optimize reproductive performance, our group has recently studied the oral administration of a short-term glucogenic treatment based on a mixture including 70% glycerol, 20% propylene glycol, and 10% water. These studies have shown that this glucogenic-based flushing treatment improves oocyte quality [237]. These effects have been related to the modification of the plasma and follicular fluid composition during the treatment period as shown above [238]. Glucogenic mixture administration, in fact, increased circulating and intrafollicular concentration of glucose, while decreasing their concentrations of NEFA and urea. These changes, together with the increase in circulating concentrations of insulin and insulin-growth factor 1 (IGF-1), lead probably to the creation of a suitable microenvironment for the final follicular growth and thus for the conception [238].

The ovary uses glucose as its principal source of energy and its well described positive effects on fertility have been related to its properties as a metabolic fuel [65]. However, during lactation, glucose requirements in the mammary tissue increase dramatically, considering that it is the main precursor of milk lactose, and it is also involved in milk protein and lipid metabolism. In ruminants, the direct uptake of glucose from the diet is usually limited as its main source is the liver *via* gluconeogenesis based on the

metabolism of rumen-derived volatile fatty acids (mainly propionic acid) and amino acids. Overall, whatever the source, the intake of glucose or glucose precursors can markedly impact on milk production in ruminants. In dairy sheep, milk yield is markedly affected by dietary non-fiber carbohydrates (NFC), such as starch, pectin, galactans, and simple sugars. During the first part of lactation, diets with high NFC concentration based on starch generally give much higher milk yield than medium-low NFC diets [54-56]. However, during mid to late lactation, feeding sheep with diet rich in starch did not improve significantly milk yield [239-241]. In contrast, Molle et al. [57] in mid-lactating dairy ewes found that grazing Italian ryegrass in afternoon rather than in morning hours increases the intake of herbage water soluble carbohydrates (mainly sugars), enhancing milk yield and body condition score. However, in all these studies reproductive performances were overlooked.

Therefore, since glucose is a key nutrient for the optimization of both reproductive and productive performance in dairy ewes, the effects of a transient rise in glycaemia in dairy ewes at their mid-to-late stage of lactation in terms of reproductive and productive performances needs to be elucidated.

Starting from these premises, the present experiment aimed at investigating whether the glucogenic mixture administration in lactating dairy ewes could create the same metabolic and hormonal milieu observed in dry ewes (as described above) [238], which would favour the final follicular growth, and thus increase fertility and prolificacy.

Another objective of this experiment is the evaluation of the effects of glucogenic treatment on food intake, milk yield and milk composition, responses previously overlooked by the literature. A further objective was the evaluation of the dynamic of glycerol concentration in the bloodstream after its oral administration and its correlation with circulating glucose, insulin, NEFA and urea. In addition, given glycerol high osmotic pressure, this experiment also investigated its effect on blood osmolarity, and circulating concentrations of total proteins as an indicator of blood volume.

4.3.2. Materials and methods

The experiment was run at Bonassai research station of Agris, located in north-western Sardinia, Italy (40°N, 8°E, 32 m a.s.l.).

4.3.2.1. Animals and treatments

Thirty Sarda ewes, aged 3-6 years (mean ± SE 3.3 ± 0.26 years) at mid-late lactation stage (4-5 months, mean ± SE, 155 ± 5 days in milk (DIM)) were used. The animal protocol and the implemented procedures were in accordance with the ethical guidelines in force at Agris and at the University of Sassari, in compliance with the European Union Directive 86/609/EC and the recommendation of the Commission of the European Communities 2007/526/EC. The research and technical personnel had been previously trained to properly handle the animals during measurement procedures.

Before being subjected to the experimental procedures, the animals were familiarized with the equipment and routine described below. The experimental protocol was run from June 11th to July 31st 2015. The experimental design was with two replicates per treatment. On June 11th, thirty Sarda dairy ewes, were selected from the farm flock, homogeneous for age and lactation stage. 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 (see below). Thereafter, the ewes were randomly assigned to two experimental groups, homogeneous for body weight (BW), body condition score (BCS), DIM, MY, and milk fat and protein. One ewe was discarded because of an acute trauma. Each group was further divided in two subsets, used as replicate.

During the experimental period, the ewes were machine milked twice daily at 07.00 and at 15.00.

Ram effect was used to synchronise ewes' ovulation during transition season (June). Vasectomised rams were introduced in the flock at the ratio of 2 rams per 15 ewes. Before the introduction, ewes had been isolated from rams for at least 6 weeks. The vasectomized rams were left in the flock from the day of their introduction (Day 0) until the presumptive starting of oestrus (Day 16). Thereafter, vasectomised rams were replaced with non-vasectomized rams at the same ram per ewe ratio. Rams were removed from the flock on Day 30.

Coincidentally with the onset of the mating period, from Day 16 to Day 20, one experimental group (glucogenic treated ewes n=15; GLU) received, orally twice daily at 08.00 in the morning and at 19.00 in the evening, 200 mL of a glucogenic mixture. The glucogenic formulation contained 70% glycerol, 20% propylene glycol (both from Sigma Chemical Co., St. Louis, MO, USA), and 10% water. The second group (controls: n=14 WAT) received 200 mL of water twice daily simultaneously to glucogenic mixture administration. Both the glucogenic formulation and the water were administered by oral gavage using an esophageal feeding tube. From Day 14 to Day 20, i.e. throughout the flushing period, the sub-groups were kept indoors in separate pens. Indoor feeding consisted of 400 g/head/day of a commercial pelleted concentrate divided in two meals and individually fed at milkings, plus c.a. 900 g/head/day of dehydrated lucerne hay and 900 g/head/day of chopped Italian ryegrass hay which were administered in two equal meals in the morning and evening.

During the remaining days, i.e. before and after the treatment period, the ewes of both the experimental groups were allowed to graze two 0.5 ha paddocks of mature (post-heading phase) Italian ryegrass (*Lolium multiflorum* Lam) pasture for 4 h/day (7:00-11:00). Paddok 1 was grazed by subgroups 1 of GLU and WAT ewes and paddock 2 by subgroups 2. During the grazing period the ewes were supplemented with 400 g/head/day of the above concentrate. The hay supplementation consisted of 1000 g/head/day of dehydrated lucerne hay before the flushing period and 900 g/head/day of chopped

ryegrass hay plus 450 g/head/day of lucerne hay after the flushing period. This increase of the amount of hay daily supplied as supplement to the grazing sheep was aimed at compensating pasture defoliation of paddocks during the grazing period preceding the flushing treatment. The hay was fed in pens at grazing turning out.

4.3.2.2. Experimental measurements, samplings and analyses

During the experimental period, both group of ewes were subjected to different measurements and their blood and milk were sampled. Table 4 shows the exact timing at which sampling and measurements were performed.

4.3.2.3. Blood samplings for metabolite and hormone assays

Blood samples, drawn from the jugular vein, were collected during the pre-experimental period, the flushing administration, in the 2 days following the end of the nutritional treatment and in the late post-flushing period to determine the circulating concentrations of glycerol, glucose, insulin, NEFA and urea (table 4). Samples were collected at 08.00 a.m., right after the milking procedures. In addition, on day 18, the 3rd day of administration of the nutritional treatment, insulin, glucose, NEFA, urea and glycerol plasma concentrations were determined in four consecutive samples collected every 30 minutes starting from the time of morning administration of glucogenic mixture or water (08.00, 08.30, 09.00 and 09.30 a.m.). In the same day and at the same time points plasma

osmolarity and total protein concentration were determined. Plasma osmolarity was measured using a freezing point micro-osmometer (Osmomat 030 - Gonotec).

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 and centrifuged at 1500g for 15 min. Plasma was removed and stored at -20°C until assayed.

4.3.2.4. Determination of plasma metabolites and hormones

All plasma samples were measured in duplicate.

Glucose, NEFA and urea were measured using commercial kit and BS-200 Mindray clinical chemistry analyzer. We used Serum I Normal (Wako) and Serum II Abnormal (Wako) 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.

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.

Glycerol concentration was measured in a single assay by a colorimetric method using a commercial Free Glycerol Assay Kit (Cell Biolabs, Inc, USA), with a glycerol standards in the concentration range of 0 mg/dL – 3.68 mg/dL. 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 0.046 mg/dL.

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 [202, 203]. 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%.

4.3.2.5. Feedstuff composition, ewe nutritional status 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 above ground level 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), ash, ether extract (EE) and CP (AOAC, 1990), neutral detergent fibre on an ash-free basis (NDF), acid detergent fiber on an ash-free basis (ADF) and acid detergent lignin (ADL, Van Soest, et al., 1991). Mean data of forage mass on offer and herbage allowance are shown in Table 5, whereas data on herbage and feedstuff chemical composition are displayed in Table 6.

Live weight and body condition score were determined in all the ewes 1 day before the vasectomized ram introduction (Day -1), the day after (Day 20) and 30 days after (Day 50) the end of the administration of the nutritional treatment. The ewes were weighed using an electronic scale before the morning meal. Body condition score [170] ranging from 1 (extremely thin) to 5 (obese) was estimated by two trained evaluators with an approximation of 0.25 BCS units. Their scores were averaged prior to data analysis.

During the nutritional treatment (from day 16 to day 19), 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, differences in group intake of hay were computed by weighing the offer and the orts. Total intake group was then computed, summing up herbage and supplement intake.

4.3.2.6. Milk yield and composition

The timing of the measurement of milk yield and determination of milk composition is shown in table 4. Milk yield was measured by weighting the production of each ewe in two consecutive milkings. 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). Fat normalised milk yield (FNMY) at 6.5% fat was calculated according to Pulina et al. [242].

4.3.2.7. 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.

4.3.2.8. Statistical analyses

Live weight and BCS at the beginning, during, and after the glucogenic treatment period and their changes were analyzed by a mono-factorial GLM.

Circulating concentrations of metabolites and insulin measured on day -1, 18, 20, 21 and day 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, and urea in the consecutive samples collected on day 18 (during treatment period) were analyzed by a mixed model for repeated measurements (PROC MIXED of SAS package) with treatment, sampling hour and their first-order interactions as fixed effects, and sheep as random effect. The relationship between the circulating concentration of metabolites and hormones was evaluated by correlation analysis using Pearson's correlation coefficients.

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.

Finally, χ^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=0.10.

4.3.3. Results

Weather, as assessed by a local meteorological station, was characterised by particularly hot days with temperature humidity index (THI) around 70 (severe heat stress) in the period of flushing treatment and beginning of mating [243].

4.3.3.1. Dynamics of glycerol, metabolites and insulin in the bloodstream

The administration of the glucogenic mixture determined a sharp increase in glycerol circulating concentrations, which in treated ewes reached values significantly higher than in controls 30 min after its administration, and kept raising to reach values 400-folds higher than in controls at 90 min (43.3 ± 13.4 vs 21663 ± 4568 µM in control and treated ewes respectively; $p<0.001$; Figure 8, panel A). Despite this fast response, glycerol circulating concentrations did not differ between treated and control ewes if measured at fasting both during the nutritional treatment period (day 18) and in the two days after its end (day 20 and 21; figure 8, panel B). In these days, at fasting, no significant interaction

between group and day was found, and glycerol mean concentrations did not show any differences between the two experimental groups nor during the nutritional treatment (day 18; 27.5 ± 12.8 vs 28.7 ± 7 μM in WAT and GLU group respectively), or in the two days after its end (day 20: 36.2 ± 13.7 vs 50.7 ± 8.8 μM in WAT and GLU group respectively; day 21: 61.9 ± 7.3 vs 50.4 ± 7.3 μM in WAT and GLU group respectively).

The sharp increase in glycerol plasma levels consequent to its administration was followed by a parallel increase in glycaemia and insulinemia (figure 9, panels A and B), and by a significant decrease in NEFA and urea circulating concentrations (figure 9, panels C and D).

As for glycerol, glycaemia and insulinemia did not differ in treated and control ewes when measured at fasting (before the administration of the glycogenic mixture), during the flushing treatment and in the days following its end (figure 10, panels A and B). In contrast, NEFA and urea circulating levels at fasting were significantly lower in treated ewes compared to controls ($p<0.05$; figure 10, panel C and D), on Day 18 (the 3rd day of its administration) but were undifferentiated between the groups onthe days before and after the end of the nutritional treatment.

Correlations between circulating concentrations of metabolites and hormones are shown in table 7. Presented results show that in consecutive sampling performed on the 3rd day of glucogenic mixture administration the rise in glycerol circulating concentrations was accompanied by a parallel increase in glucose and insulin circulating concentrations and

by a decrease in NEFA ones. In addition, insulin proved to be positively correlated with glucose, while being negatively correlated with NEFA and urea circulating concentrations. NEFA and urea were also positively correlated.

4.3.3.2. Plasma osmolarity and total protein concentrations

Blood osmolarity increased significantly in GLU ewes compared to WAT ones, reaching higher values starting from 30 min after the glucogenic mixture administration (figure 11, panel A; $p<0.0001$). Total protein concentration in the blood stream was significantly lower in GLU ewes compared to WAT ones both in its mean values ($p<0.0001$) and at 0 and 60 minutes from glucogenic mixture administration (figure 11, panel B; $p<0.05$).

4.3.3.3. Ewe nutritional status and feed intake

No differences were observed in body weight and body condition between groups at the beginning (Day -1), at the day after last treatment administration (Day 20) and 30 days after the end of the nutritional treatment (Day 50) (Figure 12; $P>0.05$).

Concentrate intake significantly decreased in GLU compared to WAT group during the glucogenic mixture administration while there was no difference in the intake before and after this period (figure 13). In the flushing treatment period, there was also a numeric decrease in hay intake in GLU group, measured as subgroup average (Table 8). However,

total intake did not differ between the two experimental groups if calculated including the glucogenic mixture in GLU group (Table 8).

4.3.3.4. Milk yield and composition

Before the beginning of the treatment, milk production, milk fat, total proteins, caseins, lactose and urea were within the physiological ranges [212] in all the ewes and showed no differences between GLU and WAT ewes (figure 14). During the treatment (Day 19), milk yield significantly decreased in GLU ewes compared to WAT ones (figure 14, panel A), together with its content in lactose (figure 14, panel D), and urea (figure 14, panel E). On the other hand, milk protein and casein content increased significantly in GLU compared to WAT ewes (figure 14, panel C and F). Milk fat content was not affected by the treatment, with no significant differences between groups (figure 14, panel B).

After the treatment end (Day 50), GLU group returned to physiological value of milk production and composition with no differences compared to WAT group.

4.3.3.5. Pregnancy scanning and ewe reproductive performance

As shown in table 9, no differences were recorded between treated and control ewe's reproductive performance. In control group, out of 14 ewes, 7 were diagnosed as pregnant at the ultrasonographical control and gave birth to a total of 11 lambs (3 singleton plus 8 twins). In treated group, out of 15 ewes, 9 were diagnosed as pregnant at

the ultrasonographical control. One pregnant ewe from the GLU group died because of an acute pneumonia. Thus, 8 ewes gave birth to a total of 11 lambs (5 singleton plus 6 twins).

Tables

Table 4 - Timing of measurements and sampling during the experimental period.

Period	Day	Measurements and sampling	Analysed metabolites and hormone
Pre-experimental	-1	BW, BCS, blood sampling	insulin, glucose, NEFA and urea
	12	MY, MC	
Flushing	18	blood sampling	insulin, glucose, NEFA, urea, glycerol, and osmolarity
	19	MY, MC	/
Post-flushing (short-term)	20	BW, BCS, blood sampling	insulin, glucose, NEFA and urea and glycerol
	21	blood sampling	insulin, glucose, NEFA and urea and glycerol
	25	MY, MC	
Post-flushing (residual effect)	50	BW, BCS, blood sampling, pregnancy diagnosis by ultrasound scanning	insulin, glucose, NEFA and urea

Table 5 - Forage mass at initial, mid, and final grazing day and herbage allowance in paddocks of I. ryegrass at reproductive phase grazed by milked ewes at mating period.

	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 day)		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 day)		2.8	

Table 6 - Feedstuff analyses.

		D.M. %		Ash % D.M.		C.P. % D.M.		E.E. % D.M.		N.D.F. % D.M.		A.D.F. % D.M.		A.D.L. % D.M.	
	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
Mean Paddock 1	15	71.4	11.1	6.8	2.8	5.1	1.2	1.4	0.3	75.4	6.1	45.5	5.9	4.8	1.4
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
Mean Paddock 2	15	78.2	13.7	5.5	1.6	6.3	0.7	1.2	0.3	78.5	6.0	45.6	4.9	4.9	1.0

Table 7 - Pearson correlation coefficients among circulating concentrations of glucose, insulin, NEFA, urea, glycerol, and plasma osmolarity as evaluated in consecutive samples performed every 30 min during the 3rd day of glycogenic mixtures administration (Day 18) in treated (n=15) and control ewes (n=14). Corresponding P-values are shown within parenthesis.

	Glucose	NEFA	Urea	Glycerol	Insulin
NEFA	-0.272				
	(0.049)				
Urea	0.021	0.455			
	(0.882)	(0.0001)			
Glycerol	0.686	-0.317	-0.012		
	(0.0001)	(0.019)	(0.931)		
Insulin	0.600	-0.428	-0.365	0.426	
	(0.0001)	(0.001)	(0.006)	(0.001)	
Plasma osmolarity	0.740	-0.259	-0.053	0.747	0.587
	(0.0001)	(0.057)	(0.699)	(0.0001)	(0.0001)

Table 8 - Intake of feedstuffs (g DM) and proportion of concentrates in the diet of milked ewes either dosed with water (WAT) or with a glucogenic mixture (GLU). Concentrates were individually fed while forages were group fed in pens.

	WAT	GLU	P<
Concentrate	352	241	0.001
Dehydrated lucerne	719	711	-
Ryegrass hay	721	539	-
Total intake of hay	1440	1250	-
Total feed intake	1791	1490	-
Glucogenic mixture		436	-
Total intake (glucogenic included)	1791	1926	-
Dietary proportion of concentrates*	0.20	0.35	-
Dietary proportion of glucogenic mixture	-	0.23	-

*Inclusive of glucogenic mixture

Table 9 - Reproductive performance of in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) and naturally mated during the nutritional treatment administration after being synchronized by the male's effect.

	Group		Index
	WAT	GLU	
Fertility	0.50 (7/14)	0.60 (9/15)	120
Prolificacy	1.57 (11/7)	1.38 (11/8)*	88
Fecundity	0.79	0.83	105

* One pregnant ewe from the GLU group died because of an acute pneumonia.

Figures

Figure 8 - Glycerol circulating concentrations in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during consecutive samplings performed every 30 min starting from the moment of their administration (panel A) and during different days of the experimental period (Day 18, Day 20 and Day 21; Day 16 - Day 19 = nutritional treatment administration; panel B). Asterisks indicate significant differences between groups ($P < 0.001$). [a, b] indicates significant variations within GLU group.

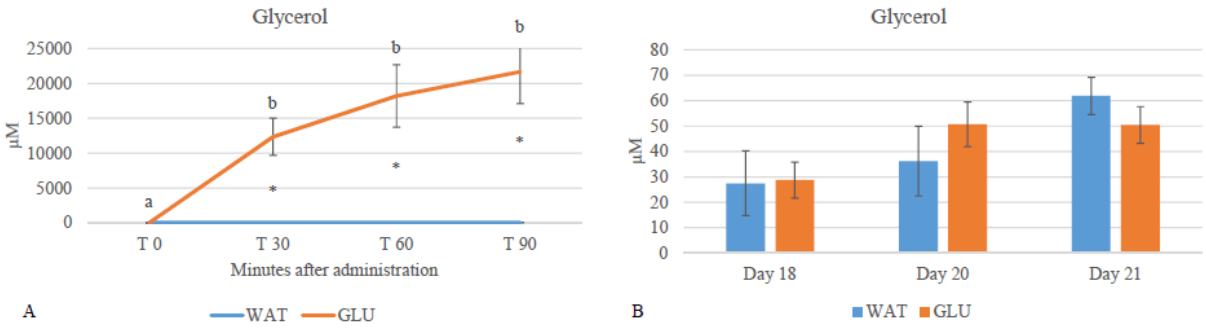
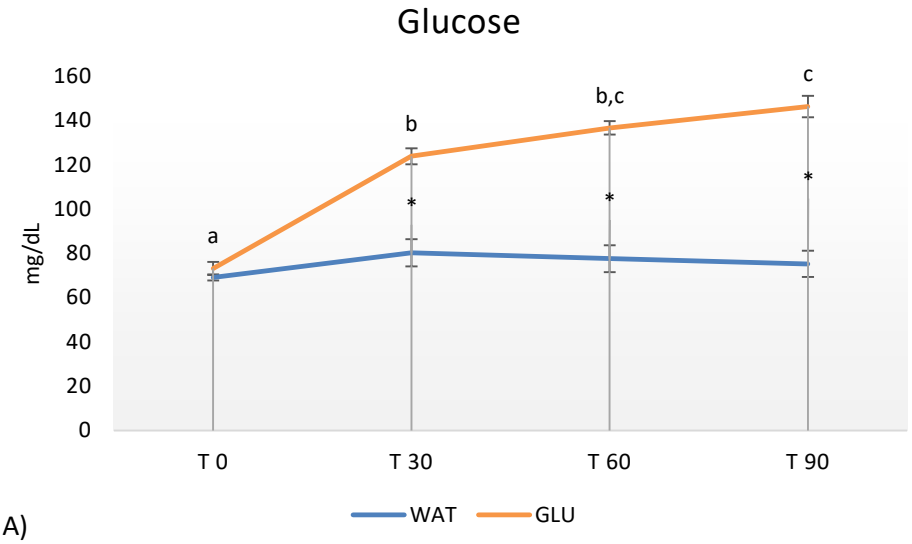
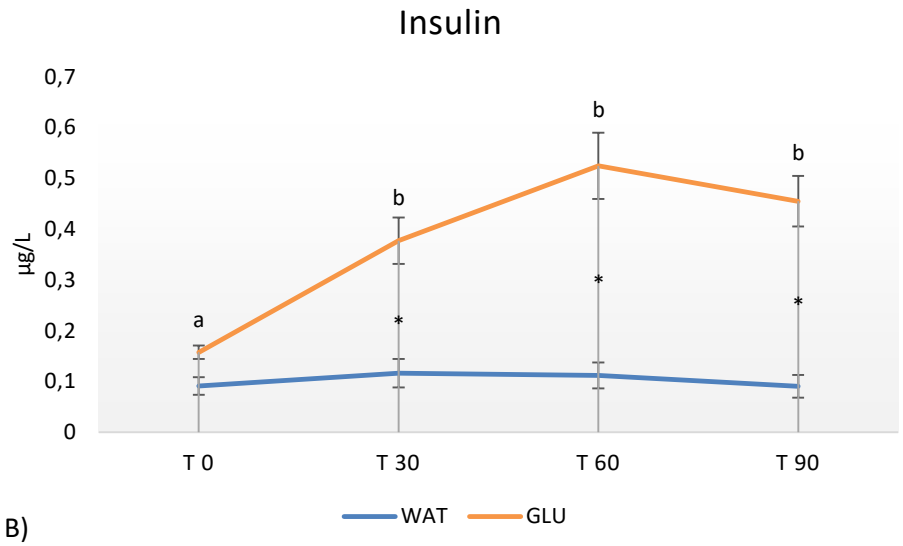


Figure 9 - Circulating concentrations of glucose, insulin, NEFA, and urea in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during their 3rd day of administration in consecutive samples performed every 30 min. Asterisks indicate significant differences between groups (P < 0.0001). a, b, c indicates daily significant variations within GLU group.



A)


Insulin
0,7
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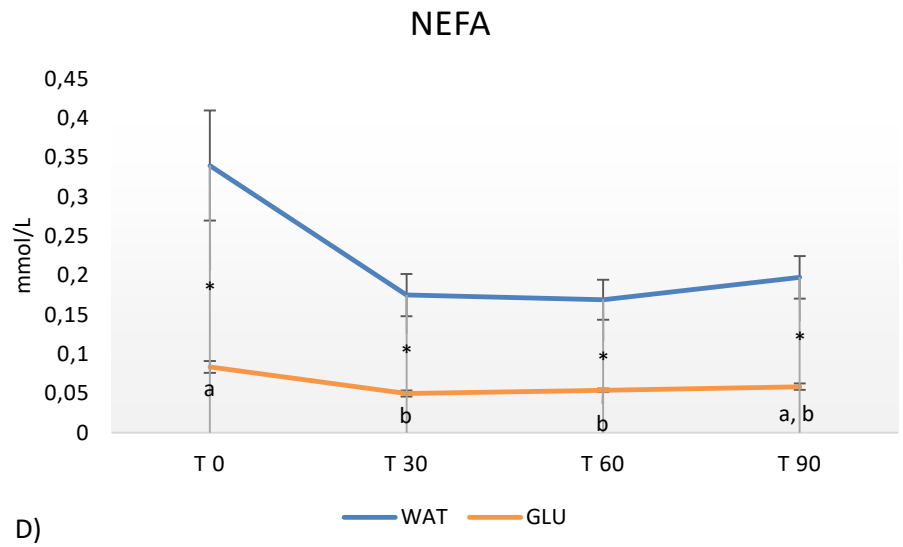
WAT
GLU

B)


NEFA
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0,35
0,3
0,25
0,2
0,15
0,1
0,05
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mmol/L
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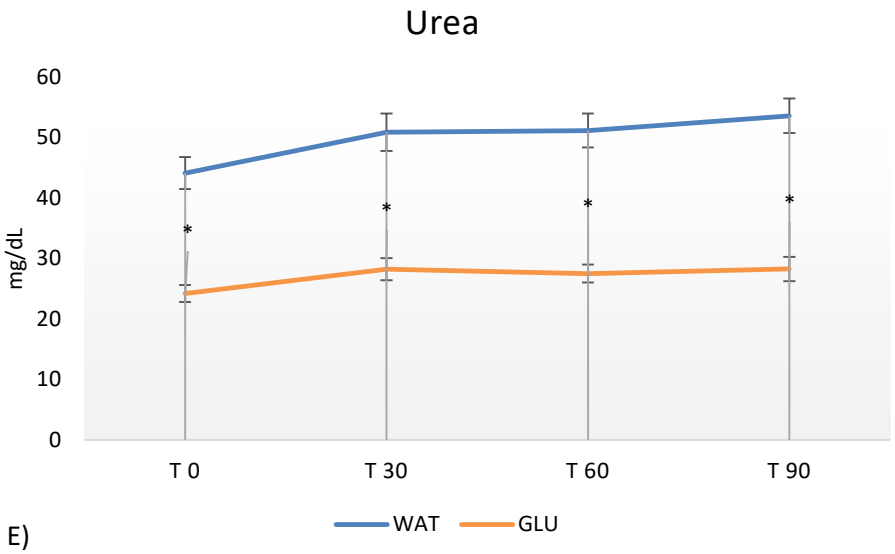
WAT
GLU

D)



E)

Figure 10 - Circulating concentrations of glucose, insulin, NEFA, and urea in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during different days of the experimental period (Day 0 = vasectomized ram's introduction; Day 16-Day 19 = nutritional treatment administration). Asterisks indicate significant differences between groups ($P < 0.0001$). [a, b, c] indicates daily significant variations within GLU group. [A, B, C] indicates daily significant variations within WAT group.

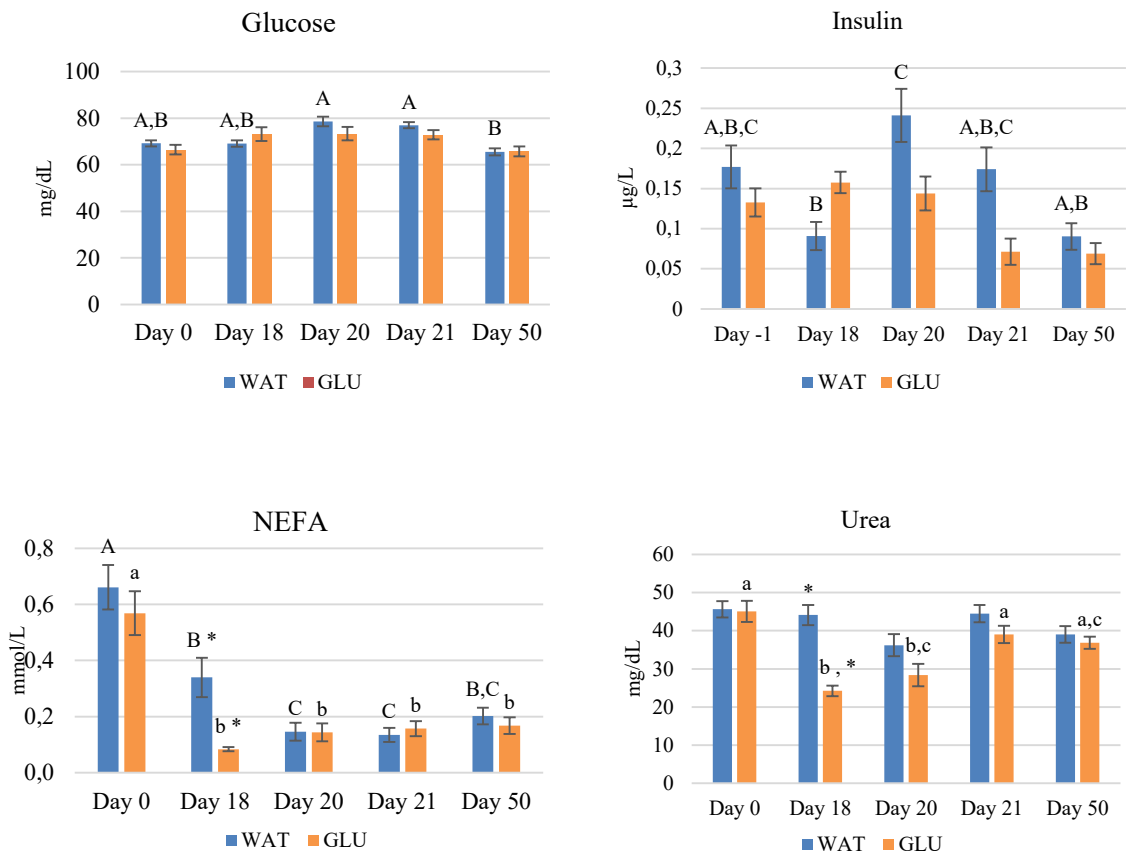
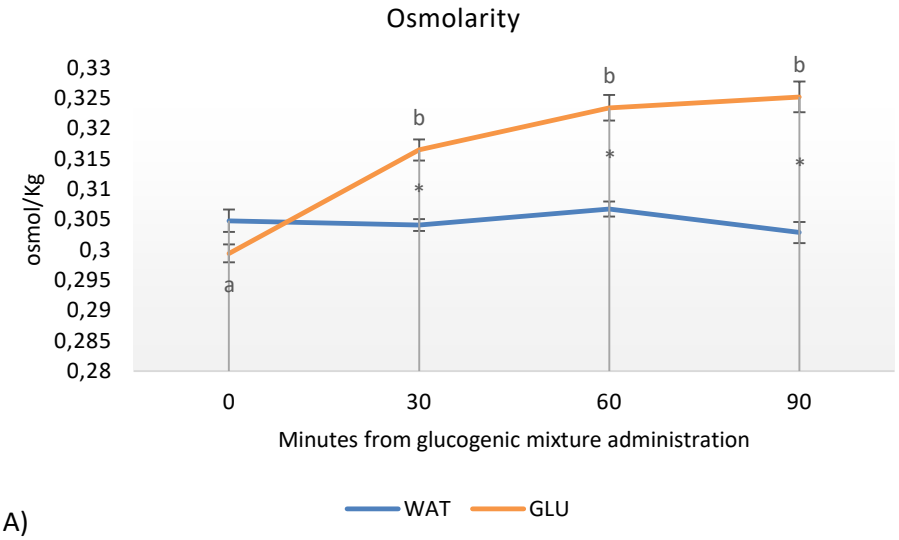
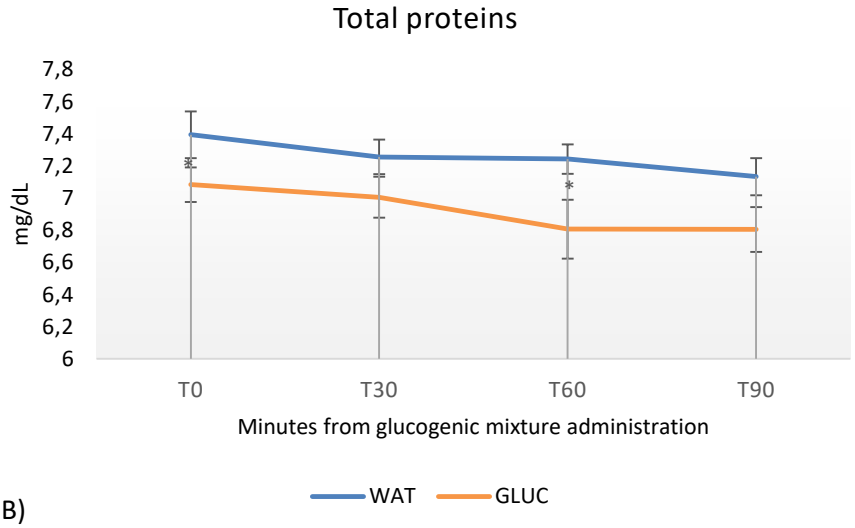


Figure 11 - Blood osmolarity and total circulating proteins in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during their 3rd day of administration in consecutive samples performed every 30 min. Asterisks indicate significant differences between groups (P < 0.0001). a, b, c indicates daily significant variations within GLU group.



A)



B)

Figure 12 - Live weight (panel A) and body condition score (BCS; panel B) in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during different days of the experimental period (Day 0 = vasectomized ram's introduction; Day 16 - Day 19 = nutritional treatment administration; Day 50 = pregnancy diagnosis).

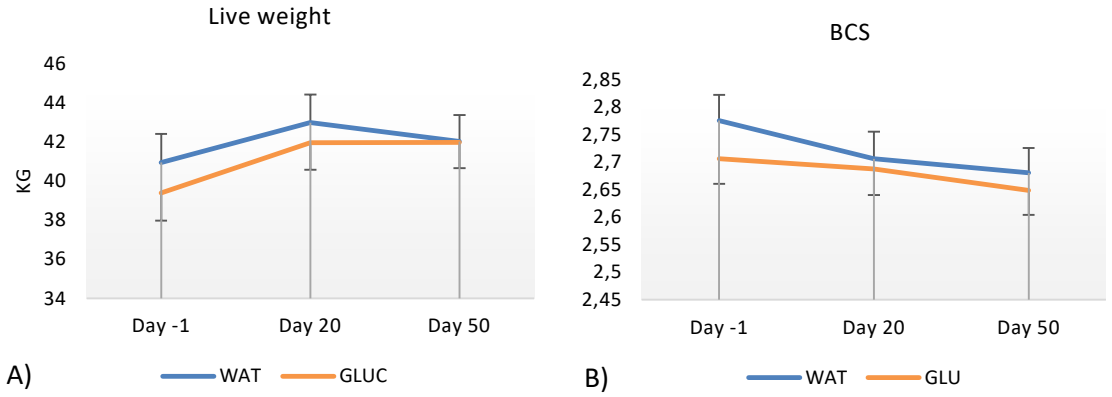


Figure 13 - Intake of concentrate in milked ewes orally dosed with either 400 ml/d of water (WAT) or 400 ml/d of a glucogenic mixture (GLUC). Concentrate was individually offered to all ewes at a level of 400 g/d (356 g DM/d). Bars denote SE. *** Difference between groups at level of P <0.001.

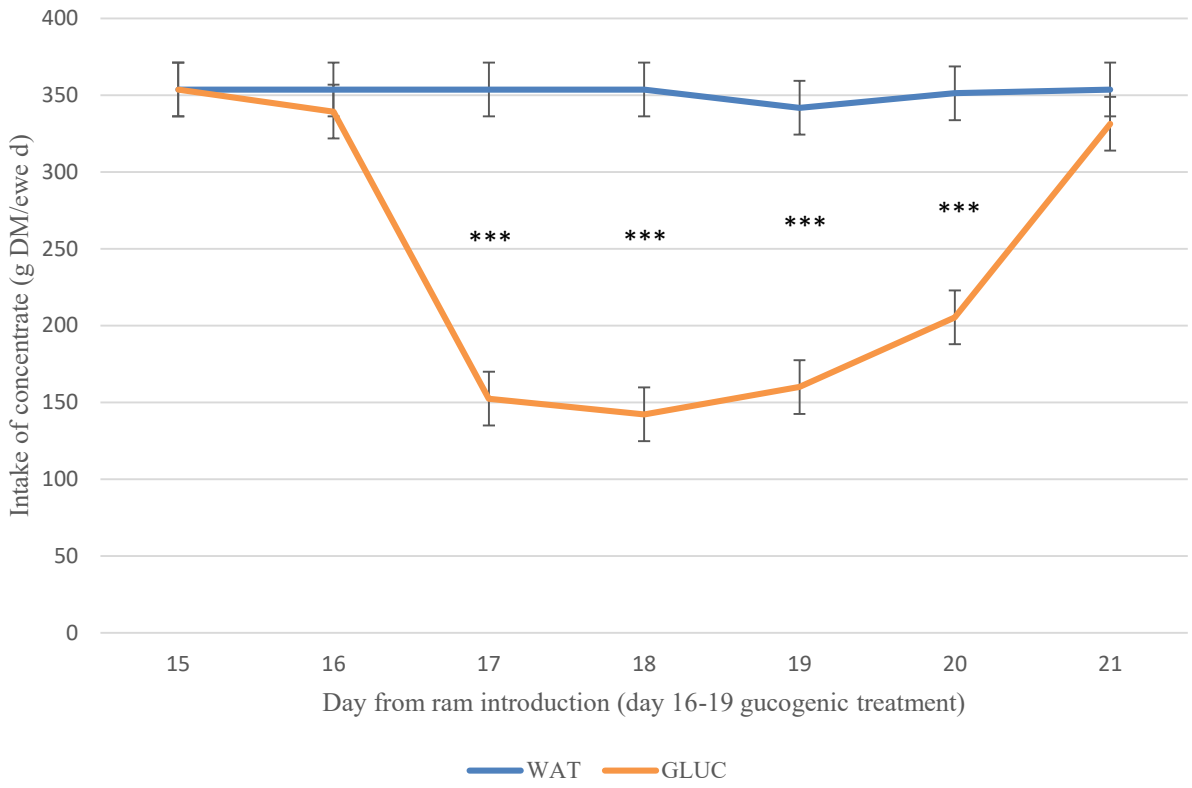
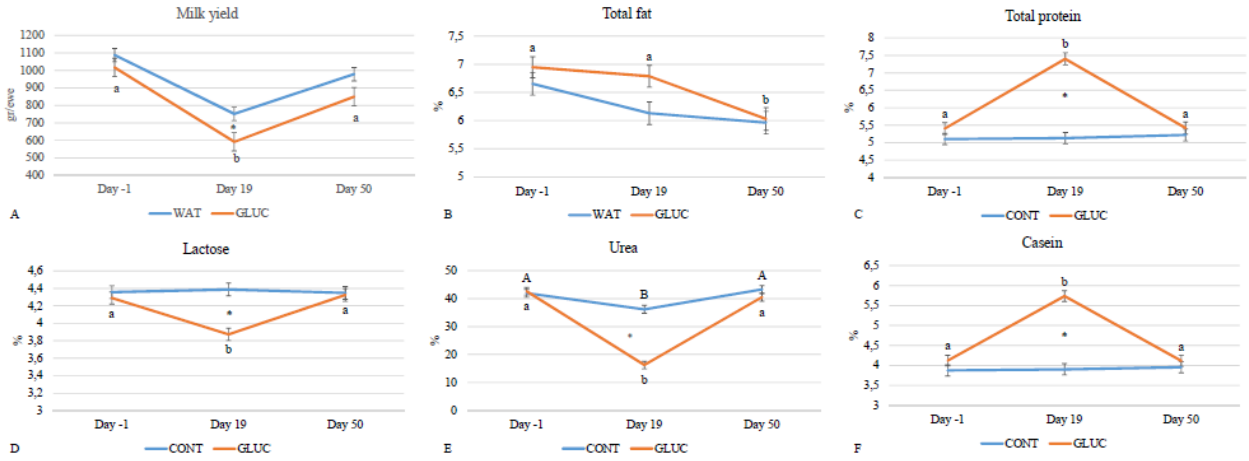


Figure 14 - Milk yield (panel A) and composition (panels B - F) in ewes orally drenched with a glucogenic mixture (GLU n = 15) or with water (WAT n = 14) during different days of the experimental period (Day 0 = vasectomized ram's introduction; Day 16 - Day 19 = nutritional treatment administration; Day 50 = pregnancy diagnosis). Asterisks indicate significant differences between groups ($P < 0.001$). [a, b] indicates significant variations within GLU group. [A, B] indicates significant variations within WAT group.



4.3.4. Discussion

The effect of a short term glucogenic mixture administration (23% DM) in dairy ewes during mid to late lactation caused: a) a 400-folds increase in circulating glycerol concentration, which in turns triggered significant modifications in the circulating concentrations of glucose, insulin, NEFA and urea during the nutritional treatment; b) a significant increase in plasma osmolarity and of blood volume; c) a significant reduction in concentrate intake, which was not accompanied by changes in ewe's nutritional status; d) significant changes in milk yield and composition, which were limited to the period of administration of the nutritional treatment; e) a lack of significant effects on reproductive performance although a slight numerical increase of ewe fertility and fecundity was detectable in the treated ewes.

After oral administration, a significant proportion of both glycerol and propylene glycol is absorbed by the rumen without fermentation through a passive diffusion and reaches the circulation to serve directly as substrates in the liver for glucose synthesis [188, 244]. The other proportion is either fermented by the rumen causing a reduction in the propionate to acetate ratio or may pass through the rumen without transformation [193, 245]. In the present experiment, oral drenching with the glucogenic mixture determined an immediate rise in circulating glycerol concentration, which in GLU ewes reached values 400-folds higher than in controls. However, glycerol clearance from the circulation was fast enough to allow the normalization of its level within 12 hrs form the oral drench, and

we did not observe any residual effect of the treatment in the two days following its end. Both in GLU and WAT ewes glycerol plasma level at fasting were in fact within similar, or even below, values reported for lactating ewes [246].

Its fast clearance from the bloodstream was due to its transformation to glucose by the liver. The rise in glycerol concentrations was, in fact, accompanied by an increase in circulating concentrations of glucose and hence of insulin, and by a decrease in NEFA and urea ones. These data confirm results reported in a previous experiment in which dry ewes were given the same nutritional treatment during an induced oestrus cycle [238]. However, the insulin-glucose system responded differently to the glucogenic mixture administration in lactating compared to dry ewes. In the present experiment, in GLU ewes, glucose peak value was 146.3 ± 4.9 mg/dL with corresponding values of insulin equal to 0.5 ± 0.1 µg/L. At the same time point (3rd day of treatment, 90 minute after the oral drench), glucose circulating concentration in dry ewes drenched with the same glucogenic mixture was 94.2 ± 4.7 mg/dL with corresponding values of insulin equal to 1.2 ± 0.1 µg/L [238]. The same pattern was observed in WAT group, with lactating ewes having higher level of glucose (75.9 ± 6.0 vs 60.5 ± 4.6 mg/dL in lactating and dry ewes respectively) and corresponding lower levels of insulin (0.09 ± 0.02 vs 0.18 ± 0.12 µg/L in lactating and dry ewes respectively) compared to dry ewes of the previous experiment. Thus, the glucose to insulin ratio was approximately 3 folds higher in lactating ewes compared to dry ones.

These differences may be due to difference in dietary energy intake, but may be also directly linked to difference in the metabolic status between physiological stages (dry and lactation period). Provision of glucose for mammary utilization is a metabolic priority for the lactating ewes, disruption of which can lead to lactation ketosis. This imperative has led to the evolution of metabolic adaptations in maternal non-mammary tissues which are regulated and coordinated to ensure that glucose supply to the lactating mammary gland is buffered against variations in maternal nutrition and other environmental influences. These homeorhetic adaptations are at least partly mediated by development of insulin resistance in maternal peripheral tissues during lactation. Insulin is a powerful regulator of nutrient partitioning. Besides its effect on glucose homoeostasis, insulin acts as antilipolytic in adipocytes [247] and depresses the hepatic ketone body formation [248]. Thus, low level of insulin or insulin resistance in the peripheral tissues promotes impaired glucose supply to the skeletal muscle and adipose tissue, increases lipolysis and enhances ketone body synthesis. Use of glucose for adipose tissue lipogenesis, which is already low during late pregnancy, is suppressed to minimal levels after the onset of lactation in ewes and is almost totally unresponsive to insulin at this time [249]. These adaptations result in a positive relationship between milk yield and insulin resistance, as demonstrated by low responsiveness of insulin-dependent tissues to the actions of insulin in cows with high milk yield [250, 251].

Thus, the different ewe's physiological status may explain differences in the response of the glucose-insulin system to the glucogenic treatment between the present experiment and the previous one [238]. In the same way, NEFA and urea circulating levels are higher in the present experiment than in the previous one, and confirm that during lactation lipolysis and proteolysis are increased to meet the energetic requirements of the mammary gland. Nevertheless, the glucogenic treatment applied significantly lowered their circulating levels, even if this effect was limited to the treatment period. Lower circulating levels of NEFA and urea have been reported in several studies aimed at assessing the effect of glycerol/glycerine supplementation in lactating cows [193, 252].

In the present experiment, total dry matter intake (DMI) did not differ between groups if calculated including the glucogenic mixture. However, GLU ewes ate less concentrate and less hay than WAT group. This result disagrees with previous findings showing no changes to DMI of Merino ewes fed up to 12% DM crude glycerin [253]. Authors reported that ewes supplemented with crude glycerin spent less time eating than the control group; however, these ewes did not consume less as a result. However, previous studies reported that DMI decreased linearly when crude glycerol replaced dry-rolled corn up to 30 or 45% DM in lamb diets [254] or when crude glycerine was given at high levels (201 g/Kg DM) to dairy cows fed corn silage-based diets [255]. A reduction in DMI was also observed in lactating cows when glycerol exceeded 10% of the diet DM [178] or when cows are fed up to 30% crude glycerine [256]. In the latter study, corn silage was top

dressed with crude glycerine and Authors speculated that it may have caused a decreased palatability of the diet. In the present experiment, the glucogenic mixture was administered by oral drenching, thus diet palatability cannot be the cause of the decreased DMI. Another possible explanation is a putative lower fiber digestibility in glycerol supplemented diet, as described in previous studies in which cows were fed with glycerol [178] or crude glycerine [255, 257]. In fact, low fiber digestibility is usually associated with low transit through the gastro-intestinal tract and hence limits DMI.

There is a paucity of information regarding sheep feeding behaviour and intake when supplemented with glycerine/glycerol, and further studies are needed to address this issue.

The present experiment also showed a significant modification in milk yield and composition following the short-term administration of the glucogenic mixture. Milk yield was significantly reduced in GLU 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 reporting that the dietary inclusion of crude glycerine for longer periods linearly decreased milk yield in dairy cows fed corn silage-based diets [255]. However, other studies on dairy cows showed that milk yield did not change [256, 257] or increased [252] when feeding glycerol supplemented diets. These studies often differ for glycerol fed, for the duration of the supplementation, and for the lactation stage.

Milk yield greatly depends on mammary lactose synthesis due to its osmoregulatory property for mammary uptake of water. Thus, milk yield decrease could be also a consequent of the decrease in its lactose content. Ezequiel et al. (2015) reported that milk lactose concentration and milk lactose yield tended to decrease linearly with increasing inclusion of crude glycerine in dairy cows diet [256]. However, this reduction was not accompanied by a significant reduction in milk yield. Other studies reported that milk composition was unaffected by glycerol feeding [178, 258].

We can also speculate that the observed decrease in milk yield could be due to the lower concentrate intake which may have caused a reduction in dietary CP content in GLU ewes, and may have consequently altered metabolizable protein available to be absorbed in the duodenum. The yield of solids components in milk (fat, protein and lactose) would have the same response of milk yield. However, while lactose decreased in GLU ewes compared to WAT ones, total protein and casein content increased significantly during the treatment in GLU ewes.

Another possible explanation is that in ewes given the glucogenic mixture the decline in milk yield may have been caused by a shorten in water availability needed for the dilution of the milk solid components. In GLU ewes, in fact, plasma osmolarity significantly increased compared to WAT ones. This increase triggered water reabsorption from the extracellular fluids to the bloodstream, as suggested by the decrease in plasma total protein concentration, which indicates haemodilution. Water accounts for the 82.5% of

milk volume in sheep [259], and an acute drop ofwater availability to the mammary gland may possibly explain the decrease in milk yield observed during the administration of the glucogenic mixture in GLU ewes.

Regarding milk protein, previous studies have controversial results. In cow milk, authors have reported either decreases of milk protein concentration with up to 3 kg/d of dietary glycerol [260], or increases of milk protein concentration [261, 262]. In the current experiment milk protein content increased during the treatment together with casein content. On the other hand, milk fat was not affected by the treatment. Previous studies using lower glycerol concentrations showed a reduction in milk fat [263, 264]. However, other studies showed that milk fat content showed a quadratic effect, decreasing with 15% dietary glycerin and increasing back with 30% dietary glycerin [256]. The speculation is that this U-shaped dose–response curve may result from a threshold effect depending on the level of Non fiber carbohydrates (mainly starch) in cow diet.

It is noteworthy that in the present experiment all these changes in milk yield and composition were limited to the treatment period, and thereafter no difference was recorded in milk characteristics between GLU and WAT group.

As for reproductive performances, the glucogenic treatment applied numerically increased ewe's fertility, but the difference was not statistically significant, and overall

reproductive performances did not differ between the two experimental groups. Previous findings showed an increase in oocyte developmental competence in dry ewes given the same glucogenic treatment during an induced oestrus cycle [237], most likely due the creation of a suitable environment for the final follicular growth both at the systemic and at the follicular level [238]. Other Authors showed that dry ewes given a single oral glucogenic dosage (100 mL of the same mixture of the present experiment) immediately before introducing the rams in the flock had higher ovulation rates than controls if primed with intravaginal sponges containing low MPA dose (10 mg). On the other hand, the opposite was found when sponges having high MPA dose (60 mg) were used [265]. An increase in ovulation rate was found also in a previous study on Manchega ewes given the same treatment applied in the present experiment and it was linked to an enhancement in the developmental competence of preovulatory follicles [36]. In the same way, Karami-Shabankareh et al. demonstrated that feeding glycerol to primiparous Holstein dairy cows improved reproductive performance in the early post-partum period by significantly decreasing days to first ovulation and to first oestrus and days open [266].

In the present experiment, the high external temperatures recorded in the mating days may have partially compromised ewe's reproductive performances, and mating success.

In conclusion, the present experiment extends our knowledge on the effect of a high dose glucogenic mixture administration in dairy ewes in terms of productive and reproductive performances. Homeorhetic adaptation to lactation plays a central role in determining

energy partitioning in mid-lactation dairy ewes, and the metabolic status influences the response to the glucogenic treatment. For this reason, further studies are needed to determine whether peripheral tissues, as the ovary, are able to take advantage of the transient hyperglycemia caused by the nutritional treatment when the glucose to insulin ratio is high and the tissue responsiveness to insulin is likely to be lower than during the dry period.

4.5. Conclusion

These experiments showed that short-term administration of a glucogenic mixture significantly affect circulating concentration of metabolites and hormones.

In dry ewes, the first experiment showed a raise of circulating concentrations of glucose, insulin, and IGF-1, and a drop of circulating concentration of NEFA and urea. These metabolic changes ultimately affected FF microenvironment, as evaluated 12 h after the end of the nutritional treatment. In particular, they led to an increase in FF concentrations of glucose, without affecting FF insulin and IGF-1 concentrations, nor GSH content and total antioxidant capacity. However, FF concentrations of NEFA and urea were significantly lowered. A reduced clearance of NEFA was observed within the follicle, thus underlying that the negative effects on follicular growth related with high NEFA concentrations can persist even after the re-establishment of a positive energy balance. All in all, these results suggest that short-term administration of a glucogenic mixture in dry ewes creates a suitable micro-environment for the final follicular growth and thus for the conception period in the ewe.

The second experiment extended our knowledge on the effect of a high dose of glucogenic mixture administration in lactating ewes. Although many metabolic response to glucogenic mixture administration were similar in both dry and lactating dairy ewes. However, homeorhetic adaptation in lactating ewes lead the insulin-glucose system to

respond differently in lactating compared to dry ewes, with the glucose to insulin ratio approximately 3 folds higher in lactating than dry ewes. In the same way, NEFA and urea circulating levels were higher in lactating ewes, and confirm that during lactation lipolysis and proteolysis are increased to meet the energetic requirements of the mammary gland. Nevertheless, the glucogenic treatment applied significantly lowered their circulating levels, even if this effect was limited to the treatment period.

Moreover, the second experiment showed a decrease in ewe DMI and changes in milk yield and composition related to the treatment. The DMI decrease can be related by a lower fiber digestibility of glycerol-supplemented diet. Milk yield decrease cold be related to its lactose content or to the shorten in water availability for the mammary gland consequent to the increase in blood volume triggered by the increase in plasma osmolarity consequent to the high glycerol circulating concentration.

Finally, reproductive performances and mating success could have been compromised from high external temperatures recorded even if treatment numerically increased ewe's fertility.

To conclude, the different ewe's physiological status may explain the differences in the response of the glucose-insulin system to the glucogenic treatment in the two studies. For this reason, further research is needed to determine whether peripheral tissues and organs, namely the ovary, are able to take advantage of the transient hyperglycaemia

caused by the nutritional treatment when the glucose to insulin ratio is high and the tissue responsiveness to insulin is possibly limited.

5. GENERAL CONCLUSION

5. GENERAL CONCLUSION

The first study underlined that background grazing during lactation can exert carry-over effects on ewe's reproductive performances, modulating the response to a short-term flushing and to the male effect, even if mating is prepared by a flushing treatment. Although different studies indicate that supplementation with lupin grain for 4–6 days before ovulation increases ovulation rate, in Study 1, the success of flushing with lupin grain, in terms of ovulation rates and then prolificacy, was related to nutrition management during lactation. Sheep that grazed for 2 h/d in Italian ryegrass, compared with sheep that grazed for 6 h/d, exhibited a significant reduction of prolificacy related to first heat and to the breeding season and a negative trend on lamb concentration and twin rates. The same restriction on berseem clover showed a residual effect evidenced by a lower resumption of behavioral estrus, followed by a negative concentration of lambing and a negative trend in prolificacy. The variability of the response at flushing treatments appear to find its cause on historical nutritional plans applied previously to the mating season, and possibly related to ewe's physiological status. Regardless the type of pasture offered in the experiments discussed above, treatments displayed residual effects on reproduction related to energy requirements around mating. The metabolic consequences of dietary supplementation in sheep can depend on the energy balance condition. This underfeeding can have overridden the flushing treatment effect, explaining the negative trends of reproductive performance. Energy intake is a key

limiting factor of sheep production in animals raised at pasture, hence insufficient energy resulted in lowered reproductive performance.

These results suggest that a restriction of time access to pasture during mid-lactation, besides constraining intake and productive performance in mid lactation, can subsequently impair the reproductive performance of late-lactation sheep, even if submitted to both male effect and flushing with lupin. Breeders that want to improve reproductive and productive performance of the flock, have to consider the pasture offered to the ewes, in terms of hours spent grazing and quality of the herbage. It is important to be able to assess whether the energy intake is sufficient for sheep productive state, evaluating to give nutritional supply as concentrate, to avoid that subsequent reproductive season will be compromised.

Short targeted feeding regimes, or "focussed feeding", are a common strategy to improve the reproductive performance of sheep. In Study 2 we investigated the effect of a short-term glucogenic flushing and obtained results demonstrated that it triggered metabolic and hormonal changes in both dry and lactating ewes.

In non-lactating ewes, that have a low energy requirement, the effects short-term administration of a glucogenic mixture, on hormones and metabolites, seems to creates a suitable micro-environment for the final follicular growth and thus for the conception period in the ewe. In fact glucose availability for the ovarian function increase, and so the insulin and IGF-1 system, while circulating concentration of NEFA and urea, that can exert

a toxic effect on follicles, decreased. These metabolic changes ultimately affected FF microenvironment, as evaluated 12 h after the end of the nutritional treatment.

The second experiment extended our knowledge on the effect of a high dose of glucogenic mixture administration in lactating ewes. Results confirmed a similar effect of the glucogenic mixture administration in dry ewes. However, homeorhetic adaptation to lactation lead the insulin-glucose system to respond differently in lactating compared to dry ewes, with the glucose to insulin ratio approximately 3 folds higher in lactating ewes compared to dry ones. The different ewe's physiological status may explain the differences in the response of the glucose-insulin system to the glucogenic treatment in the two studies. Moreover, a decrease in ewe DMI maybe related by a lower fiber digestibility of glycerol-supplemented diet. Also milk yield decrease cold be related to its lactose content or to the shorten in water availability for the mammary gland consequent to the increase in blood volume triggered by the increase in plasma osmolarity consequent to the high glycerol circulating concentration.

One of the goals of this experiment was to evaluate reproductive performances and mating success when lactating ewes are treated with a glucogenic mixture before mating. Results could have been compromised from high external temperatures recorded even if treatment numerically increased ewe's fertility with no significant difference.

For this reason, further research is needed to better evaluate the effect on reproduction, in lactating ewes, and to determine whether peripheral tissues and organs, namely the

ovary, are able to take advantage of the transient hyperglycaemia caused by the nutritional treatment when the glucose to insulin ratio is high and the tissue responsiveness to insulin is possibly limited.

6. References

6. References

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Publications

Part of the results of this dissertation have already been released in various conferences and journals of national and international scientific interest, as cited below. It is currently being drafted the second manuscript related to this dissertation.

Posters

S. SUCCU, V. PASCIU, S. GADAU, E. SERRA, G. LEONI, F. BERLINGUER, A. ZINELLU, C. CARRU, E. SOTGIU, V. SATTA, C. PORCU, M. E. MANCA, S. NAITANA - Resumption of atp levels and mitochondrial functionality in vitrified/warmed ovine oocytes - ICAR 2016 – Tours France – Poster

E. SERRA, S. SUCCU, F. BERLINGUER, C. PORCU, G. LEONI, S. NAITANA, S. GADAU - Tubulin posttranslational modifications in in vitro matured ovine oocytes- Resumption of atp levels and mitochondrial functionality in vitrified/warmed ovine oocytes - ICAR 2016 – Tours France – Poster

C. PORCU, S. SUCCU, V. PASCIU, M.E. MANCA, M. DATTENA, M. GALLUS, S. NAITANA, G. MOLLE, F. BERLINGUER - Short-term administration of glucogenic mixture modifies plasma and follicular fluid metabolic environment in the ewe - ICAR 2016 – Tours France – Poster

S. CHELUCCI, V. PASCIU, S. SUCCU, F. BERLINGUER, G.G. LEONI, V. SATTA, C. PORCU, M.E. MANCA, S. NAITANA - Seasonal variations of blood antioxidant capacity and spermatozoa fertilizing potential in Sarda breed buck - University of Sassari, Italy - XXII International Congress FEMESPRUM - University of Sassari, Italy - Poster

Oral communications

C. PORCU, M. DATTENA, M. GALLUS, F. BERLINGUER, S. NAITANA, G. MOLLE – 2015 - Preliminary study on the residual effect of part-time grazing in dairy ewes submitted to ram effect and flushing with lupin - XXII International Congress FEMESPRUM - University of Sassari, Italy

M. DATTENA, C. PORCU, A. RODRIGUEZ, M. GALLUS, G. BOMBOI, G. MOLLE, A. CABIDDU - Preliminary results on reproduction performances in Sarda dairy sheep supplemented with

cooked molasses licking block supplementation - XXII International Congress FEMESPRUM - University of Sassari, Italy

PORCU C*, RODRÍGUEZ Á*, GALLUS M, PILICHI S, DATTENA M. - Improving embryo survival by assisted uterine embryo migration in Sarda ewe: a preliminary study - 30th Scientific Meeting of the European Embryo Transfer Association - September 12th - 13th 2014, Dresden, Germany (*both authors contributed equally to this work)

PORCU C., RODRIGUEZ A., DATTENA M., GALLUS M., PILICCHI S., MOLLE G. - Studio preliminare sull'effetto residuo di un pascolamento razionato in pecore sottoposte a effetto maschio e flushing con lupino - XXI CONGRESSO NAZIONALE S.I.P.A.O.C. Società Italiana di Patologia ed Allevamento degli Ovini e dei Caprini 9/12 settembre 2014 – Foggia – Italy

Scientific International Journals

C. PORCU, V. PASCIU, S. SUCCU, E. BARALLA, M.E. MANCA, E. SERRA, G.G. LEONI, M. DATTENA, G.C. BOMBOI, G. MOLLE, S. NAITANA, F. BERLINGUER, Glucogenic treatment creates an optimal metabolic milieu for the conception period in ewes, Domestic Animal Endocrinology, Volume 59, April 2017, Pages 105-115, ISSN 0739-7240.

PW1551 - Short-term administration of glucogenic mixture modifies plasma and follicular fluid metabolic environment in the ewe

Authors and co-authors:

Porcu Cristian (1), Succu Sara (1), Pasciu Valeria (1), Manca Maria Elena (1), Dattena Maria (3), Gallus Marilia (3), Naitana Salvatore (2), Molle Giovanni (3), Berlinguer Fiammetta (2)

(1) University of Sassari, Department of Veterinary Medicine, 07100, Sassari, Italy

(2) University of Sassari, Department of Veterinary Medicine; Sardinian Animal Biodiversity Center (Centro di Competenza per la Biodiversità Animale- CCBA), 07100, Sassari, Italy

(3) AGRIS Sardegna- Agency of Agriculture Research of Regione Autonoma della Sardegna, Department of Research for Animal Productions , 07040, Olmedo - Sassari, Italy

This study aimed at determining changes in plasma and intrafollicular milieu consequent to short-term glucogenic treatment (70% glycerol, 20% 1,2-propanediol glycerol, 10% water). Oestrous cycles were synchronised in 20 Sarda ewes by the insertion of one intravaginal progestagen impregnated sponge left in situ for 6 days. After removal (D 0), the ewes were randomly allocated into two experimental groups (treated (T) and control (C) ewes). From D 7 to D 10, T ewes received oral administration of a glucogenic mixture, whereas C animals received water. Follicular development was stimulated by FSH administration from days 8 to 10. At D 11, twelve hours after the last administration, ovaries were collected after slaughtering and follicular liquid retrieved. Differences in follicular fluid and plasma metabolite and hormone concentrations between the two groups were assessed by a general linear model and a mixed model for repeated measurements, respectively. The method used to discriminate between the means was the Tukey's test for multiple comparisons. Results showed that from D 7 to D 10 glucose mean plasma values rose significantly in T group, being higher than the controls (T=79.312±1.871mg/dL; C=64.061±1.874mg/dL; p<0.0001). A parallel increase in insulin plasma levels was detected in T group (T=0.822±0.067µg/L; C=0.134±0.067µg/L; p<0.0001). Even if mean IGF-1 plasma levels did not differ between groups (T=84.181±3.667ng/mL; C=74.549±3.634ng/mL; p=0.068), T group showed higher values than C group on D 9 (T=98.813±8.371ng/mL; C=76.721±6.211ng/mL; p<0.05) and on D 11 (T=133.063±13.152ng/mL; C=82.876±7.762ng/mL; p<0.01). A significant effect was also observed in mean plasma concentrations of NEFA (T=0.0368±0.0194mmol/L; C=0.199±0.0194mmol/L; p<0.0001) and urea (T=15.729±0.836mg/dL; C=22.157±0.836mg/dL; p<0.0001), with lower value in T as compared to C group. In the same way, in T ewes the significant rise in intrafollicular glucose concentration (T=49.217±7.875mg/dL; C=26.078±3.134mg/dL; p=0.016) was accompanied by a drop in NEFA (T=0.217±0.027mmol/L; C=0.360±0.034mmol/L; p=0.0047) and urea (T=11.524±1.936mg/dL; C=26.959±2.822mg/dL; p<.0001) levels as compared to controls. Glucose is a prerequisites for follicle growth [1], and insulin and IGF-I are metabolic hormones that stimulate follicular cells [2]. Moreover, it is known that high-circulating concentrations of NEFA and urea are associated with lower fertility [2]. Thus, we can conclude that the results obtained indicate that short-term glucogenic treatment can help to create a suitable metabolic milieu for the conception period.

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PW172 - Resumption of ATP levels and mitochondrial functionality in vitrified/warmed ovine oocytes.

Authors and co-authors:

Sara SUCCU (1), Valeria PASCIU (1), Sergio GADAU (1), Elisa SERRA (1), Giovanni G LEONI (2), Fiammetta BERLINGUER (2), Angelo ZINELLU (3), Ciriaco CARRU (3), Elisabetta SOTGIU (3), Valentina SATTA (1), Cristian PORCU (1), Maria Elena MANCA (1), Salvatore NAITANA (2)

(1) University of Sassari, Department of Veterinary Medicine, 07100, Sassari, Italy

(2) University of Sassari, Department of Veterinary Medicine; Sardinian Animal Biodiversity Center (Centro di Competenza per la Biodiversità Animale- CCBA), 07100, Sassari, Italy

(3) University of Sassari, Department of Biomedical Sciences, 07100, Sassari, Italy

Alterations in mitochondrial functionality and ATP production have been documented in cryopreserved oocytes. However the capability of vitrified-warmed oocytes to recover these damages associated to cryopreservation has not been established yet. The aim of this study was to evaluate the resumption of mitochondrial activity in vitrified ovine oocytes during the post-warming culture. Metaphase II (MII) oocytes were vitrified according to standard procedure used in our laboratory. After warming, oocytes were cultured in 5% CO_2 in air at 39°C. At fixed time-points (0, 2, 4 and 6 hours) the oocytes were processed for: 1) determination of intracellular ATP levels by capillary electrophoresis; 2) assessment of active mitochondrial distribution using MitoTracker Red CM-H_2XRos probe and analyzed through confocal microscopy. For each time-points, n=20 vitrified-warmed oocytes plus n=20 fresh MII oocytes (as a control) were used for the analyses above described. Differences in mitochondrial activity and ATP concentration between control and vitrified oocytes were assessed by ANOVA, while differences in mitochondrial distribution patterns were analyzed by Chi square test. ATP levels were higher (P 0.01) in fresh than in vitrified-warmed oocytes. Moreover differences have been found in vitrified oocytes during post-warming culture. Indeed, from 0 to 2h of culture we observed a decline of ATP levels without significant differences, while at 4 and 6h post- warming the ATP concentration increased significantly but did not reach the higher value seen in fresh oocytes (P<0.01). Also mitochondrial activity showed higher value in fresh oocytes compared to vitrified ones (P<0.01). In particular during the culture post-warming, the value was significantly lower at 0 and 6h compared to fresh oocytes and to 4h of culture, while at 2h the value was intermediate between the time point 0 and 4h (P<0.01). Referring to mitochondrial distribution patterns, we classified it in three groups: 1) Pattern A: homogeneous FINE; 2) Pattern B: homogeneous GRANULAR; 3) Pattern C: heterogeneous CLUSTERED. In fresh group a lower percentages of oocytes have shown the pattern A compared to vitrified oocytes (P<0.05). For pattern B no significantly differences were evidenced among groups. For pattern C, fresh and warmed oocytes at 6h have shown the higher percentages compared to warmed oocytes at 0, 2, and 4h of culture (P<0.01). No differences were evidenced between fresh and warmed oocytes at 6 h. In conclusion mitochondrial function and ATP level increased significantly in vitrified/warmed ovine oocyte within 6h of in vitro culture. Time of mitochondrial activity resumption after vitrification could be used to standardize the vitrification protocols and to improve the developmental competence in vitrified/warmed oocyte. This study was supported by MIGLIOVINGENSAR project.

PW551 - Tubulin posttranslational modifications in in vitro matured ovine oocytes

Authors and co-authors:

SERRA Elisa (1), SUCCU Sara (1), BERLINGUER Fiammetta (1), PORCU Cristian (1), LEONI Giovanni G (1), NAITANA Salvatore (1), GADAU Sergio (1)

(1) University of Sassari, Department of Veterinary Medicine, 07100, Sassari, Italy

Microtubules (MTs), polymers of alpha/beta-tubulin heterodimers, are involved in several cellular functions including shape maintaining, mitotic/meiotic spindle organization and intracellular trafficking. MTs physiology can be influenced by a variety of posttranslational modifications (PTMs), including tyrosination, detyrosination, delta 2 modification, acetylation, polyglutamylation, polyglycylation, phoshorilation and palmitoylation. In the mammalian oocyte, MTs are essential for first and second meiosis, regulating the correct formation of meiotic spindle, crucial step for chromosomes movements. In the last decades, several works investigated the role of few tubulin PTMs (acetylated and tyrosinated alpha-tubulin), especially in mouse and human oocytes. Considering that tubulin PTMs expression and function have not been investigated in ovine oocytes, this study aimed at extending our knowledge on tubulin PTMs expression in in vitro matured ovine oocytes.

Cumulus–oocytes complex (COCs) were obtained from adult Sarda ewes regularly slaughtered at the local abattoir. COCs were in vitro matured in TCM 199 supplemented with 10% heat-treated oestrus sheep serum, 10 µg /mL of FSH/LH, 100 µM cysteamine, 8 mg/mL of pyruvate for 24 h in 5% CO2 in air at 39°C. Metaphase II oocytes were fixed in PBS/2.5% paraformaldehyde solution and processed for indirect immunofluorescence and confocal microscopy analyses. Primary antibodies against the most representative tubulin PTMs were used (tyrosinated n=20, detyrosinated n=20, acetylated n=20, polyglutamylated n=20, delta 2 n=20, total alpha-tubulin n=20, total oocytes n=120). Through the fluorescence emission quantification, we found that acetylated and tyrosinated alpha-tubulin signal was well detectable in both meiotic spindle and polar body. In particular, tyrosinated tubulin was significantly higher than acetylated in meiotic spindle ($p=0.0414$), whereas no differences were found on the polar body ($p=0.5166$). In contrast, no immunopositivity were appreciable for detyrosinated, polyglutamylated and delta 2 tubulin in both meiotic spindle and polar body.

Our results provide first information about the diverse tubulin PTMs involved in oocytes spindle organization of ovine species. This data could be useful in order to better understand the role played by each tubulin PTMs in the acquisition of oocyte competence.

Studio preliminare sull'effetto residuo di un pascolamento razionato in pecore sottoposte a effetto maschio e flushing con lupino

C. PORCU, A. RODRIGUEZ, M. DATTENA, M. GALLUS, S. PILICHI, G. MOLLE

Agris, Dipartimento per la Ricerca nelle Produzioni Animali, loc. Bonassai, 07040 Olmedo, Italia

Parole chiave: ciclo ovarico, fertilità, prolificità, nutrizione.

INTRODUZIONE - Nelle pecore a fine anaestro, l'esposizione repentina all'ariete può portare ad ovulazione e sincronizzazione dei calori (Dattena et al., 2010; Rosa et al, 2002). Questo evento detto "effetto maschio" può essere influenzato da diversi fattori, che possono essere intrinseci agli animali, come razza, peso vivo (PV), stato di ingrassamento (BCS), stadio di lattazione, livello produttivo o dipendere dal sistema di allevamento, ed, in particolare, dall'alimentazione.

L'obiettivo di questo lavoro è stato valutare l'effetto residuo del pascolamento razionato (Molle et al., 1995) di pecore da latte al quinto mese di lattazione sulla loro risposta all'effetto maschio e flushing alimentare in termini di ripresa dell'attività ovarica, della fertilità e della prolificità.

MATERIALI E METODI - A questo scopo sono state utilizzate n. 36 pecore di razza Sarda ad iniziare dal terzo mese di lattazione. A partire dal 18 febbraio le pecore sono state gestite come gruppo unico ed adattate al pascolo razionato (4 ore/giorno) di loglio italico (*Lolium multiflorum)*, ricevendo giornalmente come integrazione 700 g/capo di fieno di loglio trinciato e 700 g/capo di concentrato commerciale. Dall'11 Marzo le pecore sono state suddivise in tre gruppi da dodici soggetti, omogenei per età, PV, BCS e produzione lattea assegnati a tre trattamenti che prevedevano l'accesso al pascolo a orario con il seguente programma: G2h, 2 ore/giorno dalle h. 08:00 alle h. 10:00; G4h, 4 ore/giorno dalle h. 08:00 alle h. 12:00; G6h, 6ore/giorno dalle h. 08:00 alle h. 14:00. Dal 26 Marzo 300 g/capo di concentrato commerciale sono stati sostituiti con 300 g/capo di semi di lupino.

Dal 30 Aprile gli animali sono stati messi assieme in un unico gruppo, portando tutti i capi a 6 ore/giorno di permanenza al pascolo di loglio (07:30-13:30).

Il 02 Maggio (identificato come giorno 0) per indurre l'"effetto maschio" sono stati introdotti quattro maschi adulti, interi e non tosati. Dal 18 Maggio (giorno 16) è stato effettuato un flushing alimentare con 400 g/capo di semi di lupino e 50 g/capo di concentrato commerciale per 5 giorni. Il 18 Maggio è iniziato il controllo dei calori per 8 giorni, tre volte al giorno (h 08:00, h14:00 e h16:00) con l'ausilio di pastelli. Il 29 Giugno (giorno 58) è stato rilevato il 3° e ultimo calore e dopo 8 giorni sono stati tolti i maschi dal gruppo.

Nel corso dello studio sono morte 5 pecore per Blue Tongue, distribuite in tutti i gruppi. Nello stesso periodo non sono stati registrati effetti residui significativi dei trattamenti su produzione individuale di latte, PV, BCS e loro variazioni.

RISULTATI E CONSIDERAZIONI - Tutti i gruppi hanno ripreso l'attività ovarica all'introduzione del maschio (G2h 83% = 10/12; G4h 75% = 9/12; G6h 75% = 9/12, P>0.2, Fisher Exact test a due code). La percentuale di fertilità al primo calore è stata in G2h 30% (3/10), in G4h 70% (7/10) e in G6h 64% (7/11, P = 0.17 tra G2h e G4h) (Fig. 1). La fertilità complessiva relativa alla stagione riproduttiva (tre cicli ovarici) invece è stata in G2h 70% (7/10), in G4h 80% (8/10) e in G6h 73% (8/11, P>0.2) (Fig. 1). La prolificità al 1° calore è stata in G2h 3/3 (1 agnello), in G4h 11/7 (1.6 agnelli) e in G6h 13/7 (1.9 agnelli, P<0.05 tra G2h e G6h) (Fig. 2), mentre la prolificità relativa alla stagione è stata in G2h 10/7 (1,4 agnelli), in G4h 12/8 (1,5 agnelli) e in G6h 15/8 (1,9 agnelli, P=0.11 tra G2h e G6h) (Fig. 2).

La percentuale di concentrazione dei parti* è stata in G2h 43% (3/7), in G4h 86% (7/8) e in G6h 86% (7/8) (Fig. 1). I parti gemellari sul totale delle pecore partorite sono stati rispettivamente in G2h 3/7 (43%), in G4h 4/8 (50%) e in G6h 7/8 (86%) (Fig. 1).

* Numero di parti con data riferibile al 1° ciclo ovarico/Numero di parti con data riferibile ai primi tre cicli ovarici.

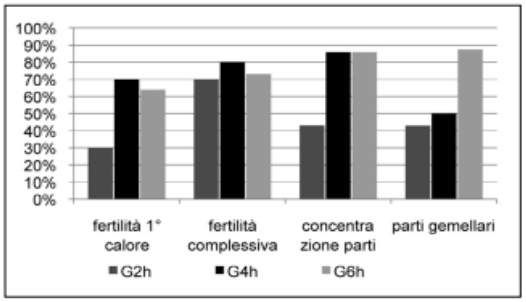

Figura 1 - Fertilità, concentrazione parti e parti gemellari in Gh1, Gh2 e Gh3.

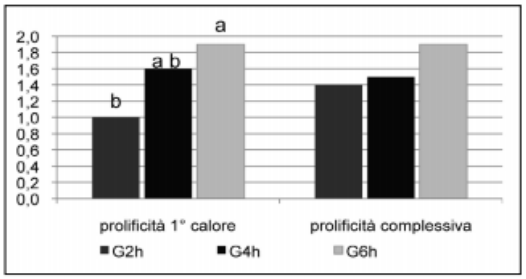

Figura 2 - Prolificità relativa al primo calore e complessiva.

In conclusione, una riduzione della durata di accesso al pascolo su loglio italico al di sotto di 6 ore/giorno in pecore a metà lattazione può manifestare un effetto residuo negativo sulla risposta all'"effetto maschio" accoppiato al flushing con lupino, con riduzione significativa della prolificità a primo calore e, tendenziale della prolificità totale. Gli effetti residui sulla fertilità non sono significativi probabilmente per il ridotto numero di osservazioni.

Preliminary study of the residual effect of a rationed grazing in ewes under male effect and flushing with lupine

Key words: ovarian cycle, fertility, prolificity, nutrition.

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Preliminary study on the residual effect of part-time grazing in dairy ewes submitted to ram effect and flushing with lupin

C. Porcu[1], M. Dattena[2], M. Gallus[2], A. Cabiddu[2], F. Berlinguer[1], S. Naitana[1], G. Molle[2]

[1]Dipartimento di Medicina Veterinaria, Università degli Studi di Sassari, Via Vienna 2, 07100 Sassari, Italy
[2]Dipartimento per la Ricerca nelle Produzioni Animali, AGRIS Sardegna, 07040 Olmedo, Italy

Abstract

The aim of this study was to evaluate the residual effect of restricted time access to pasture in dairy sheep on their response to the ram effect and flushing in terms of ovarian activity resumption, fertility, and prolificacy. Thirty-six Sarda ewes in mid-lactation were included in this study. Starting on March 13 ewes were divided into three homogenous groups and allocated to plots of berseem clover from 8:00 am with the following grazing schedule: G2h (n=12),2 hours/day; G4h (n=12),4 h/d; G6h (n=11),6 h/d. Individual feed supplementation was equal for all groups. From April 30, all ewes were allocated to a pasture of Italian ryegrass at heading stage for 6 h/d. To induce the "ram effect" on May 1 (day0), four rams were introduced in the flock. From May 18 (day16) ewes were submitted to a five day flushing with 400g/ewe of lupin seeds. Heat was visually detected three times a day. At males' introduction ovarian activity resumed in 6 out of 12 ewes in G2h, 10/12 in G4h of 11/11in G6h ($p<0.05$ between G2h-G6h). Fertility related to the first heat was 4/6 in G2h, 8/10 in G4h 9/11 in G6h ($p=0.76$). Fertility related to the breeding season was 10/12 in G2h, 10/12 in G4h,11/12 G6h ($p=0.79$). Prolificacy related to the first heat was 5/4 in G2h, 11/8 in G4h and 13/9 in G6h ($p=0.063$). Lambing concentration was 4/10 in G2h, 8/10 in G4h, 9/11 in G6h ($p<0.05$ between G2h-G6h). Twin rates were 3/10 in G2h, in 4/10 G4h and 6/11 in G6h ($p=0.51$). In conclusion, access to pasture of berseem clover for 6 h/d during mid-lactation had a positive impact on subsequent reproductive performance as compared with the shortest time access (2 h/d). This residual effect was evidenced by a prompter resumption of ovarian activity, followed by a higher concentration of lambings and a trend towards higher prolificacy. These results overall confirm those of a previous study carried out on Sarda ewes, part-time grazing a ryegrass pasture in mid-lactation, suggesting that severe restriction of time access to pasture during mid-lactation (<4 h/d) can subsequently impair the reproductive performance of sheep submitted to both male effect and flushing with lupin.

Introduction.

In sheep, during late anestrus, sudden exposure of ewes to rams, after at least 8 weeks of separation, can lead to ovulation and heat synchronization (Rosa et al., 2002, Dattena et al., 2010). This event, called ram effect, can be modulated by several factors related to the animals, such as body weight (BW), body condition (BCS), lactation stage, productive level, and animal management, with particular reference to the feeding (Mojtaba Kafi et al., 1997).Nutrition is one of the main factors of reproductive performance in ruminants. Nutrition can impact on reproduction by short-terms, pre-mating flushing (Molle et al, 1997, Stewart et al., 1986) and long-terms nutrition plan, usually implemented during lactation (Molle et al, 1995), aimed at improving the ewe status at mating. An inadequate nutrition plane in lactating ewes can have a negative influence on ovarian activity resumption and ewe fertility (Shevah et al., 1975). Unlike long-terms nutrition plan, a short-term supplementation with lupin, have an immediate effect on ovulation (Stewart et al., 1986). Flushing with different concentrates have been studied (Molle et al, 1995; Letelier et al., 2008 a/b) and several studies have proven that flushing with lupin can increase ovulation rate (Downing et al., 1995; Leury et al., 1990; Stewart et al., 1986).

A previous study carried out at the Bonassai research station, on mid-lactation Sarda ewes, submitted to male effect and flushing with lupin, showed that a time access to ryegrass pasture lower than 6 hours/day can lead to a negative residual effect on reproductive performance, such as prolificacy related to the first heat.

The aim of this study was to evaluate the residual effect of different time access to pasture of berseem clover in dairy sheep at 5th month of lactation on their response to the ram effect and flushing with lupin in terms of ovarian activity resumption, fertility, and prolificacy.

Materials and Methods

This experiment is part of a wider study conducted at the Bonassai research station of Agris DiRPA, in northwestern Sardinia, from February to November 2014. The study was branched into two experiments, experiment 1 to assess the direct effects of part-time grazing treatments on intake and performance of mid-lactation dairy ewes (Molle, 2015), experiment 2 aimed at evaluating the carry over effects of the above treatments on the reproduction performance on the same animals. The experimental period of experiment 2, described in this manuscript, ranged from April 30 to the end of lambing season (November.

At the beginning of the study thirty-six Sarda ewes in mid lactation were selected from the Bonassai flock but one died during pregnancy due to abortion and therefore its record is discarded from data analysis.

During the pre-experimental period the sheep were managed as follows: from February 22 to March 11, the animals were adapted to graze all together for 4 h/day plots of berseem clover , receiving concentrates and ryegrass hay as supplements.

On March 13, (beginning of experiment 1) the ewes were divided into three groups, homogenous for age, body condition score (BCS, 2.39 + 0.12), body weight (BW 42.31 + 3.98), milk yield, and quality and lambing period.

Groups had access to three plots of berseem clover, of equal dimension, divided into subplots which were rotationally grazed for a restricted daily time: G2h (n=12), 2 hours/day, from 08.00 to 10.00; G4h (n=12), 4 hours/day, from 08.00 to 12.00; G6h (n=12), 6 hours/day, from 08.00 to 14.00. Indoor feeding consisted of 400 g/ewe d of a commercial supplement split into two meals, 300 g/ewe d of whole corn and ryegrass-based hay offered at an equal amount to each group (c.a. 700 g/ewe d). From April 30 (beginning of experiment 2), all ewes were managed as a unique flock and were allocated to a pasture of Italian ryegrass at heading stage for six hours/day, with indoor supplementation of 500 g/day/ewe of concentrate and 700 g/day/ewe of ryegrass hay.

On May 1 (identified as day 0 for the experimental purpose) to induce ram effect, four adult, entire and not shorn rams were introduced in the flock (ratio 1/9). Starting from May 17 (day 16) to May 21(day 20), the ewes were submitted to a five days flushing, replacing 400 g/day of concentrate with 400 g/day of lupin seed. On May 18 (day 17) heat detection started two times a day (07:00, 15:00), using crayons on the rams, and checking the mark on the ewes at milking parlor. Heats and mating were detected for two more reproductive cycles.

Pregnancy were monitored by ultrasonography at 60 days of pregnancy. Lambing period were distributed from October to November. From March to November BW and BCS were checked fortnightly during mating period and monthly since then. The following reproductive data were collected: ovarian activity resumption at male introduction, heat distribution during three reproductive cycles, fertility, pregnancy rate and twinning rate.

Results

In experiment 1, when ewes were exposed to the direct effects of part-time grazing, Gh2 displayed a lower energy intake than Gh4 and GH6, which resulted in lower milk yield during mid-lactation period (March to end of April) and a trend to lower recovery in BW and BCS (Molle, 2015).Therefore, when experiment 2 began, (30 April) the sheep which had access to pasture for 2h/d (G2h) showed lower BW ($P<0.06$ as compared with G4h/d and $P<0.11$ as compared with G6h, Figure 1) and BCS ($P=0.14$ as compared with G6h, Figure 2). During pre-flushing period and afterwards , until early pregnancy (26 June), G2h group showed a progressive recovery in BW as compared to the others groups, with significant differences between groups only in the pre-flushing period (30 April – 14 May: 1.3 vs – 0.3 and -0.4 kg per ewe in G2h, G4h and G6h, respectively $P<0.05$, Figure 1). In a similar way, BCS showed a recovery trend, numerically higher in G2h than the counterparts ($P>0.15$ between groups, Figure 2). After males' introduction, during the first reproductive cycle, heat behavior was detected in 6 out of 12 ewes coming from G2h group, 10 out of 12 from G4h and 11 out of 11 from G6h ($p<0.05$ between G2h-G6h) (Figure 3 - a). Fertility related to the first heat was 4 out of 6 ewes from G2h, 8 out of 10 from G4h and 9 out of 11 from G6h ($p=0.76$) (Figure 3 - a). Fertility related to the breeding season (three reproductive cycles) was 10 out of 12 from G2h, 10 out of 12 from

G4h and 11 out of 11 from G6h (p=0.79) (Figure 3 - a). Prolificacy related to the first heat was 5/4 in G2h, 11/8 in G4h and 13/9 in G6h (p=0.063) (Figure 3 - b). Prolificacy related to the breeding season was 13/10 in G2h, 14/10 in G4h and 17/11 in G6h (p=0.95) (Figure 3 - b).
Lambing concentration was 4/10 in G2h, 8/10 in G4h, 9/11 in G6h (p<0.05 between G2h-G6h). Twin rate were 3/10 in G2h, in 4/10 G4h and 6/11 in G6h (p=0.51) (Figure 4).

Figure 1 – Body weight of late-lactating ewes at mating and in early pregnancy submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). All ewes were submitted to ram effect and flushing with lupin seed.

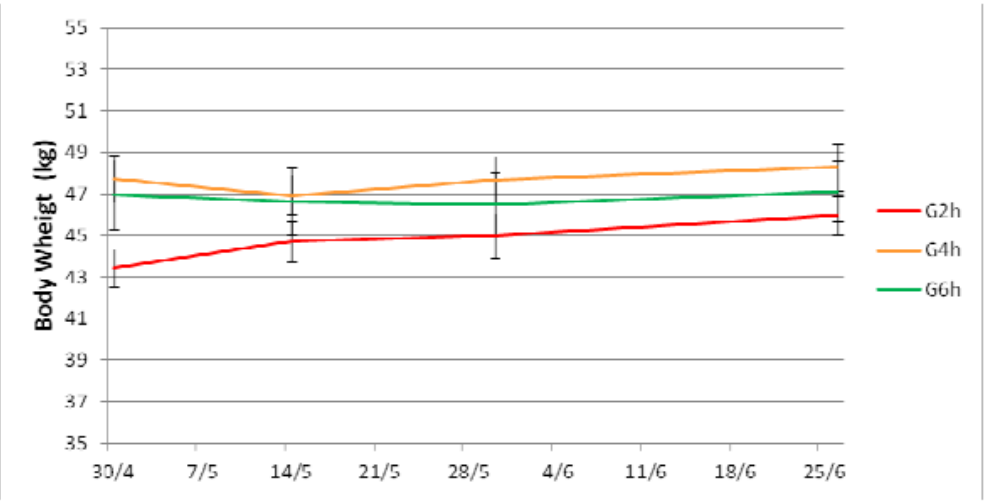


Figure 2 Body condition score of late-lactating ewes at mating and in early pregnancy submitted to the residual effect of time restricted allocation to a berseem-based pasture for 2h/d (G2h), 4h/d (G4h) and 6h/d (G6h). All ewes were submitted to ram effect and flushing with lupin seed.

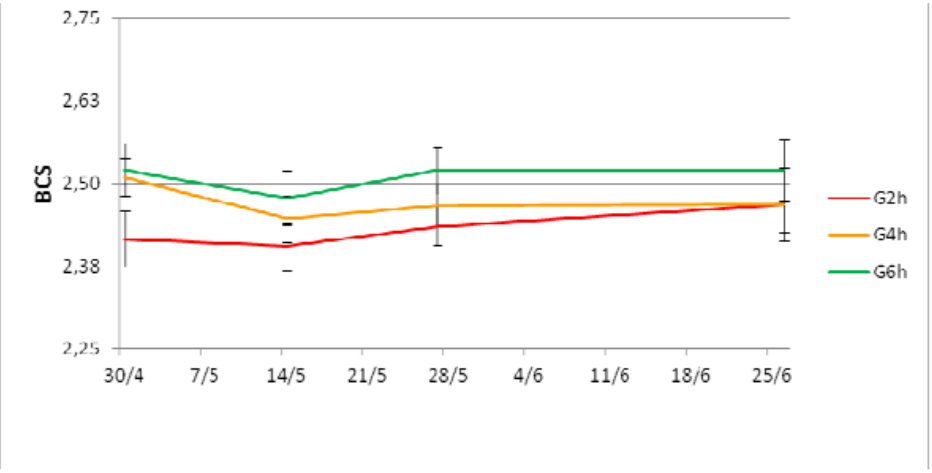


Figure 3 – a) Heat behavior detected: G2h 6 out of 12 ewes, G4h 10 out of 12, G6h 11 out of 11 ($p<0.05$ between G2h-G6h). Fertility at the first heat: G2h 4 out of 6 ewes, G6h, 8 out of 10 from, G6h 9 out of 11 ($p=0.76$). Fertility related the breeding season: G2h 10 out of 12 ewes, G4h 10 out of 12, and G6h 11 out of 11 ($p=0.79$); b) Prolificacy related to the first heat: 5/4 in G2h, 11/8 in G4h and 13/9 in G6h ($p=0.063$). Prolificacy related to the breeding season: 13/10 in G2h, 14/10 in G4h and 17/11 in G6h ($p=0.95$)

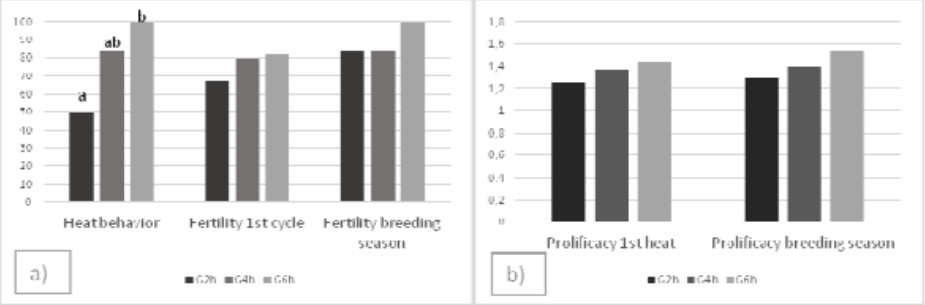
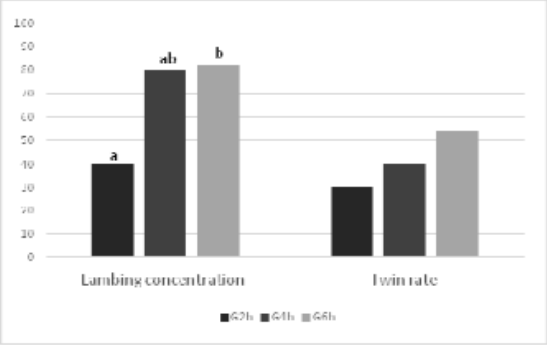


Figure 4 - Lambing concentration: 4/10 in G2h, 8/10 in G4h, 9/11 in G6h ($p<0.05$ between G2h-G6h). Twin rate: 3/10 in G2h, in 4/10 G4h and 6/11 in G6h ($p=0.51$)



Discussion

An inadequate time allocation to pasture in ewes in mid lactation, besides directly impacting on nutrient intake and milk performance, can exert detrimental residual effects on the response to male effect and flushing with lupin. This had already been showed in a previous study carried out on Sarda ewes in mid-lactation, part-time grazing, a pasture of Italian ryegrass for less than 4 h/day, on. In that case, sheep exhibited a significant reduction of prolificacy related to first heat and a negative trend on prolificacy related to the breeding season and on lambing concentration (Porcu et al., 2013).

In this study the access to pasture of berseem clover for a duration of 6 h/d during mid-lactation had a positive impact on subsequent reproductive performance as compared with the shortest time access (2 h/d). The residual effect was evidenced by a prompter resumption of ovarian activity, followed by a higher concentration of lambing and a trend towards higher prolificacy. These results overall confirm those of the previous study, suggesting that a restriction of time access to pasture during mid-lactation, besides constraining intake and performance in mid lactation, can subsequently impair the reproductive performance of late-lactation sheep, even if submitted to both male effect and flushing with lupin. This happens despite a late recovery of body weight and condition score in the ewes submitted to the most severe restriction of time at pasture during mid lactation.

Further studies are warranted to explore the mechanisms underlying the reproductive response to restricted grazing time.

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Seasonal variations of blood antioxidant capacity and spermatozoa fertilizing potential in Sarda breed buck.

S. Chelucci, V. Pasciu, S. Succu, F. Berlinguer, G.G. Leoni*, V. Satta, C. Porcu, M.E. Manca, S. Naitana*.*

Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100 Sassari, Italy. * Centro di Competenza per la Biodiversità Animale (CCBA), 07100 Sassari, Italy.

Summary

We investigated seasonal variations of plasma antioxidant concentrations, including total thiols, glutathione peroxidase (GSH), trolox equivalent antioxidant capacity (TEAC), and malondialdehyde (MDA) as indicators of lipid peroxidation. Obtained results were correlated with in vitro spermatozoa fertilizing potential. For this study 3 adult Sarda breed bucks (A, B, C) were used. Blood samples and ejaculates were collected during breeding (October) and non breeding (March) seasons. Semen samples were used to fertilize in vitro matured oocytes recovered from ovaries collected from prepubertal goats at a commercial slaughterhouse. Plasma levels of total thiols, GSH and TEAC, evaluated with spectrophotometer assay, were influenced (T student $p<0.01$) by seasonal period, being significantly higher in buck B and C in the breeding period compared to the non breeding season. In buck A, total thiols and TAC showed the highest values in October compared to March, but no significant differences were evidenced for GSH assay. Plasma concentration of MDA showed higher values (T student $p<0.01$) during the non breeding season compared to the breeding period only in buck B; no differences were recorded between breeding and non breeding season for A and C bucks. Fertilization rates did not show any differences between the breeding and non breeding season in the three bucks. However, in A and B bucks embryo output was higher ($\chi 2$ Test $p<0.01$) in October compared to March. Further studies are needed to assess the correlations between seasonal variations in blood antioxidant capacity and semen fertilizing potential.

Introduction

Oxidative stress is caused by the imbalance between the production of reactive oxygen species (ROS) and the protective action of antioxidant system responsible for their neutralization and removal. An excess of ROS causes a pathological response leading to damage of cells and tissues. Spermatozoa are particularly susceptible to the damaging effects of ROS, because their cell membrane contains large amounts of unsaturated fatty acids, which can be oxidized (lipid peroxidation), and the cytoplasm has only small concentrations of the enzyme able to neutralize ROS (Walczak–Jedrzejowska R. et al., 2013). The close association between seminal antioxidant capacity, sperm oxidative damage and seminal parameters has been widely documented (Kao et al., 2008; Khosrowbeygi and Zarghami, 2007; Patel et al., 2009; Shiva et al., 2011). On the contrary blood antioxidant profile in relation to semen antioxidant profile and semen quality has been less investigated. Correlations between blood superoxide dismutase and sperm count and between blood glutathione and progressive motility (Shamsi et al., 2010) suggest that these parameters can be important markers to assay sperm quality. The aim of this work was to assess if the blood antioxidant profile could be a valuable tool for the evaluation of sperm reproductive capacity, using Sarda buck as a model. So the present study was designed to investigate seasonal variations of plasma antioxidant concentrations, including total thiols, glutathione peroxidase (GSH), Trolox equivalent antioxidant capacity (TEAC), and malondialdehyde (MDA) as indicators of lipid peroxidation. Obtained results were correlated with in vitro spermatozoa fertilizing potential

Materials and methods

All experimental procedures were carried out during goat breeding (October) e non breeding (March) seasons at the experimental facilities of the Department of Animal Biology at the University of Sassari, Italy (latitude 40°43' N).
Ejaculates were obtained by artificial vagina from three adult Sarda male goats aged 3 years. The semen fertilizing potential of different bucks were assessed after in vitro fertilization and culture up to the blastocyst stage of in vitro

matured oocytes recovered from ovaries collected from prepubertal goats at a commercial slaughterhouse. The in vitro culture procedures were carry out according to Berlinguer et al., 2009.

For the evaluation of plasma antioxidant capacity, blood samples were taken from the jugular vein using vacuum blood evacuation tubes containing EDTA. The blood collection was collected from each male utilized for this study. Samples of blood plasma (EDTA) were centrifugated rapidly and kept frozen at -80 °C until assayed. We investigated seasonal variations of plasma total thiols, GSH, TEAC, and MDA evaluated with spectrophotometer assay.

GSH and total thiols assay: colorimetric reactions were conducted under the conditions described by Ellman (1958). Samples were suspended in phosphate buffer containing 0,25 mM DTNB. After 10 minutes its absorbance was read at 412 nm. The thiols concentration were expressed as nmol of thiols/mg proteins and it was calculated using Lambert and Beer's law with ε = 13600 (reaction A). GSH was assay (reaction B) using the same Ellman conditions in phosphate buffer and DTNB containing also EDTA 12 mM, tert-butilOH 50 mM and glutathione peroxidase (gpx) for oxidizing GSH in the sample to GSSG. The absorbance of the mixture was measured twice at 412 nm, 10 minutes after mixing the sample with the reaction solution. The thiols concentration without GSH were calculated using Lambert and Beer's law with ε =13600 and expressed as nmol of thiols/mg proteins. GSH concentration expressed as nmol of GSH/mg proteins was calculated as the total thiols (reaction A) less thiols without GSH (reaction B).

The TEAC was determined using the method described by Re et al., 1999 and modified by Lewinska et al., 2007. Briefly, ABTS•¬˙ was generated by oxidation of ABTS [2,20- azino-bis (3-ethylbenzothiazoline-6-sulphonic acid)] with potassium persulphate. ABTS•¬˙ decolouration in the presence of hydrogen-donating antioxidants was followed at 734 nm after the addition of the sample. The percentage inhibition of absorbance at 734 nm was calculated as a function of Trolox concentration, which was used as reference standard, and expressed as mM Trolox equivalent for plasma blood.

The MDA was evaluated by the TBARS assay using thiobarbituric acid according to the TBA test described by Spanier and Traylor 1991, with some modifications. The plasma blood were added to 100 µl glacial acetic acid 33%, 75 µl SDS 10%, 100 µl Tris-HCl 50 mM pH 7,4 and 250µl TBA 0,75%. The mixture was then incubated for 1 hours at 100°C and immediately cooled on ice. After 10 minutes 200 µl of acetic acid 33% were added and samples were centrifuged. The supernatant absorbance was then read 535 nm. The values of MDA in the samples were expressed in µM units and calculated using a standard curve.

The statistical analysis was carried out using the T-student for blood oxidative status, differences in fertilization rates and embryo production were evaluated by performing a χ^2 test and a probability of $P \leq 0.05$ was considered to be the minimum level of significance.

Results and discussion

Plasma levels of total thiols, GSH and TEAC, evaluated with spectrophotometer assay, were influenced (T-student $p<0.01$) by seasonal period, being significantly higher in buck B (total thiols 3.8±0.53 vs 2.5±0.75 for October and March respectively; GSH 1.1±0.17 vs 0.7±0.38 for October and March respectively; TEAC 7.5±0.92 vs 5.9±0.78 for October and March respectively) and C (total thiols 3.1±0.28 vs 2±0.48 for October and March respectively; GSH 0.5±0.03 vs 0.4±0.17 for October and March respectively; TEAC 9.7±0.63 vs 7.6±0.72 for October and March respectively) in the breeding period compared to the non breeding season. In buck A, total thiols and TEAC showed the highest values in October (2.5±0.11 and 7.9±0.85 respectively) compared to March (2.1±0.31 and 5.9±1.29 respectively), but no significant differences were evidenced for GSH assay. Plasma concentration of MDA showed higher values (T student $p<0.01$) during the non breeding season compared to the breeding period only in buck B (11.48±1.34 and 7.26±0.79 respectively); no differences were recorded between breeding and non breeding season for A (8.01±0.46 and 7.79±038 respectively) and C (7.79± 0.66 and 7.99± 0.54 respectively) bucks. Fertilization rates did not show any differences between the breeding and non breeding season in the three bucks (A= 60.21% vs 59.21%; B= 67.30% vs 59.73; C= 42.55% vs 49.67% for breeding e non breeding season respectively). However, in A and B bucks embryo output was higher ($\chi 2$ Test $p<0.01$) in October (37.50% and 41.42% for A and B bucks respectively) compared to March (15.50% and 16.80% for A and B bucks respectively).

Together with seminal oxidative stress assessment, blood oxidative status determination has been recently proposed as a tool to evaluate the sperm reproductive capacity and functional competence (Shamsi et al., 2010). In the present study, the evaluation of the blood plasma antioxidant profile revealed higher concentrations of total thiols and TEAC in the breeding season among the bucks accompanied by higher embryo production for A and B males. The antioxidant protection of semen is provided by enzymes such as superoxide dismutase (SOD), glutathione peroxidase (GPx), and catalase but also by non–enzymatic antioxidants such as vitamins E and C, and other substances contained within the sperm cell or in the seminal plasma (Lewis et al., 1997). Furthermore Benedetti et al., 2012 showed that as compared with controls, infertile men presented lower blood concentrations of TEAC, carotenoids and vitamin E. The seminal plasma antioxidant capacity is probably influenced by blood antioxidant capacity and in this way affect the spermatozoa quality.

These results suggest the prospects of using the blood serum antioxidants as helpful biochemical markers to support conventional semen analysis in sperm reproductive capacity.

So the investigation of others blood parameters such as SOD, GPx, catalase and vitamins E and C could be useful to identify valuable markers for the evaluation of sperm reproductive capacity.

Conclusions: Further studies are needed to assess the correlations between seasonal variations in blood antioxidant capacity and semen fertilizing potential.

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Cryobanking: a challenge to preserve small ruminants biodiversity

Naitana S, Berlinguer F, Porcu C, Pasciu V, Satta V, Succu S, Leoni G.
Department of Veterinary Medicine University of Sassari, Centro di Competenza Biodiversità Animale, Italy

Abstract
The possibility to maintain the potential viability of living cells indefinitely by cryopreservation open a large number of possibilities in medical, agricultural and reproductive fields. During the last years, cryobanking achieved interest but, even though the successful of both gametes and embryos was reached in a variety of animal species, the empirical approaches adopted till now added very little information on the basic knowledge of cell preservation. The use of cryoprotectants and the un-physiological reduction of temperature can cause morphological, biochemical and functional damages to cryopreserved cells. This review focuses on main advantages/disadvantages and modifications caused by cryopreservation and summarize the knowledge on biochemical resumption of cryopreserved sperm, oocytes and embryos in small ruminants.

Introduction
The first purpose of cryopreservation in mammalian species is to temporally block metabolic activity, minimize external injury, restore reversible damage after resumption of potential viability and help the regeneration of living cells and tissues. In the field of reproduction, long term storage of sperm, oocyte, and embryo is one the most important tool to improve genetic breeding and assisted reproductive technology (ART) in veterinary medicine. Agriculture intensification determined rapid and radical changes in the structure of livestock breeding causing an erosion of animal resources in the Mediterranean area where a large quantity of breeds has been selected during several centuries. Following the theory allocation resources, the resources inside of organism are limited and well balanced to respond, in natural breeding, at all several stress conditions. However, in very intensive breeding conditions characterized by hard genetic selection of few breeds with very high milk/meat production, the animal resources are re-addressed to high efficiently milk/meat production determining physiological problem to fitness. For this reason it need to maintain the biodiversity of animal ecotype in the Mediterranean area for a better sustainability of rural environments and animal production systems. Cryopreservation of both gametes and preimplantation embryos gives a new opportunity to increase genetic selection programs among the breeds, to protect population integrity of local ecotype and to preserve genetic material of endangered wildlife species. The establishing of cryobanks, associated with artificial insemination, embryo transfer programs and in vitro embryo production, could impair the erosion of animal resources and maintain biodiversity in the global world.

Cryopreservation procedures
At the moment there are two methods for reproductive cells cryopreservation: conventional freezing and vitrification. There are considerable differences between conventional freezing and vitrification in term of phase transition (ice crystal vs glassy), cryoprotectant agents and efficient concentrations (5% vs 40%), cooling rates (10 C°/m vs 23.000 C°/m) and execution time (2 hours vs 10 minutes). The most common CPAs used to vitrification include dimethyl sulfoxide (DMSO), ethylene glycol (EG), glycerol (G) and propylene glycol (PROH) which show low toxicity and high permeation. Usually a mixture of two CPAs is used to limit their specific toxicity and to combine the protection ability of each CPA. In contrast to conventional freezing the vitrification method gives the solidification with glasslike formation preventing the ice crystallization by extreme elevation of viscosity during cooling. Improving the cooling and warming rates is the most important factor that affects the probability of successful vitrification. It has been suggested that the vitrification process can be improved by decreasing the volume of liquid to be cooled to less than 1 µl. The success of vitrification procedures has been enhanced by using new cryo-device system to load the embryos with the Minimun Volume Size of vitrification solution, such as open pulled straws, cryotop, cryoloop.

Researchers preference for vitrification method could be due to its high potential to future development on the cryopreservation field in particular for oocyte and embryo.

Spermatozoa

Which cryobanking via to ART ?. Sperm collection is very easy and cheap. It is also possible to sperm collection postmortem from the epididimis within 24-36 hours in relation to the ambient temperature. The flushing collection must be carried on only from cauda epididymis because only in this part the spermatozoa acquire fertilizing potential. Only few males can be used to sperm cryobanking building, while hundred off-springs can be potentially obtained with very low economic cost. The main changes that occurs during freezing are mainly ultrastructural, biochemical and functional, which impair sperm transport and survival in the female reproductive tract, reduce in vitro fertilizing potential and in synchronized goat and sheep. In frozen-thawed goat and ram semen there is a decline in motility, viability and forward progression in the female reproductive tract. These alterations may not affect motility but reduces lifespan, ability to interact with the female reproductive tract causing a reduction in fertilizing potential of spermatozoa. In light of these information from the literature, frozen/thawed spermatozoa deposited in the female genital tract undergo a series of events that alter the stability and permeability of the cell membrane and triggers internal signaling cascades ending with functional and structural changes termed 'capacitation process', which render the spermatozoa competent to fertilize. At the same time capacitation process can also be induced in vitro by incubation in suitable medium for in vitro fertilization of oocytes and embryo production. Computer-assisted-semen-analysis (CASA) provides objective and reproducible data on a number of sperm viability and motility parameters. In recent years there has been an increase in the use of these systems to evaluate semen quality resulting in high correlations between several CASA parameters and the fertilizing potential of frozen-thawed sperm. The viability is better in bull (70-80%) than ram and buck spermatozoa (50-60%). Greater damages have been observed in cytoplasma, acrosomal and mitochondrial membranes and nuclear DNA integrity. The membrane status of spermatozoa (intact, capacitated and acrosome reacted) can be evaluated using specific stain and ATP concentration. The damage of cytoplasmic membrane determines a great reduction of sperm viability while the alteration of acrosome membrane arouse low fertilization rate. Energy metabolism is a key factor supporting sperm function. ATP is one of the basic components in a sperm cell and is used not only as a energy source but also for protein phosphorylation in cell signaling. The functional integrity of mitochondria is believed to be important for sperm survival in the female genital tract. Among other sperm tests, the evaluation of DNA integrity (comet assay) has been considered important as early embryo development depends on the presence of normal DNA by. After cryopreservation spermatozoa are particularly susceptible to DNA damage since freezing and thawing procedures lead to significant reduction in the level of spermatozoa antioxidant. Therefore the assessment of DNA integrity is of high value in determining frozen/thawed semen quality. Studies on sperm demonstrated that semen cryopreservation is associated with increased generation of Reactive Oxygen Species (ROS).The excessive production of ROS might have serious implications on sperm structure and functionality, because spermatozoa are particularly susceptible to damage induced by ROS represented by superoxide anion, singlet oxygen. For these reasons, different exogenous antioxidants as glutathione, superoxide dismutase, L-carnitine, vitamin E, melatonin, have been included in the freezing extender to restore the equilibrium between ROS and antioxidants Their ability to effectively improve sperm quality and function after thawing has been reported in several experimental studies, thus providing indirect evidence that oxidative stress during cryopreservation harms these cells. Spermatozoa incubated under antioxidant molecules have revealed numerous biochemical and physiological changes that accompany the capacitation process thus minimizing premature acrosome reaction. Previous our studies in the ovine species confirmed the protective effect of melatonin, on the mitochondria sperm and ATP production. Male animals can be classified as ''good freezers or ''bad freezers'' depending on the capacity of their semen to support freezing and recover cryopreservation injuries. There are some characteristics of the membrane structure, which may depend on individual characters determining better resistance of spermatozoa to cryopreservation. This supports the idea that cryopreservation could be under genetic control.

At moment sperm cryopreservation stays in static condition due to low viability and fertility of cryopreserved spermatozoa and low economic value cost of small ruminants. There are available two method to insemination. The

cervical insemination is very easy and quick, but at the same time it shows low fertility and lambing rate (15-35%) using very high number of spermatozoa 200-400 x106. This is unacceptable to low cost of the animals. The intrauterine insemination have high cost determined by specific equipment and well qualified technicians for laparoscopy with good fertility and lambing rate (60-80%) using low number of spermatozoa 40 x106. However, in Norway and Sweden, for animal welfare reasons, not allowed and in these countries the prevailing AI technique is based on cervical insemination with better results. Although most in vitro fertilization (IVF) studies use freshly ejaculated spermatozoa, buck and ram frozen-thawed spermatozoa have been used in IVF trials for embryo production. Usually in our lab we used in vitro fertilization, during embryo production procedures, a concentration of 1x106 frozen-thawed ram sperm having a range of 70-90% fertilization rate and of 45-55% blastocyst rate.

Oocyte

Which cryobanking via to ART ?. Oocyte is less attractive than sperm because small ruminant species are mono or bi-ovulatory. For this reason several females are needed to obtain a satisfactory number of oocytes. The investigation on the oocyte cryopreservation of small ruminants is relatively recent. To increase oocytes number, the female donors must be treated with gonadotrophin for superovulatory treatment requiring high competent technicians for laparoscopic ovum pick-up (LOPU). For this, at moment, oocyte cryobanking is very expensive. Oocytes are extremely sensitive to slow freezing, therefore, vitrification could be an alternative approach to oocyte cryobanking. Female gamete shows considerable morphological and biochemical modifications during the cryopreservation procedure which are highly variable among and within the species, depending by the procedures, cryoprotectant agents, sources and developmental stages (germinal vescicle or metaphase II). It is know that some species are more resistant than others depending on the quantity and quality of lipid inside of oocytes. In order to reduce total lipid content, mechanical or chemical delipidation using oocyte centrifugation has been proposed; also to change membrane composition it has been suggested to increase the ratio of unsaturated to saturated fatty acids by dietary modifications. A further difficulty to preserve this reproductive cell derives also by the volume of the mammalian oocyte in the range of three to four orders of magnitude larger than that of the spermatozoa, thus substantially decreasing the surface-to-volume ratio and affecting cryoprotectant flow across membranes. In this direction cryodevices used to hold oocytes during chilling and storage affect drop volume and contact to liquid nitrogen. Our group observed the different effect of distinct cryodevices on oolemma integrity, developmental potential after IVF, as well as MII spindle morphology and some expression of specific transcripts activity of vitrified MII ovine oocytes. Additional cryoinjuries have been observed after vitrification of ovine oocytes at GV stage, as fracture of ZP adjacent to cumulus cells, rupture of gap junction and loss of the communication between oocytes and cumulus cells altering maturation process consecutive to oocyte vitrification and consequently their developmental competence. At moment, embryo development, after vitrified oocytes at M II, is very low with only 17% of the oocytes developing to blastocysts, compared with 55% for untreated oocytes. More recently, better results are obtained with vitrified oocytes at germinal vescicle stage with 29% of blastocysts.

Embryo

Which cryobanking via to ART?. Embryo cryobanking summarizes both gametes maintaining complete genome and mtDNA for population reconstitution. The source of in vivo embryo production requires superovulation protocol to improve a good number of embryos. A competent team collect 80% of superovulatory responses from uterine flushing. Obviously all these aspects improve the cost of this technique giving it more expensive. Also this technique requires surgical procedures that impair repeated embryo collection from same donors. Oocytes source can be recovered from female of high genetic value by LOPU without adhesions and fibroses with low invasive procedure. After collection the cumulus oocytes are in vitro matured (22h) and in vitro fertilized (27h) and cultured up to blastocyst stage (7days). In vitro embryo production is less efficiently with 55% of blastocyst, also embryos are more sensitive to low temperature and have less cryotolerance than those collected in vivo, due to deficiencies in the in vitro culture conditions. Therefore, advances in embryo survival following vitrification could be achieved by improving their culture conditions, or by selecting embryos for vitrification based on the kinetics of their development. A single exposure to a CPA subjects the embryos to an increased risk of osmotic shock, particularly

when the concentration is extremely high. Depending on the duration of exposure, a single immersion may not allow enough time for adequate CPA permeation into the blastocoelic cavity. Survival rates after vitrification improved with the evolution of two-step protocols. In the two-step protocols, the embryos is allowed to equilibrate for a small time at a lower CPA concentration before a short exposure to the vitrification solution at a higher concentration. In ovine species has been observed that IVP embryos showed a low survival rate in term of hatching rates (80 vs 95), pregnancy rates (60 vs 80) and lambing rates (35 vs 80) compared to those in vivo produced. The developmental stage, at which the embryos are cryopreserved, is considered to be a critical factor for the viability of the embryo after vitrification. Among early stage embryos produced in vitro, those cultured to the blastocyst stage before cryopreservation had the highest rate of survival rate after warming. The higher cryotolerance of ovine blastocyst compared with those at earlier stages might be related to the higher resistance of their cellular membranes to osmotic and toxic stress after the formation of the blastocoelic cavity. The increase of Na/K ATPase activity which occurs during blastocoelic formation in trophoblastic cell membranes may determine more active transport mechanisms of cryoprotectants leading to a decrease exposure time and to low concentration of cryoprotectants needed during cryopreservation. Furthermore, the blastomeres of the blastocyst have a higher surface area to volume ratio than early stage embryos which may contribute in the former to higher permeability coefficient to water and cryoprotectants in the latter. Therefore, blastocysts are more tolerant to osmotic stress than early stage embryos. Indeed, developmentally compromised or low competent embryos may be lost during early development in vitro, such that the latter stages represent a selected group of more competent embryos. In our previous studies we observed that mitotic activity, measured as BrDU incorporation in neo-synthesized DNA, was delayed in vitrified-warmed blastocysts compared to those not vitrified. BrDU incorporation was evidenced in a small portion of nuclei after 3 hours of post warming culture, but only after 9-12 hours the rate of BrDU positive nuclei was similar to not vitrified embryo. It has been proposed that other aspects like chromosomal abnormalities can influence the viability of embryonic cells.

Conclusion

Results obtained with different procedures confirm that reproductive cell cryopreservation have to be seen not just a way to preserve cell lineages but as a more complex method to enhance genetic survival and developmental capacity. An appropriate equilibrium must be adopted among all factors involved in the cellular response to cryopreservation including quality of embryos, permeable and non permeable CPAs, time of exposure in order to minimize structural/functional injuries and to improve the potential capacity to use in the ART. It is needed to improve our knowledge on the cryopreservation procedures of reproductive cells, which is often derived from empirical experimentation with a low understanding of the cryobiological basic process and using only survival and developmental rate to assess the quality of cryopreserved spermatozoa, oocytes and embryos. Cell preservation by lyophilisation will be an interesting alternative to traditional method for cryopreserve cells without the use LN, but more studies are needed to increase efficiently and effectively of this new procedure for practical application, with the aim to facilitate the storage at room temperature and promote germplasma exchange among the breed of small ruminants in Mediterranean area. The Mediterranean environment has permitted a high expression of animal genetic diversity with large number of breeds, proportionally greater than those found on a world scale for this it need to preserve this biodiversity of small ruminants for the future generation. However, to low cost of small ruminants the building of cryobanking will be possible only through public support

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Glucogenic treatment creates an optimal metabolic milieu for the conception period in ewes

C. Porcu [a], V. Pasciu [a], S. Succu [a], E. Baralla [a], M.E. Manca [a], E. Serra [a], G.G. Leoni [a], M. Dattena [b], G.C. Bomboi [a], G. Molle [b], S. Naitana [a], F. Berlinguer [a,*]

[a] Department of Veterinary Medicine, University of Sassari, Via Vienna 2, 07100 Sassari, Italy
[b] Department of Animal Production, AGRIS Sardegna, Loc. Bonassai, 07100 Sassari, Italy

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ABSTRACT

This study determined the influence of a short-term glucogenic nutritional treatment on circulating concentrations of glucose, insulin, insulin-like growth factor 1 (IGF-1), nonesterified fatty acids (NEFA), and urea, and on their correspondent levels in follicular fluid (FF) collected 12 h after the end of the treatment. After estrous synchronization with intravaginal progestagen-impregnated sponges, 20 Sarda ewes were randomly allocated into two experimental groups (GLU and WAT) and, from day 7 to day 10 (day 0 = day of sponge removal), the GLU group was gavaged with a glycogenic mixture, whereas the WAT group was gavaged with water (control group). Follicular development was stimulated by FSH administration from day 8 to 10. At day 11, ovaries were collected and follicular fluid processed. Plasma changes were assessed from day 6 to 11. In GLU group, circulating concentration of glucose ($P < 0.0001$), insulin ($P < 0.0001$), and IGF-1 ($P < 0.01$) rose significantly, whereas NEFA and urea concentrations decreased ($P < 0.0001$), as compared with controls. In particular, in FF the higher glucose concentrations found in GLU ewes compared with controls ($P < 0.0001$) were not accompanied by any increase in insulin and IGF-1 concentrations. NEFA ($P < 0.0001$) and urea ($P < 0.0001$) were lower in FF of GLU than WAT group, although NEFA clearance in the ovary proved to be less efficient than at the systemic level. No significant difference between groups was found in FF concentrations of pregnancy-associated plasma protein A (a protease regulating the levels of free IGF-1 in follicles), glutathione, and in its total antioxidant capacity. These results suggest that glycogenic mixture administration creates a suitable follicular microenvironment for the conception period in dairy ewes.

1. Introduction

Nutrition is one of the main determinants of reproductive performance in ruminants [1]. One of the key nutrients having an effect on the ovary is glucose [2,3], since it has distinct roles in follicular function: first as a nutrient to generate ATP, and second as a signaling molecule to stimulate folliculogenesis when nutritional conditions are favorable to reproduction [4]. The role of glucose is also essential in determining the quality of the oocyte [5]. In a previous study on dairy sheep, our research group showed that short-term flushing with a glucogenic mixture based on glycerol and propylene glycol improves oocyte quality, evaluated by the kinetics of their in vitro development and by the production of blastocysts [6]. In another study, the same nutritional treatment increased the ovulation rate [7]. Glucogenic precursors such as propylene glycol and glycerol and their mixture have been used in veterinary

* Corresponding author. Tel.: +39 079 229904; fax: +39 079 229924.
E-mail address: berling@uniss.it (F. Berlinguer).

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practice to increase blood glucose and to reduce nutritional problems in dairy cows during the peripartum [8–10]. It has been reported that feeding glycerol as a top dress [8,11] or supplied in water [10] to transition dairy cows resulted in a positive energy status with higher concentrations of serum glucose and lower concentrations of plasma nonesterified fatty acids (NEFA) [9].

Glucose can be metabolized within the follicle by the pentose phosphate pathway to provide precursors for the synthesis of purine nucleotides and NADPH which are in turn used in various biosynthetic pathways, including those related with the antioxidant defense. Hence, glucose follicular fluid (FF) concentration can also influence follicular oxidative status [1].

In addition to the glucose-insulin system, another hormone playing a key role in the energy status and function of the ovary is the insulin-like growth factor 1 (IGF-1). Insulin-like growth factor 1 and gonadotropins are synergistic for growth and differentiation of the follicle [12,13]. The IGF system (receptors, ligands, and binding proteins) is expressed within granulosa and theca cells [12–14].

Starting from these premises, this study aimed at assessing the effect of a short-term administration of a glucogenic mixture on increasing the plasmatic and intrafollicular concentrations of metabolites and hormones which play a key role in follicular maturation and quality. In particular, we measured plasma levels of glucose, insulin, IGF-1, NEFA, and urea during the nutritional treatment and their corresponding levels in the FF as measured 12 h after the last administration of the glucogenic mixture. At the same time, the oxidative status of the FF was also investigated. Finally, to assess possible changes in IGF-I bioavailability in the FF, the concentration of pregnancy-associated plasma protein A (PAPP-A), a protease regulating the levels of free IGF-1 in dominant follicles [15,16] were determined in the FF.

This information is pivotal to better explain the effect of a rise in glycemia on the endocrine and metabolic milieu of sheep at mating and to set the basis for the formulation of short-term flushing treatment able to create the best conditions for the conception period.

2. Materials and methods

2.1. Animals

The experimental procedures with animals were approved by the Animal Care and Use Committee of the University of Sassari. Twenty Sarda ewes, 4–5 years old, were used. The ewes were penned outdoor with access to a sheltered area, at the experimental facilities of the Department of Veterinary Medicine at the University of Sassari, Italy (40°43′40.33″N, 8°33′1.33″E). These facilities meet the requirements of the European Union for Scientific Procedure Establishments. The ewes were group fed a maintenance ration at a level of 46 g of dry matter per kg of metabolic weight ($BW^{0.75}$) consisting of hay and concentrate fed twice daily.

The experiment was run during October 2014, within the natural breeding season (late August–late December) described for this breed at this latitude.

In brief (Fig. 1), synchronization was induced in all the animals with the insertion of one intravaginal progestagen-impregnated sponge (45-mg fluorogestone acetate, FGA, Chronogest; Intervet International, Boxmeer, the Netherlands) which remained in situ for 6 d. On the day of sponge withdrawal (day 0), the ewes received 125 μg of a prostaglandin analogue (cloprostenol, Estrumate, Essex Animal Health, Friesoythe, Germany) by i.m. injection. At the same time, ewe live weight (42.3 ± 0.9 kg) was determined. On day 0, the ewes were divided in two experimental groups at random. From day 7 to day 10 after sponge withdrawal, one group (GLU: n = 10; weight 42.2 ± 1.3 kg;) received, orally twice daily at 8.00 AM and at 19.00 PM, 200 mL of a glucogenic mixture, as previously described [7]. The glucogenic formulation contained 70% glycerol and 20% propylene glycol (both from Sigma Chemical Co, St. Louis, MO, USA) and 10% water. The control animals (WAT: n = 10; weight 42.3 ± 1.3 kg;) received 200 mL of water twice daily simultaneously to treatment administration. Both the glucogenic formulation and the water were administered by oral gavage using an esophageal feeding tube.

From day 8 to 10, follicular development was stimulated in all the ewes by the administration of 175 IU of FSH (Folltropin; Bioniche Animal Health, Bio 98, Milano, Italy) given every 12 h in 6 equal doses.

At day 11, 12 h after the last FSH administration, the ewes were weighed and then conducted to the slaughterhouse where they were sacrificed. After having collected the ovaries, follicles and corpora lutea on their surface were counted and FF from follicles ≥4 mm was aspirated with a 2.5-mL syringe fitted with a 22-gauge needle.

2.2. Blood and FF sampling

Plasma concentrations of glucose, NEFA, urea, insulin, IGF-1, and progesterone (P4) were determined from samples drawn from jugular vein at 8.00 a.m from day 6 to day

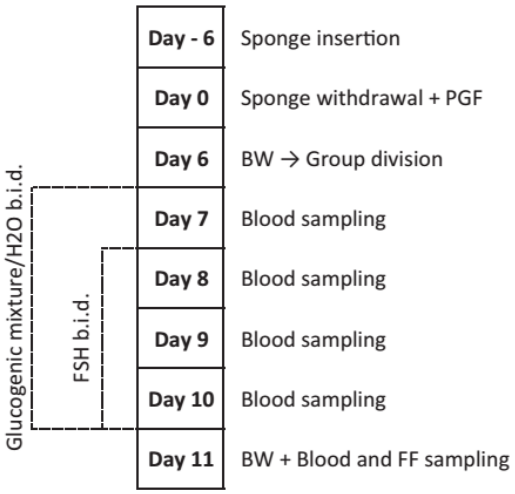

Fig. 1. Experimental protocol.

11. Glucose, insulin, NEFA, and urea plasma concentrations were also determined in three further samples collected every 30 min starting from the time of morning administration of glucogenic mixture (8.30, 9.00 and 9.30 AM) from day 7 to day 10. At each time point and from each ewe, 2 blood samples were collected, one using 3-mL vacuum collection tubes containing lithium heparin and monoiodoacetate (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 and centrifuged at 1,500 g for 15 min. Plasma was removed and stored at −20°C until assayed.

Follicular fluid concentrations of metabolites (glucose, NEFA and urea), hormones (insulin and IGF-1), and pregnancy-associated plasma protein A (PAPP-A) were determined on day 11. At the same time, to evaluate FF oxidative status, glutathione (GSH) and Trolox equivalent antioxidant capacity (TEAC) were assayed. Given the small volume recovered from a single follicle, to allow the determination of the above mentioned metabolites and hormones, the FF collected from the ovaries of a single ewe was immediately pooled and stored in Eppendorf tubes at −20°C until assayed.

2.3. Determination of metabolite concentrations in plasma and follicular liquid samples

Both FF and plasma samples were measured in duplicate.

Glucose, NEFA, and urea were measured using commercial kit and BS-200 Mindray clinical chemistry analyzer. We used Serum I Normal (Wako) and Serum II Abnormal (Wako) 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 were 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-assay and interassay CV values were 1.07% and 0.98%, respectively. UREA intra-assay and interassay CV values were 1.7% and 1.6%, respectively.

2.4. Hormone analyses

All assays were ELISA assays and were performed using the Personal Lab Adaltis (Adaltissrl, Rome, Italy), which is a tool that performs automated ELISA protocols.

Progesterone concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany), which is a solid-phase ELISA, based on the principle of competitive binding. All kit reagents, controls, and stored samples to be analyzed were thawed and warmed to 25°C at the beginning of the test. The analytical sensitivity was 0.045 ng/mL and the intra-assay and interassay CV values were <10%.

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 [17,18]. 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 on addition was 94%–114% (mean 103%). The analytical sensitivity was 0.025 μg/L and the intra-assay and interassay CV values were <7%.

Insulin-like growth factor 1 concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany) which is a solid-phase ELISA based on the principle of competitive binding. The IGF-1 assay kit used in the present study has been previously used in goats [19] and sheep [20]. The analytical sensitivity was 9.75 ng/mL and the intra-assay and interassay CV values were <8%. Before being assayed, IGF-1 was extracted following the assay procedure of the ELISA kit. Briefly, 50 μL of sample and standard solution were added to 50 μL of 0.2-N HCL. After 30 min incubation, the sample and standard solutions were neutralized with 10-μL neutralization buffer and immediately assayed. Efficiency was calculated as recovery percentage. Samples were spiked by adding IGF-1 solution with known concentrations in a 1:1 ratio. The % recovery was calculated by multiplying the ratio of the measurements and the expected values with 100. The recovery on addition was 86%–126% (mean 102%).

Pregnancy-associated plasma protein A concentration was measured in duplicate using a commercial ELISA Kit (DRG Instruments GmbH, Marburg, Germany) which is a solid-phase ELISA based on the sandwich principle. The analytical sensitivity was 0.133 μg/mL and CV values were <7%. Our PAPP-A concentrations are in accordance with those already reported in literature using RIA Kits [21,22].

2.5. Oxidative status determination in FF

2.5.1. Glutathione

Glutathione was measured after derivatization with N-ethylmaleimide (NEM) as described by Moore et al [23]. A liquid chromatographic system with triple quadrupole mass spectrometry detection (LC-MS/MS) Varian 310-MS (Varian, Palo Alto, CA, USA) was used. Glutathione was quantified in follicular fluid using thiosalicylic acid as an internal standard (IS; Table 1). Chromatographic separation

Table 1
Liquid chromatography-tandem mass spectrometry parameters.

Individual parameters	RT (min)	Capillary(V)	MRM transitions (*m/z*)	CE (eV)
GSH	2.3	50	308.3→179.1	−10
			308.3→161.9	−14
GSH-NEM	3.1	32	433.4→304.0	−11.5
			433.4→286.9	−16.5
IS	3.4	30	280.4→262	−7.5
			280.4→163.3	−20

Abbreviations: CE, collision energy; MRM, multiple reaction monitoring; RT retention time.

was achieved on a LUNA C18 column (5 μm, 100 × 2.1 mm Phenomenex, Bologna, Italy) fitted with a Phenomenex C18 security guard cartridge (4 × 2.0 mm ID). A linear gradient with 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B) was performed as follows: 1 min at 5% B; in 0.5 min solvent B was increased from 5% to 97%, and in another 1.5 min it was decreased to 95%. Then, in 0.5-min solvent B was decreased from 95% to 5% and remained constant for 2.5 min. The total run time was 6 min. The electrospray interface operated in positive mode with the following conditions: capillary voltage, 5,000 V; drying gas temperature, 200°C; nebulizer gas pressure, 50 psi; electron multiplier voltage, 1,795 V. The collision gas used was argon with a pressure of 2mTorr. The transitions related to free GSH were also monitored to verify the complete derivatization of GSH with NEM, and none was detected. Individual parameters for GSH-NEM, IS, and GSH are listed in Table 1. The values of GSH in the samples were calculated using a standard curve built in 15% methanol (range from 10 to 5,000 ng/mL) and expressed as μM [23,24]. Glutathione content was normalized for protein concentration. Total proteins in FF were measured in a multiple assay by a colored 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-assay and interassay CV values were 1.8% and 0.9%, respectively.

2.5.2. Trolox equivalent antioxidant capacity (TEAC)

Plasma TEAC was determined as described by Re et al [25] and modified by Lewinska et al [26]. Briefly, a fresh solution was prepared by dissolving 19.5 mg 2,20-azinobis 3-ethylbenzthiazoline-6-sulphonic acid (ABTS) and 3.3-mg potassium persulphate in 7 mL of 0.1 mol/L phosphate buffer, pH 7.4. This solution was stored in the dark for 12 h for completing the reaction. ABTS solution was diluted in 0.1-mol/L phosphate buffer, pH 7.4, to give an absorbance reading at 734 nm of 1.0. The absorbance of the mixture was measured twice in a spectrophotometer (Thermo-Elecrom Corporation Genesys 10 UV, Madison, Wisconsin, USA), at 734 nm, 3 min after mixing a sample with the ABTS solution. The extent of ABTS bleaching (decrease in absorbance) is proportional to the activity of antioxidants in a given sample. The antioxidant capacity was expressed as TEAC, that is, the concentration of trolox producing the same effect as the sample studied. The values of TEAC in the samples were expressed as mM Trolox equivalent normalized for volume of FF and were calculated using trolox for a standard curve.

2.6. Statistical analyses

Live weight at the beginning and end of the glucogenic-treatment period and their changes were analyzed by a mono-factorial ANOVA.

Circulating and intrafollicular concentrations of analyzed metabolites, and hormones on day 6 and day 11 were analyzed by GLM with treatment as fixed effect. Longitudinal data of plasma glucose, insulin, NEFA, and urea in the samples collected from day 7 to day 10 (during treatment period) were analyzed by a mixed model for repeated measurements (PROC MIXED in SAS Version 8, SAS Institute Inc, Cary, NC, USA) with treatment, sampling day, sampling hour and their first-order interactions as fixed effects, and sheep as random effect. In addition, to compare the composition of blood plasma and FF on the last sampling date (day 11), a GLM model was used with treatment, source of sampling and their interaction as fixed effects. Finally, on the same sample, the relationship between the concentration of metabolites and hormones in blood and FF was evaluated by correlation analysis using Pearson's correlation coefficients.

All results were expressed as mean ± SEM and a probability of $P < 0.05$ was considered to be significant.

3. Results

No differences were observed in live weight between groups ($P = 0.98$) at the beginning (day 0) and the end (day 11) of the nutritional treatment (live weight, 42.1 ± 1.3 vs 42.8 ± 1.3 kg in WAT and GLU groups, respectively).

No difference between groups was observed in ovarian follicular population, as evaluated one day after the end of the nutritional treatment (day 11; Fig. 2).

3.1. Effect of short-term glucogenic treatment on the concentrations of plasma metabolites and hormones

Before the beginning of the treatment (day 0), circulating concentrations of analyzed metabolites and hormones were within the physiological ranges [27] in all the ewes and showed no differences between GLU and WAT group (Table 2).

Progesterone plasma levels rose in both experimental groups during the experimental period ($P < 0.0001$, Fig. 3), with no differences ($P = 0.184$) between groups (GLU 3.6 ± 0.4 ng/mL; WAT 4.5 ± 0.4 ng/mL).

Short-term administration of the glucogenic mixture had an immediate effect on glycemia (Fig. 4A) and insulinemia (Fig. 4B). Glucose rose significantly in GLU group, being higher than the control both during the 4 d of

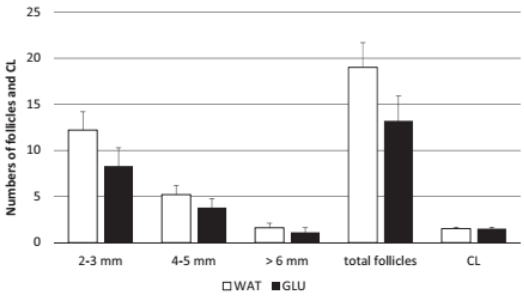

Fig. 2. Ovarian follicular and corpora lutea (CL) population in ewes orally drenched with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10), 12 h after the end of a 4-d administration. Follicles are either expressed as total count (tot) or subdivided by size categories (2–3 mm; 4–5 mm; >6 mm).

Table 2
Circulating concentration of glucose, insulin, IGF-1, NEFA, and urea in Sarda ewes before starting a 4-d nutritional treatment with a glycogenic mixture (day 0).

Analysed metabolites	Control group (n = 10)	Treated group (n = 10)
Glucose (mg/dL)	50.8 ± 2.7	51.9 ± 2.28
Insulin (μg/L)	0.095 ± 0.008	0.08 ± 0.02
IGF-1 (ng/mL)	82.6 ± 4.1	74.1 ± 3.8
NEFA (mmol/L)	0.26 ± 0.04	0.28 ± 0.03
Urea (mg/dL)	26.1 ± 2.9	29.3 ± 3.8

Abbreviations: IGF-1, insulin-like growth factor 1; NEFA, nonesterified fatty acids.
No significant differences were found between groups. Values are expressed as means ± SE.

glucogenic mixture administration ($P < 0.0001$; GLU 79.3 ± 1.9 mg/dL; WAT 64.1 ± 1.9 mg/dL) and in the day after the end of the treatment (day 11: $P < 0.05$; GLU 98.9 ± 13.1 mg/dL; WAT 69.3 ± 3.3 mg/dL). This fast response became evident from 60 min after the administration of the glucogenic mixture in the GLU group. In the same group, as a consequence of the high glucose plasma concentration, a parallel increase in insulinemia was detected from the second day of nutritional treatment. As for glycemia, insulin concentrations were significantly higher in GLU than in WAT group both during the nutritional treatment (GLU 0.82 ± 0.07 μg/L; WAT 0.13 ± 0.07 μg/L; $P < 0.0001$) and in the day after its end (day 11: $P < 0.05$; GLU 0.4 ± 0.09 μg/L; WAT 0.1 ± 0.01 μg/L).

The circulating concentration of IGF-1 (Fig. 5) was significantly higher in GLU compared with WAT group (90.7 ± 2.8 ng/mL vs 77.3 ± 2.8; $P < 0.01$). The rise in IGF-1 circulating concentration was slower compared with what observed for insulin, being detectable from the third d of nutritional treatment and reaching its highest values on the day after its end.

The glucogenic mixture administration caused a sharp drop in NEFA plasma levels in the GLU group (Fig. 4C) detectable soon after 60 min from the first glucogenic dose. Subsequently, NEFA mean circulating concentrations remained stably lower in GLU than in WAT group both during the treatment period (GLU 0.04 ± 0.02 mmol/L; WAT 0.20 ± 0.02 mmol/L; $P < 0.0001$), and after its end

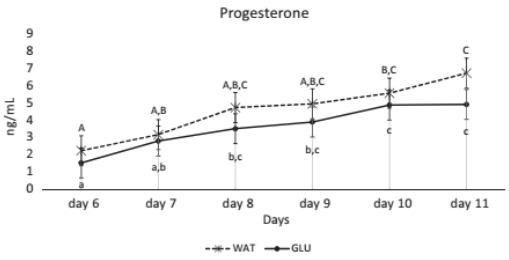

Fig. 3. Progesterone mean circulating concentrations in ewes orally drenched with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10) during the experimental period (day 6–11; day 0 = sponge withdrawal); a, b, c indicate significant variations within GLU group; A, B, C indicate significant variation within WAT group ($P < 0.0001$).

(day 11; GLU 0.02 ± 0.01 mmol/L, WAT 0.40 ± 0.08 mmol/L, $P < 0.001$).

The same pattern was observed in the concentration of urea in blood plasma (Fig. 4D). The nutritional treatment caused a significant drop in urea concentration in GLU group as compared with the WAT one. This effect was found during the treatment period (GLU 15.7 ± 0.84 mg/dL; WAT 22.2 ± 0.84 mg/dL; $P < 0.0001$) and after its end (day 11; GLU 12.4 ± 1.7 mg/dL; WAT 29.7 ± 3.1 mg/dL; $P < 0.0001$). In this case, however, the response was delayed and became evident starting from the second day of the treatment.

3.2. Effect of short-term glucogenic treatment on the concentrations of metabolites and hormones in the follicular fluid

The glucogenic supply significantly affected FF concentrations of glucose, insulin, IGF-1, NEFA, and urea, as evaluated 12 h after the end of the nutritional treatment (day 11). The observed changes in FF composition only partly mirrored those observed in the plasma. In particular, in the GLU group, the glucogenic mixture administration resulted in higher intrafollicular glucose concentrations as compared with those in WAT one ($P < 0.0001$; Fig. 6A). However, in both groups, FF glucose concentrations were significantly lower as compared with the corresponding circulating levels ($P < 0.01$).

Moreover, the higher intrafollicular glucose concentration found in the GLU group compared with WAT one was not accompanied by any increase in intrafollicular insulin concentration. On the contrary, while in WAT group no difference was observed between circulating and intrafollicular insulin concentrations, in GLU group intrafollicular insulin concentrations (Fig. 6B) were significantly lower than circulating ones ($P < 0.0001$). In other words, as confirmed by the significant interaction found between treatment and sample source (blood or FF) for insulin ($P < 0.01$), circulating and FF insulin levels varied differently in GLU and WAT group. Likewise, the higher circulating IGF-1 concentrations (Fig. 6C) in GLU group were not accompanied by significantly higher intrafollicular concentrations in GLU than WAT ewes. However, in both the groups, plasma levels were higher than intrafollicular ones ($P < 0.01$).

Changes in circulating concentrations of NEFA (Fig. 6D) and urea (Fig. 6E) were mirrored by changes in their follicular fluid concentrations. In particular, in the GLU group, a drop in mean FF NEFA (GLU 0.217 ± 0.027 mmol/L; WAT 0.360 ± 0.034 mmol/L; $P < 0.0001$) and urea (GLU 11.52 ± 1.94 mg/dL; WAT 26.96 ± 2.82 mg/dL; $P < 0.0001$) levels were observed. However, FF NEFA concentrations were higher than circulating ones only in GLU group ($P < 0.05$) with a significant interaction between group and sample source ($P < 0.05$).

No significant difference between groups was found in FF concentrations of PAPP-A, GSH, and in its total antioxidant capacity (Fig. 7).

Correlations between plasma and intrafollicular concentrations of metabolites and hormones are shown in Table 3. Presented results show that the increase in circulating glucose concentration is accompanied by a parallel

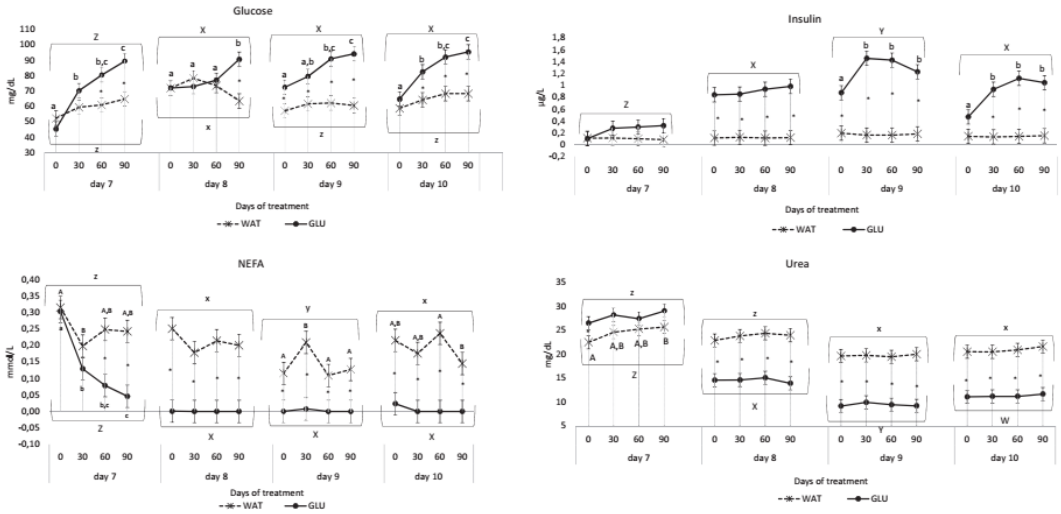

Fig. 4. Circulating concentrations of glucose, insulin, NEFA, and urea in ewes orally drenched with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10) during treatment period (D7–D10; D0 = sponge withdrawal). Asterisks indicate significant differences between groups ($P < 0.0001$). a, b, c indicates daily significant variations within GLU group. A, B, C indicates daily significant variations within WAT group. Z, X, Y indicates significant variations within GLU group on daily mean levels. z, x, y indicates significant variations within WAT group on daily mean levels.

increase in its follicular concentration, in circulating insulin, and in circulating and follicular IGF-1. In addition, insulin is negatively correlated with circulating NEFA and urea, and positively correlated with IGF-1 concentrations both in the plasma and in the follicle. NEFA and urea show a strong positive correlation both in their plasmatic and follicular concentrations.

4. Discussion

As expected, the short-term flushing did not impact on ewes' live weight. This was already found by others [7], in sheep submitted to the same flushing protocol.

The rise in circulating P4 confirmed that ovulations were efficiently synchronized, and ewes were at the beginning of the luteal phase during the nutritional treatment. Despite this, the effect of short-term flushing on follicle dynamics was below expectation. The lack of difference between GLU and WAT groups in the number of ovarian follicles and CL as response to exogenous FSH administration partially disagree with previous findings of our laboratory [6], and others [7] who found at least at some stage an increase of the number of 2- to 3-mm follicles at day 8 [6] or CL [7]. This could be partially explained by the overriding effect of FSH.

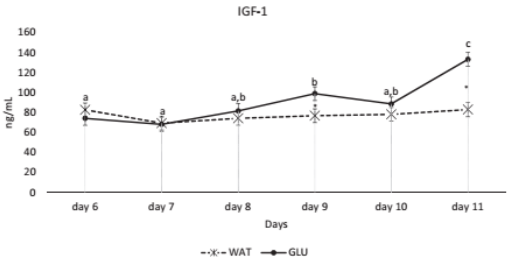

Fig. 5. Mean circulating concentrations of IGF-1 in ewes orally drenched with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10) during the experimental period (day 6–11; day 0 = sponge withdrawal); *indicates significant differences between groups ($P < 0.01$). a, b, c indicate significant variations within GLU group ($P < 0.01$).

In contrast with the lack of effect on follicular dynamics, the short-term flushing with a glycerol-propylene glycol mixture caused significant changes in the plasma and follicular fluid milieu. These changes were detectable from day 1 of administration and lasted up to the day after its end. The main impacts were on the glucose-insulin-IGF-1 system homeostasis with a positive trend of all the above levels, whereas NEFA and urea levels dropped in both the plasma and the follicular fluid.

The use of glycerol and propylene glycol as glucogenic precursors is based on in vitro and in vivo studies showing that the concentration of ruminal propionic acid increases when forage is supplemented with these organic compounds [28–31]. 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 [28,32].

In the present study, 4 d of glucogenic mixture administration had an immediate effect on glycemia and insulin plasma levels. Circulating concentration of glucose rose sharply in GLU group, reaching levels similar to those obtained in ewes by continuous glucose infusion at 10 mM/h for 72 h [33]. Consequently, in the same group, insulin circulating levels increased significantly, as showed by

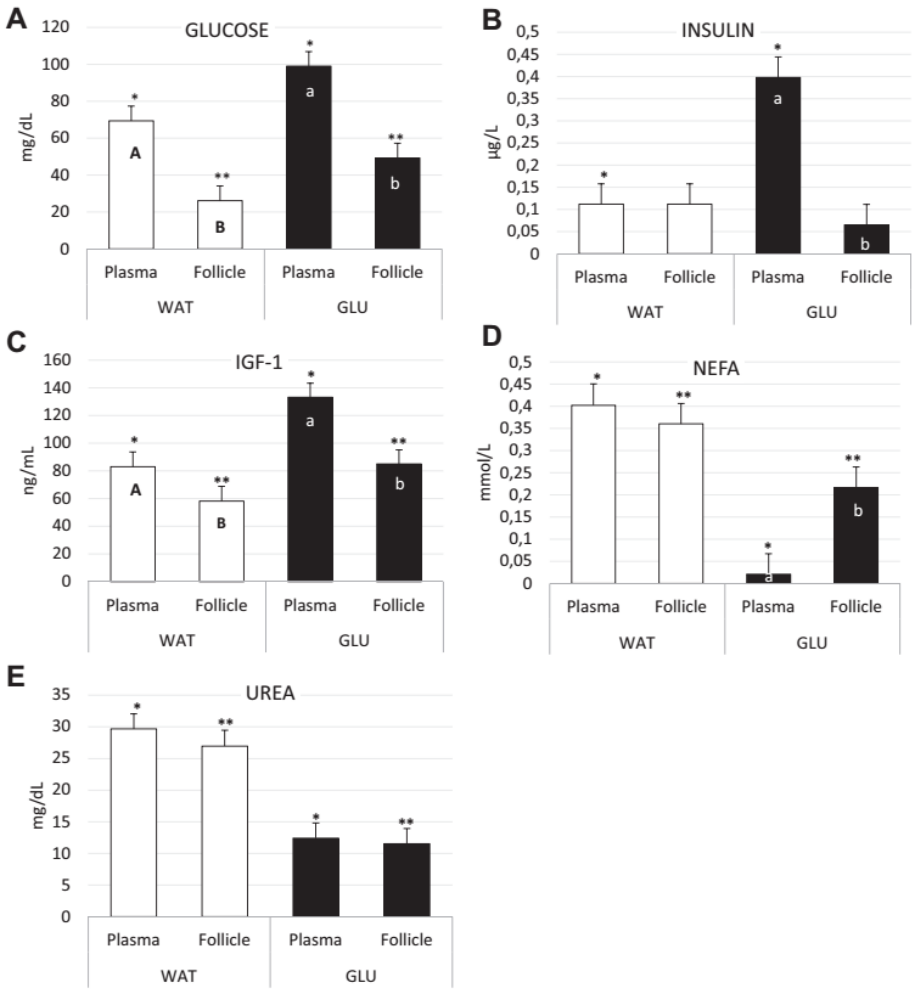

Fig. 6. Glucose, insulin, IGF-1, NEFA, and urea concentrations in the plasma and follicular fluid collected form Sarda ewes 12 h after the end of a 4-d oral drenching with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10). Follicular fluid was collected from follicles ≥4 mm in diameter. Asterisks indicate significant differences between groups in plasma ($P < 0.0001$) and FF concentrations ($P < 0.0001$). Different letters indicate statistical differences between plasma and follicular concentrations within the same group: upper case letters: WAT group; lower case letters: GLU group. Asterisks indicate statistical differences between WAT and GLU group in plasma (single asterisk) and in follicular concentrations (double asterisk). Glucose: GLM follicle vs plasma $P < 0.01$; WAT vs GLU $P < 0.0001$; treatment × source of sample $P > 0.05$. NEFA: GLM follicle vs plasma $P > 0.05$; WAT vs GLU $P < 0.0001$; treatment × source of sample $P < 0.05$. Urea: GLM follicle vs plasma $P > 0.05$; WAT vs GLU $P < 0.0001$; treatment × source of sample $P > 0.05$. Insulin: GLM follicle vs plasma $P < 0.0001$; WAT vs GLU $P < 0.01$; treatment × source of sample $P < 0.01$. IGF-1: GLM follicle vs plasma $P < 0.01$; WAT vs GLU $P < 0.001$; treatment × source of sample $P > 0.05$. IGF-1, insulin-like growth factor 1.

several studies in which nutritional treatments (lupin grain: [3,34]; a mixture of soya meal and maize: [35]; steamed corn flakes: [36]; a mixture of glycerol and propylene glycol: [7]) increased the concentrations of glucose and insulin in jugular venous plasma in ewes.

Although a previous study reported that IGF-I itself does not respond to short-term nutritional supplementation [3], in the present study, mean IGF-1 plasma levels were significantly higher in GLU than WAT group. The rise in IGF-1 plasma levels was slower than glucose and insulin's one, since it was detected from the third day of the nutritional treatment and reached its peak after its end (day 11).

The follicle has a functional insulin-glucose-IGF-1 system [37] which is affected by short-term nutritional treatments, and it is clear that components of this metabolic system are nutritionally regulated in the follicle [2,7].

The higher circulating glucose concentrations found in energy-supplemented ewes compared with controls are likely to exert a positive effect on ovarian function, especially because they were accompanied by a rise in glucose FF concentrations. It seems that the effect of glucose on fertility is primarily related to its properties as a metabolic fuel [1], glucose being the main source of energy for the ovary [37,38]. Different glucose transporters, including the

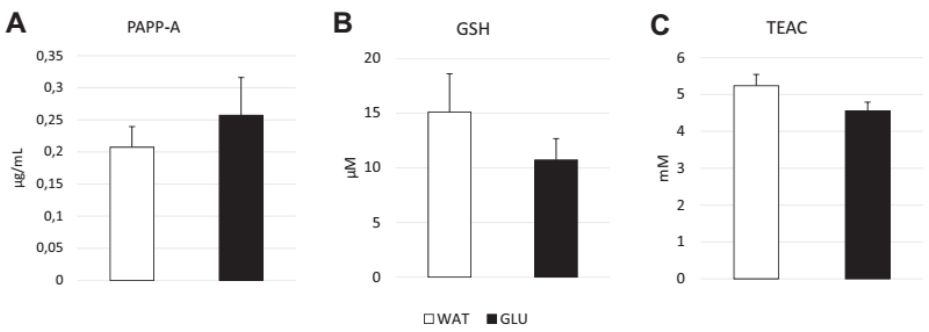

Fig. 7. Concentrations of PAPP-A (panel A) and GSH (panel B), and trolox equivalent antioxidant capacity (TEAC; panel C) in follicular fluid collected from Sarda ewes 12 h after the end of a 4-d oral drenching with a glucogenic mixture (GLU n = 10) or with water (WAT n = 10).

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 [39]. This system allows the follicle to regulate its growth and development, mainly by altering FSH-induced effects on the synthesis of estradiol by the granulosa cells, in accordance with the availability of glucose [37,40]. In addition, glucose is essential in determining the quality of the oocyte [5]. Our research group had already showed that in sheep glucogenic treatments improve oocyte quality, evaluated by the kinetics of their in vitro development and by the production of blastocysts [6]. Present findings suggest that those results were likely linked to the increased intrafollicular concentration of glucose, which was driven by the rise in its circulating concentrations.

Insulin is another key signal mediating the effects of acute changes in nutrient intake on follicle dynamics [40]. Insulin and glucose seems to act synergistically at the ovarian level, and it is likely that the effects of short-term nutrition may be mediated by direct ovarian actions of insulin and glucose [41]. *In vitro* research has shown dose-dependent stimulatory effects of insulin on the proliferation and steroid synthesis of the bovine granulosa and theca cells; these effects can be exerted via their direct stimulatory actions on the follicle as well as by increased local responsiveness of the follicle to FSH and LH [42]. An increase in insulin concentrations over a short lapse of time has been shown to exert a positive effect on the growth of small follicles before superovulatory treatment and a beneficial influence on subsequent embryonic development [43,44]. Thus, the rise in circulating insulin levels, triggered by glucose's rise, may contribute in creating a suitable systemic metabolic milieu for the promotion of ovarian function. However, no difference was found in insulin FF concentrations between the two experimental groups. Insulin likely reaches FF from the circulation by transudation, but, as observed in the present study, FF insulin concentrations do not correlate with plasma insulin [45]. Even if insulin plays an essential role in the final stage of follicular development, abnormal concentrations of the hormone can lead to follicular dysfunction, resulting in excessive atresia or the formation of follicular cysts [46]. Therefore, we can speculate that the follicle can buffer a rise in insulin circulating concentration to prevent its potential harmful effects at the local level.

Insulin-like growth factor 1 is a potent stimulator of both follicle growth and estradiol secretion [4], and the ovary is a site of IGF-I gene expression and reception [12]. In the present study, the significant rise in IGF-1 circulating concentration observed in energy-supplemented ewes was not accompanied, as for insulin, by a consequent rise in its FF concentrations.

According to the somatomedin hypothesis, nutritionally induced changes in liver IGF-1 secretion have a direct effect on the ovary through the endocrine actions of IGF-I, and thanks to its contribution to FF IGF-1 [47,48]. At this regard, Scaramuzzi et al [49] presented compelling evidence for an endocrine effect of IGF-I on ovine follicular development. They infused an IGF-I analog into an autotransplanted ovary and demonstrated increased estradiol secretion after PGF2α-induced luteolysis. Endocrine IGF-I secretion is controlled by somatotropin, and by the nutritional status (energy and protein intake relative to requirements) [48]. The nutritional treatment applied in the present study stimulated endocrine IGF-1 secretion, probably with accumulative effect that determined the insurgence of the highest values at the end of the treatment period. However, in the present study, we determined whole IGF-1 concentrations, which comprise both biologically active and inactive (bond to the IGFBP) IGF-I.

To assess possible changes in IGF-1 biological activity at the ovarian level consequent to the nutritional treatment applied, we determined intrafollicular PAPP-A concentrations, the main IGFBP protease regulating the levels of free IGF-1 in bovine dominant follicle [15,16]. However, no significant differences were observed between the two experimental groups.

The nutritional treatment applied also caused a significant drop in NEFA and urea circulating concentrations, which remained stably lower than in WAT group. Supplying meal or water with glycerol to transition dairy cow resulted in a positive energy status with higher concentrations of serum glucose and lower concentrations of plasma NEFA [8,10,11]. Lien et al [50] showed that animal treated with propylene glycol had lower levels of blood urea nitrogen respect to the control group. In ewes, supplementation of

Table 3
Pearson correlation coefficients between plasma and follicular concentrations of glucose, insulin, NEFA, IGF-1, and urea as evaluated 12 h after the end of a 4-d glycogenic mixtures administration in treated (n = 10) and control ewes (n = 10).

Analysed metabolites and hormones	Glucose		Insulin		NEFA		IGF-1		Urea	
	Plasma	Follicle	Plasma	Follicle	Plasma	Follicle	Plasma	Follicle	Plasma	Follicle
Glucose										
Plasma		0.8151 (**< 0.0001**)	0.7815 (**< 0.0001**)	−0.0918 (0.7171)	−0.3028 (0.2219)	−0.143 (0.5713)	0.7032 (**< 0.01**)	0.7458 (**0.0004**)	−0.3096 (0.2112)	−0.1589 (0.5288)
Follicle	0.8151 (**< 0.0001**)		0.7139 (**< 0.001**)	−0.4655 (0.0515)	−0.3753 (0.1249)	−0.231 (0.3563)	0.4962 (**< 0.05**)	0.5519 (**< 0.05**)	−0.4113 (0.0899)	−0.3856 (0.114)
Insulin										
Plasma	0.7815 (**< 0.0001**)	0.7139 (**< 0.001**)		−0.0546 (0.8297)	−0.5353 (**< 0.05**)	−0.3583 (0.1443)	0.5853 (**< 0.05**)	0.6294 (**< 0.01**)	−0.5486 (**< 0.05**)	−0.4171 (0.085)
Follicle	−0.0918 (0.7171)	−0.4655 (0.0515)	−0.0546 (0.8297)		0.0765 (0.7627)	0.1451 (0.5657)	0.2006 (0.4247)	0.0225 (0.9294)	0.2692 (0.28)	0.3622 (0.1397)
NEFA										
Plasma	−0.3028 (0.2219)	−0.3753 (0.1249)	−0.5353 (**< 0.05**)	0.0765 (0.7627)		0.6991 (**< 0.01**)	−0.2984 (0.2291)	−0.217 (0.387)	0.8122 (**< 0.0001**)	0.7876 (**< 0.0001**)
Follicle	−0.143 (0.5713)	−0.231 (0.3563)	−0.3583 (0.1443)	0.1451 (0.5657)	0.6991 (**< 0.01**)		−0.2226 (0.3746)	−0.0501 (0.8435)	0.6699 (**< 0.01**)	0.6777 (**< 0.01**)
IGF-1										
Plasma	0.7032 (**< 0.01**)	0.4962 (**< 0.05**)	0.5853 (**< 0.05**)	0.2006 (0.4247)	−0.2984 (0.2291)	−0.2226 (0.3746)		0.4414 (0.0667)	−0.2059 (0.4124)	−0.1062 (0.675)
Follicle	0.7458 (**< 0.001**)	0.5519 (**< 0.05**)	0.6294 (**< 0.01**)	0.0225 (0.9294)	−0.217 (0.387)	−0.0501 (0.8435)	0.4414 (0.0667)		−0.4119 (0.0895)	−0.2525 (0.312)
Urea										
Plasma	−0.3096 (0.2112)	−0.4113 (0.0899)	−0.5486 (**< 0.05**)	0.2692 (0.28)	0.8122 (**< 0.0001**)	0.6699 (**< 0.01**)	−0.2059 (0.4124)	−0.4119 (0.0895)		0.9454 (**< 0.0001**)
Follicle	−0.1589 (0.5288)	−0.3856 (0.114)	−0.4171 (0.085)	0.3622 (0.1397)	0.7876 (**< 0.0001**)	0.6777 (**< 0.01**)	−0.1062 (0.675)	−0.2525 (0.312)	0.9454 (**< 0.0001**)	

Abbreviations: IGF-1, insulin-like growth factor 1; NEFA, nonesterified fatty acids.
Corresponding *P*-values are shown within parenthesis.
Bold values indicate statistical significance.

soluble sugar reduced urea levels in serum and FF [51], and short-term high energy feeding diets lowered serum urea nitrogen levels while increasing the numbers of large follicles [52].

In the present study, glucogenic mixture administration caused a drop in NEFA and urea concentrations not only in the plasma but also in the follicular fluid. Although Rabiee et al [53] report that there was no significant uptake of NEFAs by the bovine ovary, others have reported that in cattle increased concentrations of NEFAs in blood were reflected in the FF microenvironment [54,55]. Elevated concentrations of plasma NEFA frequently observed in the FF of the dominant follicle in dairy cows were associated with reduced in vitro developmental competence of the oocyte [55–57]. In addition, high NEFAs levels can compromise the viability of the bovine granulose cells [58]. Therefore, diets or metabolic states that favor high concentrations of NEFAs should be avoided during the cycle of conception and the early post-conception period [1]. The present study while confirming the negative relationship between plasma concentrations of NEFA from one hand and insulin and glucose to the other, also showed that the drop in FF NEFA concentrations found in energy-supplemented ewes was slower than the one observed in the plasma. In other words, NEFA clearance in the ovary is less efficient than at the systemic level, and thus the negative effect of high FF NEFA concentration can persist even after the re-establishment of a positive energy balance. This could possibly explain while, under some circumstances, short-term flushing treatments based on lupins or glucogenic mixtures failed to improve reproductive performance of sheep submitted to long-term systemic underfeeding [59].

In the same way, high levels of urea in blood have been associated with lower fertility due to a changed uterine environment and poor embryo viability [60,61]. *In vitro* studies showed that urea can impair meiosis and thereby reduce the percentage of oocytes fertilized and of embryos that develop [62]. Elevated plasma urea levels reduced ovine embryo viability and development in vivo and in vitro [61,63]. Circulating urea concentrations are reflected in FF and may affect the quality of both the oocyte and the granulosa cells [54,64]. Therefore, the observed drop in NEFA and urea concentration and the systemic and follicular level can contribute to the creation of a suitable environment for follicular growth and for the acquisition of oocyte developmental competence.

The survival of follicles subjected to gonadotropin stimulation is also related to an increase in the expression of antioxidant enzymes since inadequate protection against ROS constitutes a trigger for follicular atresia [65]. Apoptosis of granulosa cells during follicular atresia is indeed preceded by oxidative stress, partly due to a drop in the antioxidant GSH. Within the follicle, glucose metabolism, via the pentose phosphate pathway, provides precursors for the synthesis of NADPH and thus for GSH regeneration. Results obtained in the present study showed no difference in FF GSH concentrations and total antioxidant capacity between GLU and WAT group, and no correlation was found between glucose FF concentration and its antioxidant defenses. Previous studies in *in vitro* cultured somatic cells showed that high glucose

concentrations (27.5 and 11 mM) in culture media decrease intracellular GSH by impairing cystine uptake capability [66]. However, this effect was not observed at lower glucose concentrations (5.5 mM), similar to those found in FF in the present study. Further studies are needed to better address the relationship between glucose FF concentration and follicular antioxidant defenses.

5. Conclusions

The present study showed that short-term administration of a glucogenic mixture significantly increased circulating concentration of glucose, insulin, and IGF-1, while reducing circulating concentration of NEFA and urea. These metabolic changes ultimately affected FF microenvironment, as evaluated 12 h after the end of the nutritional treatment. In particular, they brought about an increase in FF concentrations of glucose, without affecting FF insulin and IGF-1 concentrations, nor GSH content and total antioxidant capacity. However, FF concentrations of NEFA and urea were significantly lowered. A reduced clearance of NEFA was observed within the follicle, thus underlying that the negative effects on follicular growth related with high NEFA concentrations can persist even after the re-establishment of a positive energy balance.

Taken together, these results suggest that short-term administration of a glucogenic mixture, creates a suitable microenvironment for the final follicular growth and thus for the conception period in the ewe.

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The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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