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Does health start in the womb? Impact of maternal undernutrition during gestation on the reproductive and cardiovascular system of female offspring in dairy cattle

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LIST OF ABBREVIATIONS

ADG: Average daily gain

AFC: Antral follicle count

AGM: Aortic gonadal mesonephros

AMH: Anti-Müllerian hormone

Aot: Aortic root in transversal section

BCS: Body condition score

BL: Body length

BP: Blood pressure

BPM: Beats per minute

BW: Body weight

CL: Corpus luteum

CR%: Conception rate

CRL: Crown rump length

CTR: Control diet

DG: Day of gestation

DIAST: Diastolic pressure

DMI: Dry matter intake

DOHaD: Developmental origin of health and disease

EF: Ejection fraction

FP: Feeding program

FS: Fractional shortening

FSH: Follicle stimulating hormone

GnRH: Gonadotropin releasing hormone

GREL: Gonadal ridge epithelial-like cells

HH: Hip height

HR: Heart rate

HR: Heart rate

HW: Height at withers

IgG: Immunoglobulin G

IVSd: Interventricular septum in diastole

IVSs: Interventricular septum in systole

LH: Luteinizing hormone

LVIDd: Left ventricular internal diameter in diastole

LVIDs: Left ventricular internal diameter in systole

LVPWd: Left ventricular posterior wall in diastole

LVPWs: Left ventricular posterior wall in systole

M: Maintenance energy requirements

MAP: Mean arterial pressure

NR120: Nutrient restricted from 10 day before conception to day 120 of gestation

NR80: Nutrient restricted from 10 day before conception to day 80 of gestation

PBS: Phosphate buffered saline

PG: Prostaglandin

PR%: Pregnancy rate

SCC: Somatic cell count

SIST: Systolic pressure

T: Testosterone

TC: Thoracic circumference

TMR: Total mixed ration

ABSTRACT

Introduction. Evidence indicates that the environment encountered during fetal life influences development and post-natal risk of disease in mammals (Barker, 2007). Previous studies indicated that female beef calves born to mothers exposed to a nutritionally restricted diet in early gestation had a reduced total number of ovarian follicles (ovarian follicular reserve), enlarged aortic trunk size and enhanced peripheral arterial blood pressure independent of alterations in birth weight and postnatal growth (Mossa et al., 2013).

We hypothesized that energy restriction from shortly before conception (10 day before artificial insemination, AI) to different windows of gestation (day 80 and 120) may impair the development of the ovaries and post-natal function of the cardiovascular system of female offspring in dairy cattle.

Materials and Methods. Holstein Friesian heifers (n=42) were divided into two experimental groups: Nutrient Restricted (NR, n=32) and Control (CTR, n=10), similar in age (NR=16.1±1; CTR=16.34±1.5 mo.) and live weight (NR=366.1± 42.67; CTR= 368.8±39.1 kg). Starting 10 days before artificial insemination, NR heifers were individually fed a ration providing 0.6% of their energy maintenance needs (M), while the CTR group was fed at 1.8%M. All heifers were inseminated with sex-sorted semen from a single sire and the size of the preovulatory follicle (PO) was measured. Pregnancy was diagnosed at approximately 28 and 60 days post-AI and the number of antral follicles ≥ 3 mm (Antral Follicle Count, AFC) was assessed on the same days. At 60 days of gestation, pregnant NR heifers were divided in two subgroups and individually fed at 0.6M until either day 80 (NR80, n=11) or 120 (NR120, n=12) of gestation. CTR heifers were individually fed at 1.8M from 10 days before AI until day 120 of gestation. After the end of the differential feeding regime, all heifers were group fed ad libitum. The mean Voluntary Dry Matter Intake (DMI), body weight (BW) and BCS were recorded during the entire gestation.

Twenty-four single female calves were born (NR80, n=9; NR120, n=10; CTR, n=5). One calf (NR120) died at around 30 days of life due to omphalitis

complications, but data from her dam were included in the study. Another calf (NR80) was excluded from the study (including data from her pregnant dam) because it was considered abnormal.

Body weight and biometric measurements were assessed in healthy female calves (CTR, n=5; NR80, n=8; NR120, n=9) until slaughter at 135 days. Arterial resting peripheral blood pressure (BP) was measured with the tail-cuff system in calves every fortnight starting from 30 days of age until slaughter. The heart and aorta were examined by an echocardiographic exam when calves were 30 and 100-110 days old. Post-mortem, ovarian weight and size were measured, all visible antral follicles were counted and COCs (cumulus oocytes complexes) were retrieved. The heart and kidneys were weighed, aortic internal circumference and aortic diameter were measured.

Results. Conception rates at approximately 28 day post-AI (NR n=23, 74%; CTR n=6, 66%) and pregnancy rates at circa 60 day post-AI were similar between groups (NR n=22, 70%; CTR n=6, 66%). Heifers pregnant with a male calf were excluded from the experiment (NR, n=3; CTR n=1). Among heifers that would subsequently be pregnant (n=28), the mean PO size was smaller in NR heifers than in CTR animals ($p<0.05$); also, NR heifers that failed to conceive had a smaller PO compared to CTR animals that would become pregnant ($p<0.05$). Among pregnant heifers (n=28), the AFC was repeatable within the same individual from the day of conception to days 28 and 55-70 of gestation (n = 28; P = 0.66) and was similar between the NR and CTR groups during early pregnancy.

Heifers that would be subsequently pregnant in the three groups were similar in BW before the start of the differential feeding regime (NR120=379.3±14.5; NR80=372.9±14.5; CTR=389.6±11.1) and BW increased as pregnancy progressed in all groups ($p<0.0001$). CTR heifers were heavier than both NR80 and NR120 from day 12 of gestation to term, whereas no difference was detected between NR80 and NR120.

At birth BW was lower in NR80 than CTR calves ($p<0.05$) and tended to be lower in NR120 compared to CTR individuals ($p=0.107$). As calves grew older, BW among the three experimental groups resulted linearly similar until slaughter at 135 days. No differences were detected in arterial blood pressure and echocardiography parameters among groups.

The number of visible antral follicles (CTR=197.2±36.5; NR120=104.2±10.7; NR80=150.1±20.9) and weight of both ovaries (CTR=10.4±1.3g; NR120=6.7±0.5; NR80=7.4±0.9g) was lower ($p<0.05$) in calves born to NR120 dams compared to CTR calves. The mean number of COCs retrieved per animal was lower in NR80 and NR120 calves as compared to CTR individuals ($p<0.05$), but no difference was detected between NR80 and NR120 calves (NR80=48 ±3.5; NR120=48.2± 6.5; CTR=75.8±12.6). Volume of both ovaries (cm^3) was similar among the groups.

Conclusion. This study indicates that an energetically scarce diet during gestation in dairy heifers has deleterious effects on the development of the ovaries of the daughters, similar to previous findings in beef cattle, and potentially, on their future fertility. It should be noted that nutritional restriction resulted in an impairment of the ovarian reserve in the progeny, regardless of its duration (until day 80 or day 120 of pregnancy). Since female mammals are born with a finite number of oocytes, that progressively decreases with age, and is never replenished, the programming impact of maternal nutrition on the size of the ovarian reserve may be among the determinants of fertility in cattle.

1. INTRODUCTION

1.1. DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE

Evidence indicates that environmental factors in early fetal life may have an impact on later life, health and disease in mammals. This was reported for the first time by Barker et al., in 1986, which introduced the Developmental Origin of Health and Disease (DOHaD) hypothesis. Prof Barker and his collaborators demonstrated that "a poor nutrition in early life increases susceptibility to the effects of an affluent diet, resulting in increased mortality for coronary heart disease in later life" in humans. Following this original finding, numerous studies have tested this theory in several species. Presently, environmental factors acting from the periovulatory phase to different windows of gestation are believed to influence the expression of genes in the developing progeny, altering the epigenome of the target organism (Cushman et al., 2019).
This concept represents an incentive to investigate the impact that the environment may have during gestation and consequently on the progeny in humans using animal models.

The term "programming" has been introduced to describe the process whereby a stimulus or an insult at a critical and sensitive period of fetal or perinatal life has permanent effects on the structure, physiology, and metabolism of different organs and systems resulting in several differences in genetically similar animals due to modifications of epigenome function (reviewed in Mossa et al., 2015; Cushman and Perry, 2019). Indeed, epigenome refers to all the chemical modifications to DNA and histone proteins that regulate the expression of genes within the genome (Morgensztern et al., 2018).
External factors, such as maternal nutrition, high temperatures and other environmental stressors or internal factors, such as age and weight of the mothers, are described as being able to alter the epigenome of the developing

organism. For example, in dairy cattle, daughters born to a young dam produced more first-lactation daily milk, had higher body condition score (BCS), but experienced difficulties conceiving (Banos et al., 2007). Furthermore, gestating dams with higher BCS tended to give birth to a progeny with higher BCS, had lower return rates, but slightly lower daily milk yields (Banos et al., 2007). A study conducted in rats, described that pre-conceptional and gestational maternal obesity induced cardiac dysfunction and hypertension in offspring (Loche et al., 2018).

Initial studies were designed to identify early determinants of pathological conditions, thus, they primarily investigated the potential negative impacts of programming factors on the progeny (Cushman and Perry, 2019). The concept that controlling the conditions during gestation can induce desirable effects on the offspring is a novel area of investigation with relevant potential applications in both human medicine and livestock farming. One of the areas of application is the biology of reproduction in mammals, and how female fertility could be enhanced by prenatal life.
Before delving into these latter concepts, it is appropriate to explain the process and the phases of development of the female gonads in mammals in various species.

1.2. DEVELOPMENT OF FEMALE GONADS

Ovaries of different mammalian species largely develop in a similar manner but differences in timing and sequence of events are described (Smith et al., 2014). Ovarian development starts at the mesonephric surface epithelium, where the future gonadal ridge will be located. The surface epithelial cells differentiate into gonadal ridge epithelial-like (GREL) cells. The GREL cells proliferate, and

primordial germ cells migrate into the gonadal ridge among the GREL cells (Hummitzsch et al., 2013*)*.

This event occurs from around 7 to 11 embryonic days in mouse (Anderson et al., 2000), days of gestation (DG) 17 to 21 in sheep (Ledda et al., 2010) and DG 18 to 31 in cattle (Wrobel and Süss, 1998). In the bovine fetus, primordial germ cells can be seen in undifferentiated gonads prior to DG 35 (Fortune et al., 2013), and before DG 23 in the ovine species (McNatty et al., 1995).

Gonadal sex differentiation starts as these primordial germ cells migrate to the cortical region of the developing ovary and this occurs at day 12 in mice (Menke et al., 2003), DG 32 in sheep (Mc Natty et al., 1995) and DG 40 in cattle (Erickson 1966), although sex gene expression occurs earlier.

In bovine, primordial germ cells proliferate reaching up to 16.000 in number at DG 50, and 2.700.000 at DG 110. However, at DG 170 the number of primordial germ cells decreases down to 108.000 (Erickson et al., 1966).

Subsequently, through several mitotic divisions primordial germ cells become oogonia (Figure 1). Oogonia proliferate and the stroma penetrates toward the ovarian surface, thus enclosing oogonia and GREL cells into ovigerous cords. Cords are surrounded by a basal lamina at their interface with the stroma, but are open to the ovarian surface. The distinction into cortex and medulla becomes evident: the cortex is characterized by alternating areas of ovigerous cords and stroma; medulla is composed of stromal cells, vasculature, and tubules originating from the mesonephros, generating the rete ovarii (Hopper et al., 2015).

The stroma penetrates toward the periphery and the GREL cells at the surface of the gonadal ridge are aligned by a basal lamina at their interface with the stroma and begin to differentiate into typical ovarian surface epithelium. Ovigerous cords are segmented into smaller cords and lastly into individual primordial follicles, as the GREL cells mature becoming granulosa cells and surround the oogonium.

Finally, the surface epithelium becomes single-layered and a tunica albuginea, composed by connective tissue, develops from the stroma under the basal lamina of the surface epithelium (Hummitzsch et al., 2013).
Some primordial follicles become activated and develop into primary and preantral follicles as they enter in the first phase of meiotic division (prophase) at around day 80 post conception in bovine (Fortune et al., 2013) and day 55 in ovine (McNatty et al., 1995).
In cattle, evidence indicates that meiosis continues until approximately day 150, arresting at the diplotene stage of prophase I. Indeed, meiotic arrest, follicle formation and growth occur before birth in ruminants (Fortune et al., 2013), whereas in rats and mice oocytes proceed in synchrony through the leptotene, zygotene and pachytene stages of prophase I during the last third of gestation (Pepling, 2006).

Evidence indicates that a pool of approximately 133.000 primordial follicles remains quiescent and constant until 4-6 years old in cattle (Cushman and Perry, 2019). It is estimated that the follicular pool constantly declines undergoing atresia until the animal is 20 years-old and it runs out with the last ovulation (Cushman and Perry, 2019).
Albeit in modern farming systems, cattle are normally culled before the pool of ovarian follicles is exhausted, the biological pathways that regulate the size and establishment of the ovarian reserve (total number of healthy oocytes and follicles in the ovaries) during fetal life warrant investigation. Indeed, the size of the ovarian reserve may be among the determinants of fertility in cattle and the environment encountered by the dam during gestation may influence the establishment of the ovarian reserve in the offspring (Evans et al., 2012; Ireland et al., 2011; Cushman et al., 2009).

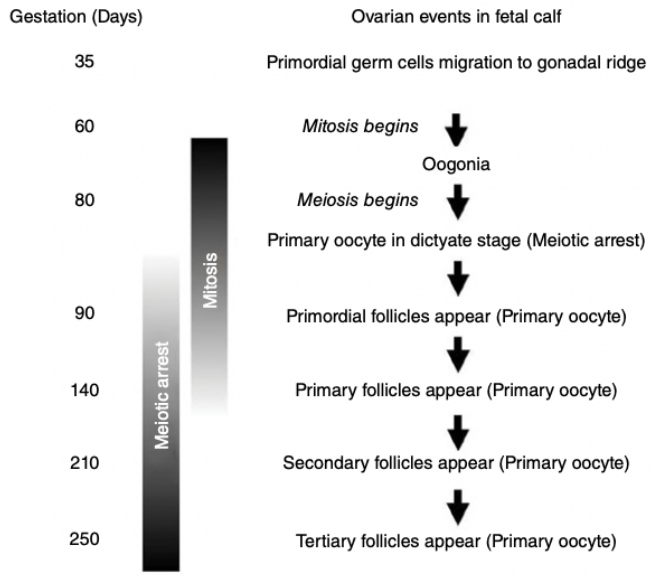

Figure 1. Bovine fetal ovarian development, chronology of early folliculogenesis and oogenesis (Hummitzsch et al., 2013).

1.3. OVARIAN FOLLICULAR DYNAMICS

Folliculogenesis is described as the development process where an activated primordial follicle grows reaching the preovulatory size until ovulation (Senger et al., 2003).Ovarian follicles are classified based on their morphologic characteristics, such as the number of granulosa cell layers surrounding the oocyte, the diameter of the follicle and oocyte or the presence or absence of a fluid-filled antrum. Generally, follicles are classified as primordial, primary, secondary, or tertiary (antral or vesicular) (Lussier et al., 1987).

Activation of primordial follicle starts when the flattened pre-granulosa cells transformed into a single layer of cuboidal granulosa (follicular) cells giving rise to the primary follicle (Braw-Tal et al., 1997). Granulosa cells proliferate and increase the number of layers that surround the oocyte, thus originating a secondary follicle (from 2 to 6 layers) and subsequently tertiary follicle or antral follicle characterized by a fluid filled antrum. The primordial follicle has a

diameter of about 0.04 mm whereas the small antral follicle reaches 0.25 mm (Lussier et al., 1987, Braw-Tal et al., 1997). Subsequently, a gonadotropin surge permits to the larger antral follicle, defined as Graafian follicle to become the ovulatory follicle (Braw-Tal et al., 1997). The time necessary for a follicle to grow from the secondary follicle stage to a mature ovulatory size has been estimated to be about 42 days (Lussier et al., 1987). In cattle, primordial, primary, secondary and tertiary follicles are described at days 90, 140, 210, and 250 of gestation, respectively (Rüsse et al., 1983).

The introduction of ultrasonography in the late 1980s and subsequent studies using ultrasonic imaging permitted to monitor follicular populations in different size categories (Pierson et al., 1987) or to monitor individually identified follicle (Knopf et al., 1989) and documented that follicular growth in cattle occurs in a wave-like fashion. Furthermore, it was demonstrated that the majority of the estrous cycles in cattle comprise two or three follicular waves (Figure 2). Follicular wave emergence in cattle is characterized by a sudden (within 2–3 days) growth of 8–41 small dimensions follicles, detected by ultrasonography, with a diameter of 3–4mm (Pierson et al, 1987a-b, 1998; Ginther et al., 1989a; 1989b). Growth rate is similar among follicles of the wave for about 2 days, after which one follicle, the dominant follicle, is then selected to continue its growth while the remainder ones, the subordinate follicles, become atretic and regress. The emergence of the first follicular wave occurs consistently on the day of ovulation (day 0) in both three and two wave estrous cycles while the emergence of the second wave occurs on day 9-10 for two-wave cycles, and on day 8-9 for three-wave cycles. In three-wave cycles, a third wave emerges on day 15-16. Under the influence of progesterone (for example during diestrus or pregnancy), dominant follicles of successive waves are anovulatory and undergo atresia (Bergfelt et al., 1991). The dominant follicle recruited at the onset of luteolysis becomes the ovulatory follicle and the emergence of the subsequent wave is delayed until the day of the ensuing ovulation. Subsequently, corpus luteum starts to regress earlier in two-wave cycles, around day 16, than in three-wave

cycles at day 19. This event result in a shorter estrous cycle (20 days in two and 23 days in three wave cycle) (Adams et al., 2008).

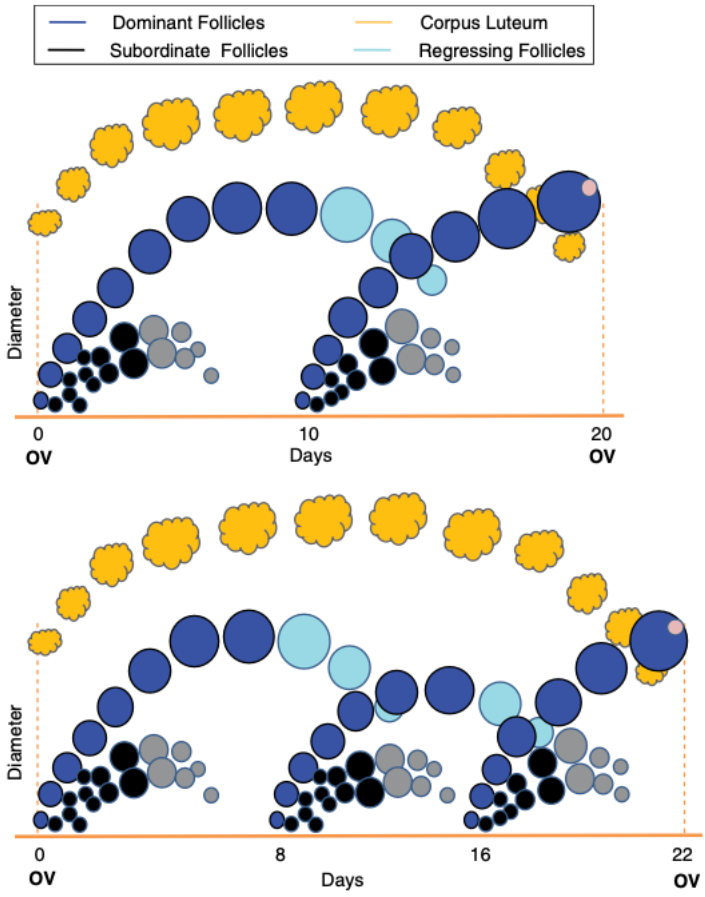

Figure 2. Follicular dynamics during a two-wave (upper panel) and three-wave (lower panel) estrous cycles in cattle. Events from both ovaries are represented, to identify the dominant follicle of each wave (growing, dark blue; regressing, light blue), the subordinate follicles of each wave (growing, black; regressing, gray), the occurrence of ovulation, and the growth and regression of the corpus luteum (dark yellow). A follicular wave in cattle is characterized by the sudden emergence (within 2–3 days) o of 8-41 small follicles, that grow at a similar rate for 2–3 days. Subsequently, one follicle is selected to continue growth (dominant follicle) while the others become atretic and regress (subordinate follicles). A 21-day estrous cycle is as an average between two-wave cycles (20 days) and three-wave cycles (22 days). (R.M. Hopper, Bovine reproduction, 2015)

The described duration of 21-day estrous cycle in cattle is an average between two- and three-wave cycles. (R. M. Hopper, 2015). There is no evidence that indicates a breed or age predilection for a specific wave pattern in *Bos taurus*

(R.M. Hopper, 2015). However, studies indicate an increase in the proportion of three-wave patterns has been associated with a low plane of nutrition (Rhodes et al., 1995) and heat stress (Wolfenson et al., 1995).

Emergence of a follicular wave and selection of the dominant follicle are temporally associated with a rise and fall in circulating concentrations of FSH (Follicle-stimulating hormone; Adams et al., 1992). Adams described in 1992 that the emergence of a follicular wave is preceded by a similar magnitude surge in plasma FSH concentrations in both spontaneous waves and induced waves: mid-cycle surges are similar in amplitude and breadth as those of the preovulatory gonadotrophin surge. FSH release and the subsequent follicular wave emergence are suppressed by follicular products (Estradiol and Inhibin), in particular those from the dominant follicle (Singh et al., 1998). All follicles of the new wave are responsible for FSH suppression, and this is supported by the positive relationship between the number of follicles emerging in response to the FSH surge and the subsequent degree of FSH suppression (Gibbons et al., 1997; Ginther et al., 2005). Inhibin-A is produced by small growing follicles of the wave and is considered the most important suppressor of FSH during the first 2 days of wave emergence, while estradiol, secreted from the dominant follicle, is defined the most important FSH suppressor thereafter (Bleach et al 2001). Circulating concentrations of FSH begin to rise over the next 2 days after the end of the dominance period (for example at ovulation), and there is a peak about 12–24 hours before emergence of the next follicular wave, when the future dominant follicle is 4–5 mm in diameter (Mihm et al., 2003).

1.4. OVARIAN RESERVE

The size of the ovarian reserve (total number of healthy oocytes and follicles in the ovaries) is established during fetal life in cattle. There is evidence of variability among age-matched animals in the size of the ovarian follicular reserve in cattle and of the positive correlation between follicle numbers and fertility (Evans et al., 2010, 2012; Ireland et al., 2011; Cushman et al., 2009). The gold standard to reliably estimate the total number of ovarian follicles in different stages of development (from primordial to antral follicles) is the histological analysis of the cortical tissue; this approach requires either ovariectomy or post-mortem recovery of the gonads. To overcome this issue, two in-vivo indicators of the size of the ovarian have been validated in cattle: 1) the antral follicle count and 2) the peripheral concentrations of Anti-Müllerian hormone.

The antral follicle count (AFC) is the total number of ovarian antral follicles equal or larger than 3 mm in diameter countable in both ovaries and it is determined by ovarian ultrasonography. Ovarian ultrasonography is a non-invasive technique which can be performed during a routine gynecological exam of the reproductive tract in heifers and cows. Calves can also be scanned by using an extension probe, when rectal examination is not feasible due to their reduced body size. The operator scans both ovaries from side to side and counts the total visible antral follicles equal or larger than 2-3 mm in diameter. The **AFC is positively correlated with the total quantity of healthy follicles and oocytes in ovaries of cattle** (Burns et al., 2005; Ireland et al, 2011). Further, a positive correlation between the number of visible antral follicles evaluated post-mortem and the numbers of healthy primordial, secondary and total follicles was described in Bos indicus cows (De Vasconcelos et al., 2020).

Burns (et al., 2005) conducted a study in dairy Holstein heifers at different ages, stage of lactation, season of the year. A single operator performed an ultrasound analysis on fourteen 10-12 mo. heifers and sixteen 4-7 yr. old cows (9 cows at late stages of lactation, 7 cows not lactating), twice a day, to count the total number of follicles (3 mm in diameter or greater) throughout 138 follicular waves. Results demonstrated that the repeatability of AFC is high in the same animal (range between 0.8 to 0.9, where 1 is perfect). Subsequent studies confirmed the repeatability of AFC in cattle independent of breed, age, season, stage of lactation and span of time between AFC evaluation in the same individual (Ireland et al., 2007, Ireland et al., 2008, Ireland et al., 2009, Jimenez-Krassel et al., 2009, Mossa et al., 2012, Succu et al., 2020). Recent studies described AFC repeatability: i) between an unknown stage of follicular growth and the day of wave emergence in lactating dairy cows (0.37; Gobikrushanth et al., 2017); ii) from puberty to yearling age in Bos taurus indicus beef cattle (0.89-0.92; Morotti et al., 2017) and iii) following weaning to pre-service in Hereford and Braford heifers (0.72; Santa Cruz et al., 2018).

The AFC was reported as highly variable among individuals (range 8 to 54) (Burns et al., 2005). Several studies confirm that AFC has a high variability among heifers/cows. For example, in Holstein heifers AFC ranged from 3 to 36 (Succu et al., 2020) and 18 to 110 (Baldrighi et al., 2014), in lactating Holstein cows it varied from 4 to 61 (Mossa et al., 2012), from 6 to 45 on the expected day of follicular wave emergence, from 10 to 53 at an unknown stage of follicular growth (Gobikrushanth et al., 2017).

In cross-bred beef heifers AFC ranged from 4 to 56 (Cushman et al., 2019) and 7 to 54 in Angus (McNeel et al., 2017). In Bos taurus indicus beef cattle AFC varied from 3 to 64 in (Morotti et al., 2017), whereas in multiparous Bos taurus indicus dairy Nelore cows the AFC ranged 21-51 (De Lima et al., 2020) and 2-50 (Santos et al., 2016). A variability in the detection of the antral follicles can differ among operators, settings and frequency of assessment of AFC.

Another validated indicator of the size of the ovarian reserve in cattle is the peripheral concentration of Anti Müllerian hormone (AMH). AMH is a protein hormone, that has a crucial role during differentiation of gonads. Before sexual differentiation, mesoderm starts to proliferate giving rise to two genital duct pairs, The mesonephric duct or Wolffian, and paramesonephric duct or Müllerian. Sertoli cells of the fetal testis produce AMH, which has an inhibitory effect on Müllerian duct, enabling proliferation of the Wolffian ducts and the subsequent differentiation into epididymis, seminal vesicles and other structures of the male reproductive tract. Ovaries, in fetal life are not able to produce AMH, so this allows Wolffian duct to differentiate in female tract structures, such as uterus, cervix, oviducts and vagina (Spencer et al., 2012).

In females during post-natal life, AMH is predominantly produced by granulosa cells in growing follicles in ruminants and women (Vigier et al., 1984, Bézard et al., 1987, La Marca et al., 2006). Similar to AFC, it is considered an indicator of the size of the ovarian reserve in cattle; AMH is not produced by primordial follicles, whereas it is secreted when follicles are recruited, from the primary follicular stage of development (McGee and Hsueh, 2000), it reaches a peak when follicles are in the preantral and small antral follicular stages, and then decreases as the selected follicle reaches the preovulatory stage under the FSH stimulus (Monniaux et al., 2008, Veiga-Lopez et al., 2012). Moreover, evidence indicates a high positive association between AMH serum levels and the antral follicle population in cattle (Ireland et al., 2008, Rico et al., 2009, Baldrighi et al., 2014), sheep and goats (Monniaux et al., 2014), thus both indicators have been used to estimate the size of the total number of healthy oocytes in these species. Indeed, similar to AFC, AMH serum concentrations vary greatly among individuals, but are highly repeatable in the same animal (Ireland et al., 2011).

1.5. CAN REPRODUCTIVE FUNCTION BE PROGRAMMED?

Scientific reports indicate that in dairy and beef cattle the majority of reproductive traits are lowly heritable (Berry et al., 2014). AFC is considered a moderately heritable genetic trait in dairy cows (0.31 ± 0.14) and heifers (0.25 ± 0.13; Walsh et al., 2014). Subsequently, in a study conducted on 2,905 Holstein heifers, the AMH pedigree based heritability was 0.43 ± 0.07 (heritability estimate) and AMH genomic heritability was 0.36 ± 0.03 (Nawaz et al., 2018).
A similar study evaluated the genomic heritability of AFC in 198 Canadian Holstein cows (0.46 ± 0.31; (Gobikrushanth et al., 2018). These estimates (Walsh et al., 2014, Gobikrushanth et al., 2018, Nawaz et al., 2018) are considered the highest for any trait associated with reproduction in female cattle (Berry et al., 2014), so is possible to use genetic selection to increase the size of the ovarian reserve in dairy cattle.

On the other hand, evidence indicates that the environment during fetal life may impact the development and future function of the female reproductive apparatus. Specifically, there is evidence that numerous factors acting during gestation can have an impact on the daughter's future reproductive performance in ruminants (Cushman and Perry, 2019). Most studies focused on the impact of the exposure to high environmental temperatures and humidity during different stages of pregnancy, as well as nutritional status of the dam.. A study conducted by Succu et al. 2020 showed that heifers born to mothers exposed in early gestation to high environmental temperatures and humidity had smaller ovarian reserve compared to herd-mates conceived in winter.
Several studies show that a poor fetal growth due to alterations in maternal diet suppresses gonadotropin gene expression in the pituitary and the number of ovarian follicles in lambs (Da Silva et al., 2002).
Furthermore, a restriction in maternal diet inhibits follicular development of the offspring. A study conducted by Mossa et al. (2013) showed that providing a

restricted diet to beef heifers to 0.6 of their maintenance energy requirements a few days before conception and for the first trimester of pregnancy (110 days) had an impact on daughter follicular reserve. Despite the weight of the daughters resulted unaltered at birth, AFC (evaluated at 7,18 and 35 weeks of age) was lower (60%) in female calves born to mothers fed with restricted diet compared to calves born to control mothers (Mossa et al., 2013).

A study conducted on dairy cows showed that a pathological condition, such as the high number of SCC (more than 200.000), considered an accurate index of recurrent udder infections (Green et al., 2004), could determine a diminished ovarian function and suboptimal fertility in the offspring (Ireland et al., 2008).

This emerging area of research that investigates how maternal environment could determine embryonic and fetal development and the future productive e reproductive life is of fundamental importance, especially in dairy cows, as it could lead to prenatal approaches (such as nutrional, sanitary) to improve fertility and health of the offspring.

Farm animals may often be exposed to nutritional deficiencies during gestation and lactation. Large ruminants, in particular periparturient and lactating dairy cows, are predisposed to metabolic diseases, decreases in production, difficulty in reproductive recovery and uterine involution. Breeding of small ruminants is often a peculiarity of marginal areas so there is less attention to the food ration. Furthermore, in ruminant farms, there is a growing demand for the role of the nutritionist, who accurately studies the food ration based on all physiological needs. Given the current distance from the constant contribution of this professional figure, it is good to study the effects of an unbalanced nutrition to raise awareness on this issue.

Overnutrition has often been studied in rodents as a model for humans, even though fewer studies have been conducted on the effects of undernutrition on the offspring. Moreover, in literature are described several works in which a specific feeding regime was supplied to different species in different stages of

gestation and the effects evaluated on the reproductive system of their offspring (Table 1).

Undernutrition

In ewes fed a low energy (50% M) compared to high energy (150%M) diet from mating to early gestation, a reduced number of oogonia was detected at 47 DG high energy diet compared to low energy progeny, but at 62 d the degeneration of germ cells was less advanced in low energy than high energy offspring. These results indicate a delay in ovarian development in daughters born to mothers underfed from mating and for early gestation (Borwick et al.,1997).

The negative effects of maternal undernutrition were also confirmed in a study conducted by Abecia et al., (2014 a; 2014 b) in which ewes were underfed from mating to DG 7 and 15 resulting in an increased number of oogonia in one and two month old female lambs, respectively. A similar study (Rae et al., 2001) described that ewes born to mothers underfed (50%M) compared with ewes born to mothers fed a control diet (100%M) during different periods of mating to DG 100, showed a delay in ovarian development.

A greater FSH response to GnRH and a greater number of follicles (3mm) was evaluated in pubertal ewes born to mothers underfed during the first month of gestation. Contrarily, the same treatment from DG 31 to 100 determined a lower number of corpora lutea and reduced number of ovulations in ewes (Kotsampasi et al., 2009).

Rae et al., (2002) compared offspring born to underfed mothers vs control mothers from mating to DG 95. The adult offspring born to underfed mothers had a reduced ovulation rate compared to control resulting in reduced reproductive performances.

A nutrient restriction in sheep from mating to DG 55 (0.6 M) determined a higher number, but a reduced proliferation rate of germ cells in daughters, two

months after individual feeding, compared to daughters control fed in utero (Smith et al.,2019).

In sheep overfed in utero during the third trimester of gestation (from DG 47 to 147) ovulation rate was similar to the progeny of to control fed ewes (Gunn et al., 1995).

Hoffman et al., (2018) reported that ewes undernourished in utero during the last trimester of pregnancy (from DG 100 to parturition) had smaller secondary and tertiary follicles and less endometrial glands.

In cattle, a low protein diet provided during the first trimester of gestation and subsequently replaced with a high protein diet during the second trimester of pregnancy determined small follicles and a reduced number of primordial and primary follicles as adults (Sullivan et al., 2009).

In rats it was confirmed the same negative effect of maternal undernutrition on the offspring. Sloboda et al., (2009) showed that maternal undernutrition during the entire pregnancy and lactation cause an advanced pubertal age in the offspring.

Comparing these studies in different species, it is evident that, in ruminants, further studies are necessary, whereas more information is available in murine models. No studies have investigated the impact of unbalanced nutrition for the entire pregnancy, particularly in dairy cattle; this is probably caused by the length of gestation and the cost necessary to support them.

Overnutrition

Other studies showed the effects of maternal overnutrition on female reproductive performances in the offspring. In sheep fewer follicles in the ovaries of female fetuses exposed to high compared with moderate diet from 4 to 130 DG were reported (Da Silva et al., 2002) or from mating to 130d of gestation (Da Silva et al., 2003). Weller et al. (2016) compared the effects of a maternal moderate diet to a high intake diet in cattle from 0 to 199 and from 0

to 268 DG. They demonstrated that a restricted diet is responsible for a lower number of primary, preantral and antral follicles in the offspring compared to a moderate diet.
Increased maternal nutrient intake during the third trimester increased the proportion of daughters that calved in the first 21 d of their first calving season (Cushman et al., 2014).

In rats, maternal overnutrition from 5 day before mating until the end of lactation caused in female offspring a reduced number of small follicle and an increased serum concentration of estradiol compared to rats born to mothers fed moderately. Sloboda et al., (2009) tested also the effect of maternal overnutrition in rats for the entire pregnancy and it caused an advanced pubertal age in the offspring. Neonatal rats exposed to high fat diet during the entire gestation had an advanced puberty onset, irregular estrous cycles, a lower number of secondary follicles in the ovary (Zhou et al., 2019)

These results confirm the negative effect of overnutrition on development of fetal ovary and reproductive performances as already demonstrated in the effects of maternal undernutrition. Furthermore, it would appear that there is less scientific interest in overnutrition as it is a nowadays a less common problem in livestock than undernutrition.

Table 1. Effects of individual diets on dams during gestation on reproductive development in female offspring (Modified from Mossa et al., 2015).

Period of gestation	Species	Maternal diet	Period of diet	Effect on the offspring	Reference
1st third of gestation	Sheep	**Undernutrition**	Mating to early gestation	Delayed fetal ovarian development at 47 and 62d of gestation	(Borwick et al.,1997)
	Sheep	**Undernutrition**	Mating to 7 d mating to 15d	Increased total number of oocytes at 1 and 2 mo old	(Abecia et al.,2014a, b)
	Sheep	**Undernutrition**	Mating to 30 d	Increased FSH response to GnRH and small follicles at 10 mo old	(Kotsampasi et al.,2009b)
	Sheep	**Undernutrition**	31 to 100 d	Decreased number of corpora lutea at 10 mo old	(Kotsampasi et al.,2009b)
	Cattle	**Undernutrition**	-11 d before insemination-110 d	Decreased number of follicles, lower AMH and higher FSH concentrations	(Mossa et al., 2013)
	Cattle	**Low-high protein**	Low protein in first trimester, high protein in second trimester	Smaller largest follicle before puberty, lower densities of primordial, primary and healthy antral follicles as adults	(Sullivan et al.,2009)
	Sheep	**Undernutrition**	Mating to 55 d	Increased number of germ cells, Less proliferation of germ cells	(Smith et al.,2019)
	Sheep	**Undernutrition**	Mating to 55 d	Increased number of germ cells, Less proliferation of germ cells	(Smith et al.,2019)
2nd and 1st-2nd third of gestation	Sheep	**Undernutrition**	Mating to 95 d	Reduced ovulation rate in adults	(Rae et al.,2002a)
	Sheep	**Overnutrition**	Mating to 130 d4 to 130 d	Fewer follicles in fetuses	(Da Silva et al.,2002, 2003)
	Cattle	**Overnutrition**	Insemination to 199d Insemination to 268d	Lower number of primary, preantral and antral follicles	(Weller et al.,2016)
3rd third and entire gestation	Cattle	**Overnutrition**	Third trimester	Higher proportion of heifers calved in their first calving season	(Cushman et al.,2014)
	Sheep	**Undernutrition**	47 to 147 d	No difference in ovulation rate in adults	(Gunn et al.,1995)
	Rats	**Undernutrition**	Entire pregnancy and /or lactation	Advanced pubertal age	(Sloboda et al.,2009)
	Rats	**High fat diet**	Entire pregnancy and/or lactation	Advanced pubertal age	(Sloboda et al.,2009)
	Sheep	**Undernutrition**	From 100d to parturition	Decreased diameter of secondary and tertiary follicles	(Hoffman et al.,2018)
	Rats	**Overnutrition High fat diet**	-5 d before mating until the end of lactation	Decreased number of small follicles Increased E2	(Li et al.,2017)
	Rats	**High fat**	From mating to parturition	Lower number of secondary follicles Advanced puberty onset Irregular estrous cycle	(Zhou et al., 2019)

1.6. ROLE OF MATERNAL HORMONES ON OVARIAN AND CARDIOVASCULAR DEVELOPMENT OF THE FETUS

Evidence suggests that maternal nutrient restriction is correlated with increased androgens level. A study conducted in beef heifers, showed that circulating maternal testosterone (T) level was higher in underfed (NR) heifers compared to heifers fed with a control diet for the first 110 days of gestation (Mossa et al., 2013). It was observed a T rise at day 7 of gestation in NR and it persisted until the end of the differential diet at 110 days.

Moreover, leptin, a metabolic hormone produced by adipose tissue, can inhibit production of androgens modulating the secretion of GnRH (gonadotropin releasing hormone). In fact, circulating leptin concentration are low in healthy, when there is a adipose tissue loss or overweight individuals (Thompson et al., 2015).

Androgens and testosterone were hypothesized to play a determinant role on reproductive and cardiovascular system differentiation and function.

A connection between reproductive and cardiovascular system has been described. Aorta and gonads have a common embryonic precursor, AGM (aortic-gonadal mesonephros) anlage (Kumaravelu et al., 2002) and androgen receptors are present in embryonic stem cells, pre-implantation stage embryos (Chang et al., 2006), fetal cardiovascular (Sajjad, Quenby, Nickson, Lewis-Jones, & Vince, 2007) and reproductive systems (Fowler et al., 2011; Sajjad, Quenby, Nickson, Lewis-Jones, & Vince, 2004; Yang & Fortune, 2006). These findings allow us to speculate that AGM may have been influenced by maternal feeding restriction in beef cattle (Mossa et al., 2013), leading to an impairment on ovarian and cardiovascular development of the progeny.

1.7. FETAL PROGRAMMING AND EFFECT ON CARDIOVASCULAR SYSTEM

Several studies conducted in humans indicate that a nutritional imbalance can have deleterious consequences on the cardiovascular system of the progeny (Barker et al., 1986, 1988).

Indeed, it was demonstrated that measurements taken at birth such as birthweight, length body proportions and placental weight, are correlated strongly with either the later incidence of disease, including coronary heart disease and hypertension in later life (Barker et al., 1988, 1989, 1993, 1995, 1999; Godfrey et al., 2000; Martyn et al., 1996).

Barker and Osmond (1988) found a relation between low birth weight and a raised diastolic blood pressure conducting several investigations in different age categories in Britain. Other scientific investigations have been conducted with an epidemiological approach in marginal areas of the world, where maternal malnutrition is common. A study conducted in Jamaica found that children of mothers who were thin in early pregnancy had increased blood pressure at 10 years of age (Godfrey et al., 1994). In South India the prevalence of coronary heart disease was higher in men and women whose mothers had low weight in pregnancy (Stein et al., 1996). The incidence of stroke is associated with a pattern of retarded fetal growth that is found in mothers with a 'flat' bony pelvis, a deformity that is caused by nutritional deficiencies in childhood (Martyn et al., 1996).

Fetal nutrition is determined by the combination of the mother's dietary intakes and nutrient stores, together with nutrient delivery to the placenta and the transfer capabilities of the placenta (Owens et al., 1989). The placenta could contribute to altered fetal cardiac development altering total peripheral resistance because of alterations in vasculature or vascular reactivity (Tappia et al., 2006).

A nutritional restriction during pregnancy has been the purpose of various scientific works that have used the animal model. For example, a study conducted on rats, revealed that maternal restriction from day 10 until parturition determine hypertension in the offspring. This research hypothesized that maternal undernutrition inhibits angiogenesis, thus increasing their susceptibility to develop cardiovascular disease such as hypertension in adult life (Khorram et al., 2007).

Another study employed a rat model in which pregnant mothers were fed with low protein (LP) and a normal protein diet from 2 weeks before mating and for the entire pregnancy. The left ventricular (LV) internal diameters were increased until 12 weeks of age in the low protein diet group. Furthermore, between 3 days and 2 weeks of age the LV wall of the heart in the LP group were thinner in low protein than in normal protein diet group. At 40 days reduced mean arterial pressure was observed in LP (Cheema et al., 2005).

Mossa et al. (2013), using beef cattle as a model, described an aorta trunk dilation and increased peripheral blood pressure in female offspring, born to mothers underfed for the first 110d of gestation.

2. AIM OF THE STUDY

We hypothesized that energy restriction from shortly before conception (10 day before artificial insemination, AI) to different windows of gestation (day 80 and 120) may impair the development of the ovaries and post-natal function of the cardiovascular system of female offspring in dairy cattle.

In particular this research aims to:

1) Evaluate the effect of an acute dietary restriction (from 10 days before estrus) on the development of the preovulatory follicle and pregnancy rates (pregnant heifers/inseminated heifers) of the dams;

2) Identify the windows of exposure to maternal nutrient restriction that can program the size of the ovarian reserve and influence the cardiovascular system in female progeny by:

- Evaluating weight at birth and the growth in female progeny;
- Assessing the number of follicles in prepubertal offspring;
- Assessing the progression of aortic trunk dilation, cardiac morphology and peripheral arterial blood pressure in pre-pubertal offspring.

3. MATERIALS AND METHODS

3.1. ETHICAL APPROVAL

All animal experiments were performed in accordance with DPR 27/1/1992 (Animal Protection Regulations of Italy) in conformity with European Community regulation 86/609 and were approved by the local Committee for the Animal Welfare of the University of Sassari (prot. n 0001848 of 2/5/2019).

3.2. DESCRIPTION OF THE FARM

This study was conducted from April 2021 to July 2022 in a commercial dairy farm located in North Sardinia, Italy (40°35'29.8" N 8°53'19.9" E). The farm is equipped with technological and automated systems. The herd consists of 650 Holstein cattle, 310 of which are lactating cows. All animals are housed in paddocks, groups are based on the physiological and productive phase of the animals and there is access to pasture. Calves are housed in individual igloo boxes from approximately 0 to 70 days of age. Igloos are designed for rearing calves outdoors, with a sheltered areas and an open area surrounded with a gate equipped with two feed openings. Male calves are usually sold within one month after birth, whereas female calves are group housed in a dedicated paddock from 70 days to 10-12 months of age. The other paddocks host: pubertal nulliparous heifers (from 12 mo. to 2-3 weeks before calving), lactating cows (from 1-2 weeks post-calving to approximately 60 days before parturition), dry cows and heifers/cows in peri-partum (from 2-3 week before calving to 1-2 weeks post-partum). This organization allows different feeding regimes for each group and reduces competition among individuals.

Each cow is milked twice a day using an automatic herringbone milking machine for a mean of 210 days of lactation. The mean production per animal/day is 35

kg, for a total amount of 11.500 liters per year (Composition: 4,20% lipids; 3,45% proteins).

Artificial insemination (AI) is performed by the farmers, with limited use of protocols to induce ovulation. Estrus is detected with activometers (equipped in neck collars) in cows and by visual observation in heifers. The first insemination is performed approximately at 15 months of age and the average period of days open (the interval between calving and the subsequent conception) is around 140 days.

3.3. PROCEDURES IN THE DAMS

3.3.1. SELECTION OF THE HEIFERS

Heifers to be enrolled in experiment were selected within the herd. There were 55 replacement Holstein Friesian heifers, aged 14 to 18 months in the herd, that had never been subjected to artificial insemination. Heifers were selected based on their body weight: they were weighed, in a chute equipped with a digital scale. Heifers with homogenous body weight were selected. Further, tame animals that manifested low resistance to handling were selected.

A gynecological exam was performed on the selected heifers on a random day of the estrous cycle. The heifers were captured in self-capturing racks and the reproductive tract was visualized by transrectal ultrasound (MyLab™Omega, Esaote, Italy, equipped with a 4-10 MHz linear transrectal transducer) by the farm vet, Dr Sebastiano Sale. Heifers with a physiological development of the reproductive tract and that had a corpus luteum were considered reproductively mature and enrolled in the study.

Based on the abovementioned selection criteria, 42 Holstein Friesian heifers were recruited; their mean age was 16.2±1.22 mo., ranging from 14 to 18 mo.; their mean body weight (BW) was 366.2 ±41.1 kg (range 465-305 kg).

3.3.2. EXPERIMENTAL PROTOCOL IN THE DAMS

The previously selected 42 Holstein Friesian heifers were randomly assigned to two experimental groups balanced on body weight and age: Nutrient Restricted, **NR** (n=32; BW=366.1±42.67 kg; Age=16.1±1.13 mo.) and Control, **CTR** (n=10; BW=368.8±39.1 kg; Age=16.34±1.55 mo.). Heifers in the NR group were assigned a diet providing 0.6 of their maintenance energy requirements (M), whereas the CTR group was fed at 1.8M. All heifers were individually fed (details are provided in "Experimental diets and individual feeding") starting ten days before artificial insemination. As described in the following paragraphs, pregnancy was diagnosed via ultrasonography of the reproductive tract around 28 days after AI and confirmed 55-60 days post insemination; heifers diagnosed as non-pregnant at 28 or 55-60 days post-AI were excluded from the study. At 55-60 days post-AI fetal sex was also determined, and heifers pregnant with a male calf were excluded from the study and returned to the farm herd.

Following pregnancy confirmation at 55-60 days post-AI, pregnant heifers in the NR group (n=23) were divided into two subgroups, omogenous in body weight: the **NR80** group (n=11; BW= 388.5±14.5 Kg) was fed the Nutrient Restricted diet (0.6M) until day 80 of pregnancy, whereas the **NR120** group (n=12; BW= 404.3±11.6 Kg) received the experimental diet until day 120 of gestation. Pregnant heifers in the CTR (n=6) were individually fed the 1.8M diet until day 120 of gestation. After the end of the differential feeding regime, all heifers were group fed a total mixed ration ad libitum until calving (Figure 3).

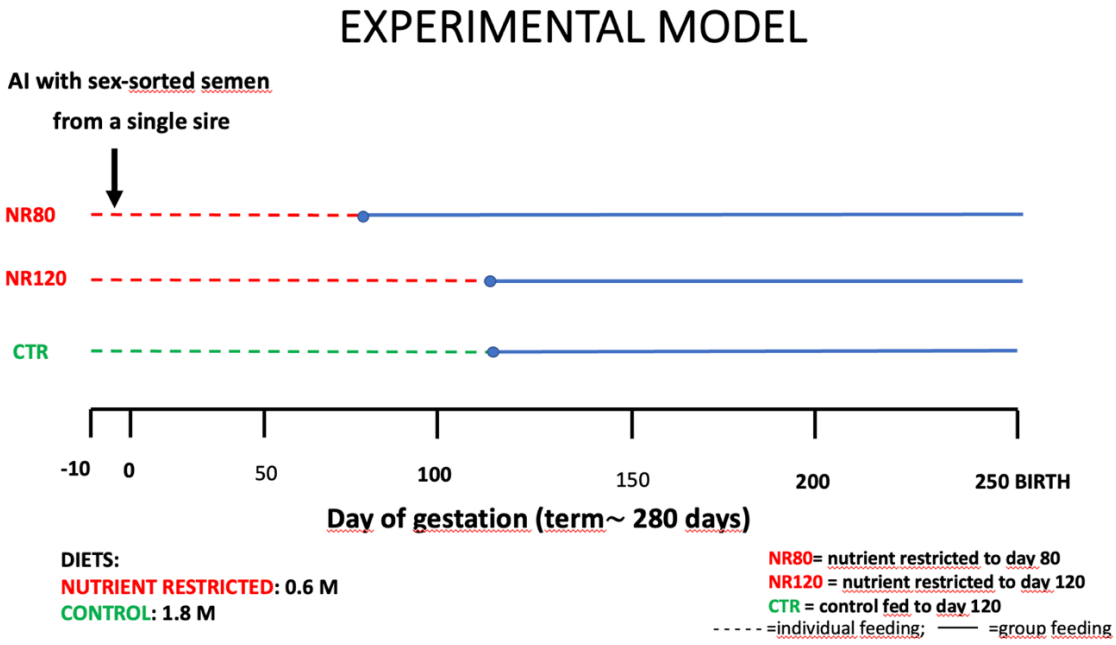

Figure 3. Experimental model. Holstein Friesian heifers (n=42) were divided into two experimental groups: Nutrient Restricted (NR, n=32) and Control (CTR, n=10), similar in age (NR=16.9±1; CTR=17±1.6 mo.) and live weight (NR=365.4± 42.2; CTR= 368.8±39.1 kg). Starting 10 days before artificial insemination, NR heifers were individually fed a ration providing 0.6% of their energy maintenance needs (M), while the CTR group was fed at 1.8%M. All heifers were inseminated with sex-sorted semen from a single sire and pregnancy was diagnosed at approximately 28 and 60 days post-AI. At 60 days of gestation, pregnant NR heifers were divided in two subgroups and individually fed at 0.6M until either day 80 (NR80, n=11) or 120 (NR120, n=12) of gestation. CTR group was individually at 1.8M from 10 days before AI until day 120 of gestation. From day 120 of pregnancy to calving all heifers were group fed ad libitum.

3.3.3. EXPERIMENTAL DIETS AND INDIVIDUAL FEEDING

The selected heifers were initially divided into 4 paddocks, pending the start of the individual diet and artificial insemination. Control heifers were located in a paddock with 10 stalls per self-capturing rack, NR80 and NR120 heifers were placed in paddocks with 8, 16 and 16 stalls per self-capturing rack, respectively. After being housed in the paddocks, all heifers were allowed a 10-days adaptation period prior to the start of the individual feeding regime.

The individual ration was supplied in boxes drilled to the ground in the feed lane. Heifers were restrained in the self-capturing rack, so that they could only access their individual box (Figure 4) and were immediately released after feeding. All heifers had ad libitum access to water. The rest area was organized either in single cubicles or on straw bedding.

Figure 4. Individual feeding method. The total mixed ration was individually calculated for each heifer and placed in boxes drilled to the ground in the feed lane. Heifers were restrained in the self-capturing rack and allowed to only feed from their individual box.

The Total mixed ration (TMR) was formulated based on the energetic intake of the diet. The ration consisted of a commercial complete feed *(FiberFeed*, Cooperativa Produttori Arborea). The Wet and dry TMR diets (Table 2) and were prepared using the large ruminant nutrition system (LRNS).

Dry TMR contained: 34.5% grass hay, 19.2% steam flaked corn, 3.9% cane-beet molasses blend, and 42.4% grain mix {29.6% wheat bran, 29.4% sorghum grain, 21.6% soybean meal, 14.7% flaked soybean, 2.2% calcium carbonate, 1% sodium chloride, 0.4% magnesium oxide, 0.9% sodium bentonite, and 0.3% vitamin and mineral premix [provided 40,000 IU of vitamin A, 4,000 IU of vitamin D3, 30 mg of vitamin E 92% α-tocopherol, 5 mg of vitamin B1, 3 mg of vitamin B2, 1.5 mg of vitamin B6, 0.06 mg of vitamin B12, 5 mg of vitamin K, 5 mg of vitamin H1 (para-aminobenzoic acid), 150 mg of vitamin PP (niacin), 50 mg of choline chloride, 100 mg of Fe, 1 mg of Co, 5 mg of I, 120 mg of Mn, 10 mg of Cu, and 130 mg of Zn]}. Wet TMR (kg/DM) contained 4.01 kg ryegrass hay, 2.40 kg of ryegrass silage, 0.43 kg of ground fine corn, 0.88 kg Soybean meal 48%, 0.10 kg 4020 optimizer complex, 2.18 kg mineral premix.

Table 2. Chemical composition of experimental diet (%DM).

Item	**Dry TMR[1]**	**Wet TMR**
DM (Dry matter)	96.6	95.2
CP (Crude protein)	14.5	10.9
NDF (Neutral detergent fiber)	45.3	48.7
ADF (Acid detergent fiber)	30.2	32.6
EE (Ether extract)	1.4	2.5
ADL (Acid detergent lignin)	4.3	4.2
Ash	9.7	9.0
ADIP (Acid detergent insoluble protein)	1.0	0.9
Sugars	5.2	4.6
SOLP (Soluble protein)	4.2	4.9
NDIP (Neutral detergent insoluble protein)	3.2	1.8
NDF24 (NDF determined after 24h incubation)	48.8	48.3
Starch	16.3	11.6
[2]NFC (Non-fiber carbohydrate)	29.1	28.9
PSPS (Mean±SD[3])		
19 mm (%)	12.9±1.99	28.3±9.09
8 mm (%)	26.9±4.29	42.4±7.77
4 mm (%)	19.1±0.99	14.9±1.1
Bottom (%)	41.2±2.67	14.4±4.13
peNDF(physically effective NFD)	27.44	41.60

[1]TMR, Total mixed ration
[2]Calculated as NFC = 100 − CP − ash − NDF − ether extract.
[3]Penn state particle separator (PSPS)

Diet composite samples were used to determine particle size distribution on an as-fed basis using the Penn State Particle Separator (Lammers et al., 1996). Diet peNDF (physically effective NDF, the fraction that stimulates rumination and contributes to proper ruminal digesta mat consistency) was calculated as the product of the total diet NDF content and its physical effectiveness factor (Mertens, 1997).

DMI at the end of individual diet program was calculated based on predicted equations (NRC, 2001; Hoffman et al., 2008) below:

1. DMI=12.91×(1 − e(−0.00295 × BW) [(Hoffman et al. (2008); end of individual diet program to day 250 of gestation]
2. DMI=1.71 − (0.69× eˆ(0.35 × DP − 280))/100 × BW [NRC, 2001; >250 to calving]

where e is the Euler number (e=2.718), BW is the body weight and DP is the day in pregnancy.

To ensure freshness of the rations, the feed was weighed daily and stored in individual plastic bags, marked with the farm identification number of each heifer. At the time of the meal, each heifer was restrained in the self-tapping rack and was allowed to eat exclusively from her box (Figure 4). The daily ration was individually offered in two portions to the NR group (8:00; 15:30 h) and three portions per day (8:00; 12:00; 15:30 h) for the CTR group. Residual feed was individually collected from CTR heifers and weighted daily to measure DMI by subtracting TMR refusals from supplied. All heifers received an additional 1 kg of hay per day, to maintain adequate ruminal activity. After the end of individual feeding, heifers were group fed with ad libitum access to feed (1.4M) until calving (Table 3).

Table 3. Example of food ration (1.4M) calculated and provided to heifers pregnant with a single female calf from the end of the experimental individual feeding period (day 80 of gestation for NR80, day 120 for NR120 and CTR groups) to calving. Heifers were group fed with ad libitum access to feed.

Item	**DM amount (Kg)**	**Conc. S.S. (%)**
Volume	7.812	100.000
DM (Dry matter)	4.328	55.404
Moisture	3.484	44.596
CP (Crude protein)	1.183	15.143
Sol Prot. % of CP SOLP (soluble protein)	29.305 ratio	29.305 ratio
Forage products	6.405	81.989
NDF (neutral detergent fiber)	4.042	51.744
Digestible NDF	1.442	18.440
ADF (acid detergent fiber)	2.134	27.319
Fat	0.917	2.517
Rumen Sol Sugar	0.355	4.546
Ash	0.751	9.618

3.3.4. ESTRUS SYNCHRONIZATION PROTOCOL AND ARTIFICIAL INSEMINATION

To best manage AIs, calving assistance and calves management, heifers were divided into two estrous synchronization and AI groups that were inseminated 21 days apart (1st AI group: CTR n=5, NR n=15; 2nd AI group: CTR n=5, NR n=17).

On the first day of differential feeding regime, the reproductive tract of the heifers was examined by transrectal ultrasound (MyLab™Omega, Esaote, Italy equipped with a 4-10 MHz linear transrectal probe) by the farm vet, Dr. Sebastiano Sale. Heifers with a corpus luteum (CL) received two prostaglandin (PG) treatments administered 10 days apart (Cloprostenol, PGFVeyx™, Bayer; 2ml IM). Heifers without CL and without follicles >10 mm in diameter received Gonadotropin Releasing Hormone, GnRH (Gonadorelin, Enagon™, Intervet productions; 2ml IM) followed by prostaglandin (Cloprostenol, PGFVeyx™, Bayer; 2ml IM) after 10 days. Starting 12h after the second injection, heifers were visually monitored to detect signs of estrus and the presence of a preovulatory follicle was confirmed by reproductive ultrasonography. Approximately 8h after heat was detected, heifers were artificially inseminated with frozen-thawed semen from a single sire (Barbaro, *In Seme,* Italy), to minimize the variables in the experimental trial. Sex-sorted semen was used to increase the number of female calves born.

3.3.5. PREGNANCY DIAGNOSIS AND FETAL SEX ASSESSMENT

Pregnancy was diagnosed and monitored by the farm vet, Dr. Sebastiano Sale, by ultrasonography of the reproductive apparatus (MyLab™Omega, Esaote, Italy equipped with a 4-10 MHz linear transrectal probe). Pregnancy status was assessed at approximately 28 day post-AI (Figure 5); non-pregnant heifers were

excluded from the trial and returned to the herd. Positive signs of pregnancy were the presence of a CL (an echogenic, ovoid-shaped structure in the ovary), anechogenic fluid in the ipsilateral uterine horn, the embryonic vesicle (circa 20 mm in length) and the embryo heartbeat evidenced by a rapid sparkling of echogenic points (180-200 points per minute; G. Gnemmi, 2004).

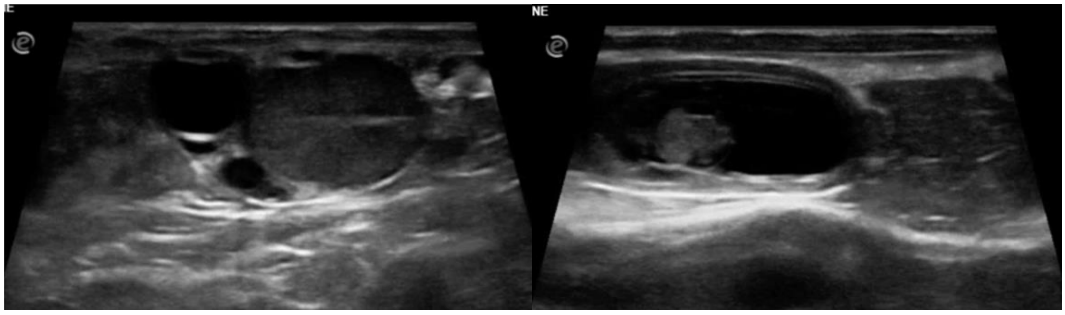

Figure 5. Pregnancy diagnosis 28 days post-AI. The image on the left shows an ovary bearing a 7 mm antral follicle on the top left, three smaller follicles and a CL on the right. The image on the right shows a section of the uterine horn: the horn is fluid-filled and the conceptus is visible at the bottom left of the uterine cavity. Ultrasound images were acquired from My Lab Omega, Esaote™ equipped with a 4-10 MHz linear transrectal probe.

Pregnant heifers were examined as previously described at 55-70 days of gestation to determine fetal sex. At this stage of gestation, the fetus has normally reached 6-7 cm in length from the head to the lower part of the body (crown-rump length, CRL), some organs such as the omasum and abomasum and the ossification centers of the skull and vertebrae are visible. Fetal sexing is based on the localization of the genital tubercle, that is first visible around day 41-44 of gestation. Both in male and female fetuses, the genital tubercle is bilobed, hyperechoic and brilliant. Around day 58 the tubercle reaches its final position (Figure 6), just caudal to the navel in the male and under the tail in the female. In the male fetus after day 65 the genital tubercle assumes a quadrilobed shape following the formation of the urogenital folds; the external genitals (scrotum and foreskin) will be visible from the day 70 of gestation. In the female fetus,

the genital tubercle (which will give rise to the clitoris) appears bilobed and the mammary papillae appear. in the period between 80-130 days of gestation the diameter of the mammary glands is 0.6-3 mm (L. DesCoteaux, J. Colloton, G. Gnemmi et al.,2009).

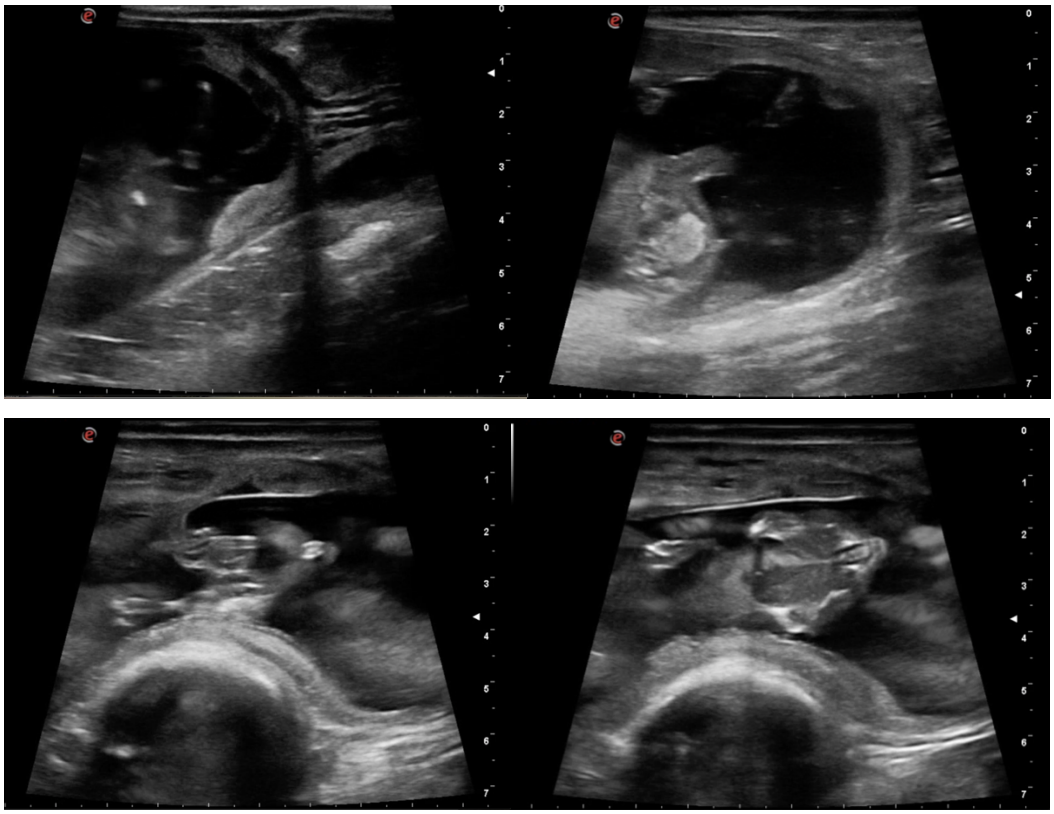

Figure 6. Pregnancy confirmation and fetal sexing at 55-65 days post-AI. The upper panels show images of male calves, as the genital tubercle is visible just caudal to the navel. The lower panels show female calves, with the genital tubercle located under the tail. Ultrasound images were acquired with a My Lab Omega, Esaote™, equipped with a 4-10 MHz linear transrectal probe.

3.3.6. ASSESSMENT OF THE SIZE OF THE PREOVULATORY FOLLICLE AND OF THE ANTRAL FOLLICLE COUNT (AFC)

Ultrasonographic exams of the reproductive tracts conducted on the day of AI, day 28 and day 55-70 post-AI were recorded to allow for their subsequent analysis. To remove investigator bias and enhance accuracy, videos were subsequently transferred on a laptop and analyzed by a single operator, who was blinded of the heifer ID. Videos were analyzed with Windows 10® and data were recorded on Excel®. The internal diameter of the preovulatory follicle (maximum internal diameter, Figure 7), was assessed in all heifers that showed estrus and were inseminated (n=40; CTR n=9; NR n=31).

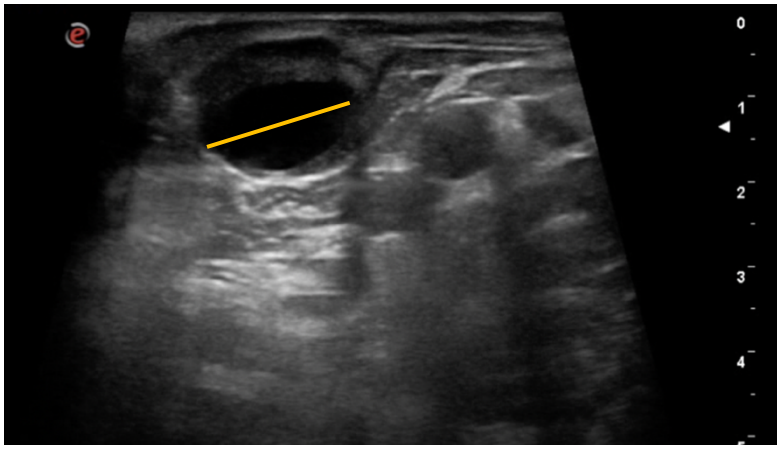

Figure 7. Measurement of the internal diameter (yellow line) of the preovulatory follicle (PO). In this picture, the PO is an egg-shaped, anechoic structure, located in the bottom-center of the ovary. Ultrasound images were acquired with a My Lab Omega, Esaote™, equipped with a 4-10 MHz linear transrectal probe.

The total number of antral follicles ≥ 2mm in diameter (Antral Follicular Count, AFC) was determined at AI, day 28 and 55-70 in pregnant heifers (N=28; CTR n=6; NR n=22; Figure 8). The entire surface of both ovaries was scanned and the total number of antral follicles ≥ 2mm in diameter was counted (Figure 8).

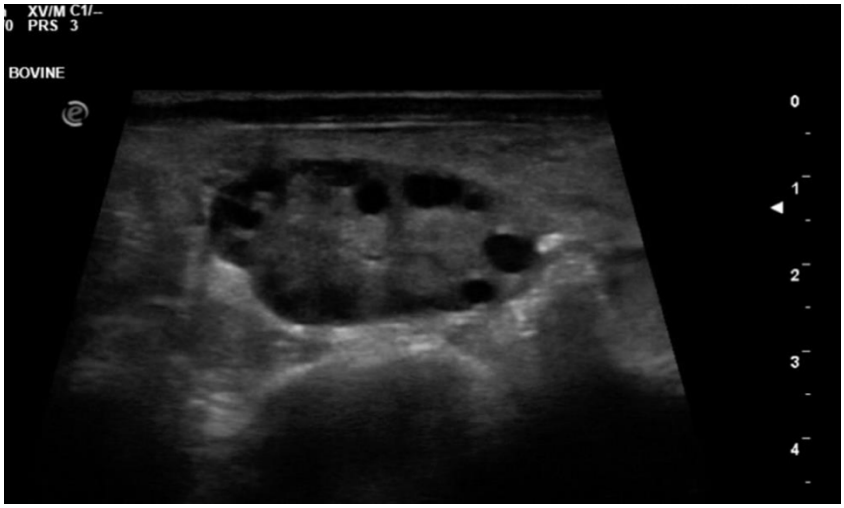

Figure 8. Example of antral follicle count (AFC) assessment. The entire surface of both ovaries was scanned and the total number of antral follicles ≥ 2mm in diameter was counted. The image shows one ovary with approximately 15 antral follicles (circular anechoic structures) ranging from 2 to 4 mm in diameter, located in the gonad circumference. Ultrasound images were acquired with a My Lab Omega, Esaote™, equipped with a 4-10 MHz linear transrectal probe.

3.4. SAMPLING PROCEDURES IN THE DAMS

3.4.1. BLOOD SAMPLING IN THE DAMS

Blood samples were collected from heifers during gestation on a monthly basis starting one day before the start of the differential feeding regime. Blood was collected from the coccygeal vein, using 20G needles with the vacutainer system red cap (added with Clot activator) and purple cap (added with K3EDTA) 9 ml tubes (Vacutest™). Each tube was marked with the heifer ID and transported to the laboratory in a refrigerated container. The red tubes were centrifuged at 4°C at 2500 rpm x 15 minutes to obtain serum, whereas the purple tubes were centrifuged at 3000 rpm x 15 minutes to retrieve plasma. Finally, both serum and plasma samples were stored in 2ml Eppendorf tubes at -20°C, pending subsequent analysis.

3.4.2. BODY WEIGHT AND BCS ASSESSMENT

The heifers were weighed monthly with a digital scale, positioned inside a manual chute.

Every two weeks, the weight of the heifers was measured through the thoracic circumference (TC), considered one of the most accurate indirect ways to estimate the weight in livestock. TC is measured in centimeters (cm) using a measuring tape (Animeter ™, Kerbl) by encircling the chest cavity behind the scapular bone joint (Hasan et al., 2020). The tape has two sides, one divided in centimeters and on the opposite is reported the corresponding estimate weight in kilograms.

BCS was also assessed, on occasion of gynecological examinations for five measurements, at different days of gestation (d): i) -14 d from AI; during the first gynecological examination (two weeks before AI); ii) 0 d, when performing AI; iii) 28 d at pregnancy diagnosis; iv) 55 d at pregnancy confirmation; v) 265d,

of gestation; vi) two weeks after calving. The evaluation of BCS provides a gross, but accurate measure of energy reserves in cattle (Roche et al., 2009).

3.5. PROCEDURES IN THE OFFSPRING

3.5.1. CALVING AND CALVES MANAGEMENT

Three weeks prior to the expected calving date, heifers were moved to the calving pens with straw bedding and access to water. Each pen had been set up in an area that was easily accessible to personnel, in order to facilitate assistance during calving and timeliness in the first calf care.

The calves were separated from their mothers within 4 hours after birth and placed in individual pens with abundant straw bedding. The navel was promptly disinfected with a dipping container for the udder, filled with a 50% solution of iodopovidone (LH iodo10™, Lombarda H S.r.l, Italy); navel disinfection is a fundamental operation for the hygiene and sanitary management of calves.

The calves were kept for the first 4 days of life in individual boxes and then housed for approximately 30 days in group paddocks (each of them housed 4 animals), organized by birth order. When calves were approximately 30 days old they were moved to individual igloos (Calf-o-Tel, Grifovet, Italy) located in the sheltered area inside the barn (Figure 9). The straw bedding, was renewed at least twice a week or more frequently if necessary.

Figure 9. Organization of the calf farm.

3.5.2. CALVES FEEDING PROTOCOL

The same feeding regime was used for all single female calves (NR80 n=9, NR120 n=9, CTR n=5) regardless of the experimental groups of the dam during gestation.

COLOSTRUM EVALUATION

Colostrum was collected from multiparous cows that had recently calved (within 12h) in the herd. The colostrum was measured with the refractometer and only used if the Brix scale was 22% or greater. Good quality colostrum was either immediately administered to the calves or stored in hermetic containers at -20°C.

The administration of colostrum is the most important and delicate operation in the management of the calf because it is essential to prevent the onset of neonatal diseases. Indeed, provision of a good quality colostrum (Immunoglobulin G (IgG) concentration ≥50 g/L)) is the first step toward ensuring proper passive transfer of immunity to the newborn calf (Buczinski et

al., 2016). The colostrum IgG concentration cannot be measured on farm, thus the most common system is to estimate it is the Brix scale, measured with a refractometer. This procedure has been described as highly correlated with the IgG concentration in the colostrum and a value of 22% or higher has been estimated to define a good quality colostrum (Buczinski et al., 2016).

COLOSTRUM ADMINISTRATION

Colostrum was administered promptly within 6 h after birth and with a minimum quantity of 3-4 liters per calf, followed by two administrations of 2 liters twice a day (8:00 and 19:00 h) for the first 4 days of life. The calves were fed with a bucket connected to a teat to facilitate sucking.
If the calves were born when fresh good quality colostrum was available (i.e. during milking time of a fresh cows in the herd), they received fresh colostrum, whereas calves born away from milking time (i.e. at night) were fed previously frozen colostrum; colostrum was slowly thawed by inserting the hermetic container in water at high temperatures, until reaching 37°C, the optimal administration temperature. Furthermore, in the first two days the colostrum was always supplemented with 2g of freeze-dried colostrum (Bayern Genetik™) in both daily meals. In case of emergency, when quality of colostrum was poor (Brix scale between 18 and 22%), it was further supplemented with a double dose (4g) of freeze-dried colostrum.

MILK FEEDING PHASE

From day 5 of life calves were fed milk replacer (Servatec™, Excell) twice a day (8:00 and 19:00 h.; Table 4). The milk was reconstituted with a Milk Bar (Milk Shuttle™, TDM Total dairy management) consisting of a stainless-steel tank (capacity of 200 liters) with electric movement. Furthermore, this tool allowed us to directly supply the milk in the bucket (Figure 10), thanks to a dispensing

gun that allowed us to automatically calibrate the necessary quantity of milk. To simplify the cleaning phase after each use, the Milk Bar was equipped with a drain system that allowed us to conveniently empty both any leftover milk and the washing water.

Table 4. Schedule of liquid feeding phase. During the first 4 days of life, calves were fed with colostrum, necessary to reduce pathologies in the first months of age. Subsequently, the diet was milk-based, and the quantity increased as the days progressed.

Age (day)	**Liquid food**	**Quantity, twice a day (L)**
1	Fresh/frozen + freeze dried colostrum	2
2-4	Fresh/frozen colostrum	2
5-10	Milk replacer	2.5
10-30	Milk replacer	3
30-60	Milk replacer	3.5

Figure 10. Calf feeding system. Liquid feed was supplied inside a bucket.

WEANING PHASE

To stimulate the development of rumen and forestomaches, milk-based nutrition was supplemented with small quantities of starter concentrated feed from day 5 of life, with a gradual increase in the quantity as the calves grew. From day 60 to 70 of life, each calf was weaned. The quantity of milk was gradually reduced starting from day 60 to day 70 of life with the complete interruption of the liquid feeding. On the other hand, the amount of starter feed was gradually increased in order to compensate for the liquid feed and gradually allow the calf to adapt to solid feed (Table 5).

Table 5. Weaning protocol. The weaning process was completed between day 60 and 70 of life. Calves were fed twice daily at 7:30 and 19:00. The amount of milk replacer decreased as days progressed, while the quantity of solid feed (Starter concentrated feed) increased to compensate the nutritional requirements and stimulate the development of the rumen and forestomaches.

Time (h)	**7:30**		**19:00**	
Age (day)	Milk replacer, L	Starter concentrate feed, Kg	Milk replacer, L	Starter concentrate feed, Kg
60	3	1.5	3	1.5
61	3	1.5	2.5	2
62	2.5	2	2.5	2
63	2.5	2	2	2.5
64	2	2.5	2	2.5
65	2	2.5	1.5	2.5
66	2	2.5	1	2.5
67	1.5	2.5		2.5
68	1.5	2.5		2.5
69	1	2.5		2.5

3.5.3. CALVES HEALTH MANAGEMENT

When calves were around 10 days old, some cases of neonatal diarrhea occurred (*Cryptosporidium spp.)* and were diagnosed with rapid antigenic tests (Kerbl). All calves were treated with a Paromomycin-based drug (Parofor crypto™, Huvepharma; 2 ml/10 kg IM of body weight for 7 days.

Furthermore, when calves where one month old, six of them manifested cough, nasal discharge, and sometimes fever. The calves were carefully examined, nasal swabs were collected and *Pasteurella* infection was diagnosed. The calves with fever and nasal discharge received antibiotic treatment (Alamycin LA 300™, oxytetracycline dihydrate 1ml/10 kg every three days for one week).

In addition, a thoracic ultrasound exam was performed to monitor the health of the calves. This technique has recently gained great importance in herd management and permits to identify several pathologic conditions as atelectasis,

edemas, emphysema, and the pneumonias as are frequent causes of death in perinatal calves (Jung et al., 2004). Evidence also indicates that consolidated areas ≥ 3 cm diagnosed by ultrasound in early life are related to a decrease in milk production (-525 Kg milk in fist lactation; Dunn et al., 2018).

Each calf was gently restricted in standing position, thoracic areas where not clipped or shaved but was only used ethyl alcohol (90°) diluted with water, and ultrasound gel. Lung areas were examined performing ultrasound by screening dorsal to ventral intercostal spaces from the right 2nd through 10th and left 3rd through 9th intercostal spaces (Teixeira et al.,2017). The examination was performed by ultrasonography (MyLab Alpha™, Esaote, Italy, connected with a phased array probe; center frequency 2.8MHz) designed for cardiologic examination (details are subsequently provided in the paragraph"Echocardiography") or another device (MyLab One™, Esaote) connected to a linear transrectal probe (4-10MHz).

3.6. IN VIVO PROTOCOL IN CALVES

Blood samples were collected from the calves, following the method described for the dams. The sampling regime was: 1) within 24h after birth, 2) every two weeks in the first month of life and 3) monthly for the last two months before slaughter.

Calves growth was also monitored at birth and every two weeks until slaughter, by measuring BW and other body measurement: Thoracic Circumference (TC, cm), Height at wither (HW, cm), Hip height (HH, cm), Back length (BL, cm). Female calves were weighted in a chute, designed for small ruminants and calves, and all body measurement were assessed using a meter.

3.7. EXAM OF THE CARDIOVASCULAR SYSTEM

3.7.1. ARTERIAL BLOOD PRESSURE MEASUREMENT

Arterial resting peripheral blood pressure (BP) was measured in calves every fortnight starting from 30 days of age. The tail-cuff system was used with a non-invasive electronic sphygmomanometer (Cardell Veterinary Monitor 9401BP, SHARN Veterinary) that had been previously validated in cattle (Mossa et al., 2013). This instrument consists of a monitor connected to a cuff, on which the following values (mmHg) are reported using the oscillometric technique: systolic pressure (SYST), diastolic (DIAST), mean of diastolic and systolic (MAP) and heart rate (BPM). Based on the tail circumference, a cuff size was selected: 8-12 cm cuff was used for all calves. The calf was gently restricted in standing position by an operator and the cuff was applied the base of the tail. A minimum of five consecutive BP measurements (each including SYST, DIAST and MAP) per calf was recorded. Three consecutive measurements with MAP variation < 10mmHg were selected and included in the study.

3.7.2. ECHOCARDIOGRAPHY

To investigate the long-term impact of maternal nutrition on the offspring cardiovascular system, an echocardiographic exam was conducted. The heart and aorta were examined when heifers were 30 and 100-110 days old. The calf was gently restricted in standing position while the head was extended upward. Cardiovascular system was evaluated using an ultrasound machine (MyLab Alpha™, Esaote) connected to Phased array Probe (center frequency 2.8MHz) designed for Cardiologic examination. The probe was placed, on the right side of the calf, cranially to the sternum. To better visualize all ultrasound images and different prospective of the heart, the probe was rotated. Each exam was recorded in B-mode and M-mode in order to subsequently measure different anatomical portions in more detail. Accuracy of the data was assured by

measuring every parameter three consecutive times from the same ultrasound image and subsequently calculating the mean of these three values. Several parameters were considered (Crippa et al., 1992):

IVSd: Interventricular septum in diastole
LVIDd: Left ventricular internal diameter in diastole
LVPWd: Left ventricular posterior wall in diastole
IVSs: Interventricular septum in systole
LVIDs: Left ventricular internal diameter in systole
LVPWs: Left ventricular posterior wall in systole
HR: Heart rate
FS: Fractional shortening (calculated by measuring the percentage change in left ventricular diameter during systole)
EF: Ejection fraction (calculated by measuring the amount of blood the left ventricle of the heart pumps out with each contraction)
Aot: Aortic root in transversal section (early diastole)

3.8. POST-MORTEM SAMPLING

Calves were slaughtered in a commercial slaughterhouse when they were 19,4 weeks old (4.5 mo.). The animals were slaughtered in four different groups that were similar in age and included NR80, NR120 and CTR calves:

Group 1: 135.33± 0.81 days, NR80 n=3, NR120 n=2, CTR n=1;

Group 2: 136.17±1.36 days, NR80 n=2, NR120 n=3, CTR n=1;

Group 3: 135.7± 4.66 days, NR80 n=2, NR120 n=2, CTR n=2;

Group 4: 136,2± 4,86 days, NR80 n=2, NR120 n= 2, CTR n=1.

3.8.1. SAMPLING OF THE REPRODUCTIVE TRACT

After the incision of the abdominal wall, the entire reproductive tract was extracted. Ovaries were isolated and ovarian length, height and depth were measured with a caliper. Ovarian volume was calculated using ellissoid volume formula ($V=4/3\ \pi abc$). Subsequently, pairs of ovaries were labelled and transported from the commercial slaughterhouse to the Uniss Obstetrics and Gynecology Laboratory (within 1-2 hours), in PBS (Dulbecco's phosphate buffered saline with 0.1 g/L penicillin and 0.1 g/L streptomycin) at 27-30 °C.
The oviduct was isolated from the mesovary and sampled for a length of two centimeters, in the portion of the isthmus. One uterine horn was opened via a full-thickness cut of the uterine wall. An endometrial sample was stripped from the tip of the horn (the portion closest to the oviduct), taking care not to include the caruncles, myometrium and perimetrium. Uterine and oviductal samples were stored in RNA later at -20° C for subsequent gene expression analysis. In addition, the tip of the controlateral uterine horn and the isthmus of the oviduct were sampled and stored in formalin (8%) for subsequent histological studies.

3.8.2. OVARIAN PROCESSING

In the lab, both ovaries were washed in fresh PBS and subsequently weighted with a precision scale. The antral follicles visible on the surface of both ovaries were counted (Surface follicles), and the largest visible antral follicles (≥3 mm). were measured. Cumulus-oocyte complexes (COCs) were collected from each pair of ovaries. The ovarian cortex was gently sliced in CM (collection medium:9.5 g of Tissue Culture Medium (TCM) 199 in powder with 1 L of Milli-Q water supplemented with penicillin (0.1%) and streptomycin (0.1%), with 25 mM HEPES, 0.4 g/L bicarbonate and 0.1% (w/v) polyvinyl alcohol (PVA) (pH 7.3, osmolality 290 mOsm/kg) using a micro-blade to release follicular content. COCs were selected using a stereomicroscope (60x magnification) and stored in 2μl of cell protectant (Qiagen; 10 COCs/tube) at -20°C waiting for a subsequent gene expression analysis.

Following COCs retrieval, one ovary was stocked in formalin for the histological examination, whereas 1 cm^3 of tissue was stocked in RNA later at -20° C from the controlateral ovary for subsequent gene-expression analysis.

3.8.3. KIDNEYS PROCESSING

Both kidneys were individually weighted. Immediately after the slaughtering procedure, a portion of left kidney, that included cortical and medullary tissue, was sectioned using a scalpel blade and stored in a 15ml tube previously filled with RNA later solution and stored at -20° C for subsequent gene expression analysis. A second sample was collected for the histological analysis. The left kidney was sectioned in the center, in its full thickness, including the renal artery, and the sample was stored in a prefilled jar with formalin at 8% (Contenitore zero 500 D, Meccanica G.M. S.r.l., Italy)

3.8.4. HEART PROCESSING

Immediately after slaughtering the heart was extracted from the thorax and a specimen of the apex (left ventricle) in all its full thickness was stored in a 15 ml tube previously filled with RNA later solution and stored at -20°C for subsequent gene expression analysis. Subsequently the heart was weighted, and the internal aortic diameter was measured with a caliper. An opening of the atrium and the ventricle was created to permit the measurement of the internal aortic circumference. A tape meter was used to measure the circumference at the base of the aorta, between the extremes of the openings.
A second sample was stored in a prefilled jar with formalin for the histological examination. The sample included the base of the heart and 1-2 cm of the base of the aorta. The cut was made full thickness under the atrioventricular septum between the apex of the heart and the atrioventricular septum.

3.9. STATISTICAL ANALYSIS

All data were analyzed using R statistical software (R Core Team, 2020) and are expressed as mean ± SEM. Differences in means were considered significant when the p value of the ANOVA test was less than 0.05. According to Murtaugh (2014), a probability value > 0.05 and < 0.10 was considered suggestive, although not conclusive.
Differences in pregnancy rates between the NR and CTR heifers were analyzed with Pearson chi-square test.
The normality of the data distribution was verified using the Shapiro-Wilk test. When the data were not normally distributed, they were transformed into log base 10; however natural numbers are reported in the text.
Differences in the diameter of the pre-ovulatory follicle and the repeatability of the AFC in the dams were analyzed using the LMER package of the R software (ANOVA, Type 3).

Each dependent maternal variable studied as biometric measurement (DMI, BW, BCS) was analyzed as repeated measure within treatments using the multivariable linear regression model:

$$Y_{ijkl} = \mu + FP_i + D_j + FP*D_k + ID_l + e_{ijkl}$$

Y_{ijkl} was the independent variable; μ was the overall mean; FP_i was the fixed effect of gestation feeding program of pregnant heifers (3 levels, Control (CTR) Nutrient restricted for 120 days (NR120); Nutrient restricted for 80 days (NR80)); D_j was the fixed effect of gestation day of pregnant heifers; $FR*D_k$ was the interaction between the effects of FP_i and D_j; ID was the random effect of individual heifers; e_{ijk} was the residual error. When the predictor effects were considered significant, the least squares means were separated using Tukey's test for honestly significant differences (significance declared for $p<0.05$).

Each dependent variable studied as biometric measurements in the calves was analyzed as repeated measure within treatments using the multivariable linear regression model:

$$Y= \mu + FP_i + age_j + FP * time_k + e_{ijk}$$

Y was the independent variable; μ was the overall mean; FP_i (feeding program) was the fixed effect of maternal feeding program on daughters (3 levels, Control (CTR) Nutrient restricted for 120 days (NR120); Nutrient restricted for 80 days (NR80)); $time_j$ was the fixed effect of age of daughters; $FP*time_k$ was the interaction between the effects of FP_i and $time_j$; e_{ijk} was the residual error. Differences in means were considered significant when the P value of the ANOVA test was less than 0.05.

Each dependent variable studied as echocardiographic and arterial blood pressure parameter was analyzed as repeated measure within treatments using the multivariable linear regression model:

$$Y = \mu + FP_i + age_j + FP * time_k + e_{ijk}$$

Y was the independent variable; μ was the overall mean; FP_i (feeding program) was the fixed effect of maternal feeding program on daughters (3 levels, Control (CTR) Nutrient restricted for 120 days (NR120); Nutrient restricted for 80 days (NR80)); $time_j$ was the fixed effect of age of daughters; $FP*time_k$ was the interaction between the effects of FP_i and $time_j$; e_{ijk} was the residual error.
Data collected post-mortem were analysed with One -way analysis of variance, ANOVA and mean contrast separated with Tukey post hoc test. A correlation test was necessary to find association between body weight at slaughter and weight of the ovaries, heart and kidneys.

4. RESULTS

4.1. REPRODUCTIVE RESULTS IN DAMS

4.1.1. CONCEPTION AND PREGNANCY RATES

Out of the 42 heifers initially enrolled in the study (NR n=32; CTR n=10), 40 showed estrous behavior (NR n=31; CTR n=9) within 48 hours after the administration of estrous synchronization treatment. Two heifers (Control n=1; NR n=1) did not show any sign of estrus (Table 6); the absence of a pre-ovulatory follicles was also confirmed via reproductive ultrasonography; thus they were returned to the herd.

Following AI with sex-sorted semen from a single sire (Barbaro, InSeme), 29 heifers were diagnosed pregnant at approximately 28 day post-AI and the conception rate was similar between groups (NR n=23, 74%; CTR n=6, 66%; Table 6). Non-pregnant heifers were returned to the herd.

At approximately 60 days post-AI, 28 heifers (70%) were diagnosed as pregnant, and pregnancy rate was similar between groups (NR n=22, 70%; CTR n=6, 66%; Table 6). Fetal sex was assessed and heifers pregnant with a male calf were excluded from the experiment (NR, n=3; CTR n=1; Table 8)

After day 60 of gestation, NR heifers pregnant with a single female calf (n=19), were divided in two subgroups, homogenous in weight and individually fed at 0.6M until either day 80 (NR80, n=9) or 120 (NR120, n=10) of gestation.

Table 6. Conception (CR) and pregnancy rates (PR) in dairy heifers that were individually fed a ration providing 0.6% (Nutrient Restricted, NR) or 1.8% (Control, CTR) of their energy maintenance needs, starting 10 days before artificial insemination (AI). All heifers were inseminated with sex-sorted semen from a single sire, pregnancy was diagnosed by reproductive ultrasonography at approximately 28 (CR) and 60 (PR) days post-AI. At 60 days post-AI fetal sex was determined. No differences were detected by the Pearson chi-square test in CR and PR between NR and CTR heifers.

Experimental groups	Synchronized heifers, n	Inseminated heifers, n	Heifers pregnant 28 days post-AI, n (CR%)	Heifers pregnant 60 days post-AI, n (PR%)
Total animals, n	42	40	29 (72%)	28(70%)
Nutrient Restricted (NR)	32	31	23 (74%)	22 [A](71%)
Control (CTR)	10	9	6 (66%)	6 [B] (66%)

[A] Calf sex: female n=19; male n=3

[B] Calf sex: female n=5; male n=1

4.1.2. SIZE OF THE PREOVULATORY FOLLICLE

The size of the preovulatory follicle diameter was measured in all heifers that showed estrous behavior and were artificially inseminated (n=40; CTR n=9; NR n=31; Figure 11). The mean size of the PO follicle in all heifers was 12.47±0.42 and ranged from 23 to 8 mm.

The PO follicle measured on the day of artificial insemination was smaller (p=0.047) in NR heifers that would subsequently be pregnant (12.6± 0.47mm, n=22) compared to CTR animals that would conceive (15.7 ±1.4 mm, n=6). Similarly, NR heifers that failed to get pregnant (11.8±0.53 mm, n=9) had a smaller PO follicle than pregnant CTR animals (p=0.028).

The size of the PO follicle, regardless of the experimental diet, was not associated with the probability of pregnancy at day 60 post-IA (p = 0.3).

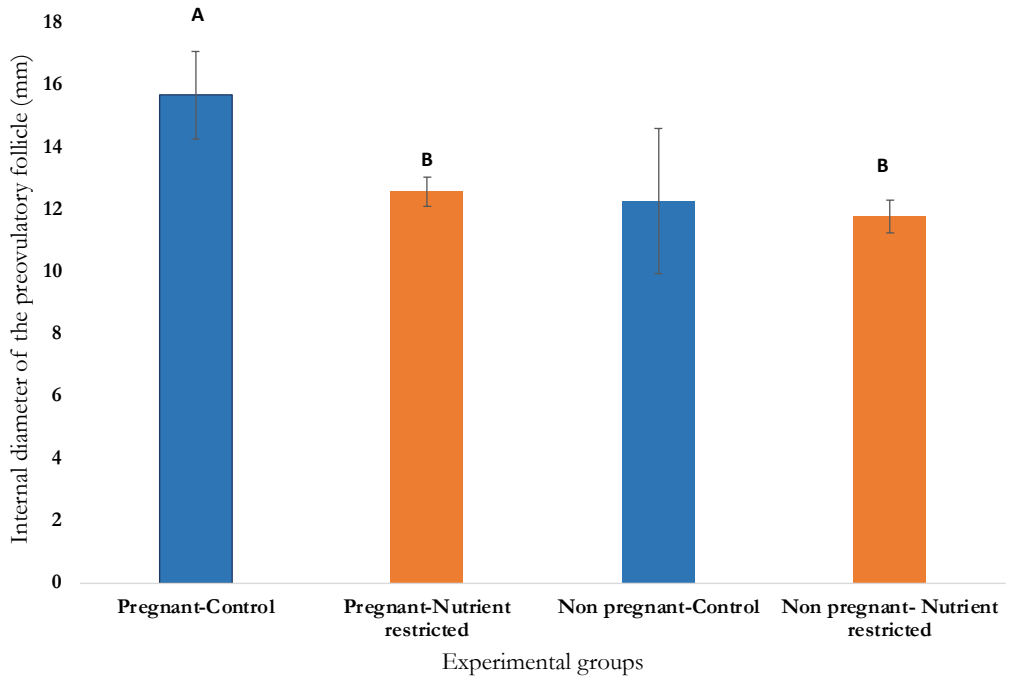

Figure 11. The internal diameter of the preovulatory follicle (mm) was measured on the day of artificial insemination (n=40; CTR n=9; NR n=31), corresponding to day 10 of the differential diet. Pregnancy was subsequently diagnosed on day 28 and 60 post-AI and heifers were retrospectively divided into: Pregnant Control heifers (n=6); Pregnant Nutrient restricted heifers (n=22); Non pregnant Control heifers (n =3); Non pregnant Nutrient restricted heifers (n=9). The Nutrient Restricted group was fed with ration at 0.6% of the maintenance requirement (M), while the Control group received a ration that provided 1.8% M starting 10 days before artificial insemination. Different superscripts indicate statistical differences ($p<0.05$).
A vs. B= $p<0.05$.

In heifers that would subsequently be pregnant with a male or female calf, the mean AFC was measured (Figure 12). The average AFC in all heifers was: 14.7±1.13 ranging from 26 to 8 antral follicles at AI (1 DG); 15.64± 0.99 ranging

from 26 to 7 antral follicles at 28 DG (pregnancy diagnosis); 15.82±1 ranging from 23 to 7 antral follicles at 55-70 DG(pregnancy confirmation). The AFC was repeatable within the same individual from the day of conception to days 28 and 55-70 of gestation (n=28; p=0.66) and was similar between the NR and CTR groups during early pregnancy (p=0.74).

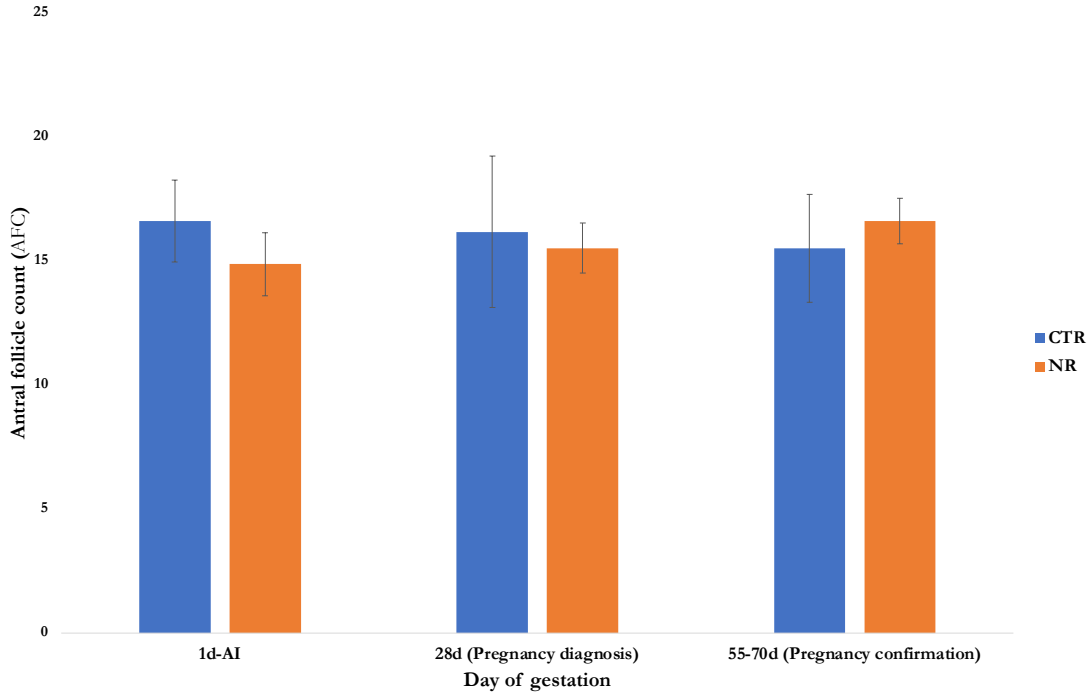

Figure 12. Mean total number of ovarian antral follicles ≥ 2 mm in diameter (Antral follicle count, AFC) in Holstein-Friesian heifers pregnant with a single female or male calf (Nutrient Restricted; NR, n=22) and Control; CTR, n=6) assessed on the day of artificial insemination (corresponding to day 10 of the differential diet), and on day 28 and 55-70 of gestation. The NR heifers were fed with ration at 0.6% of their maintenance requirement (M), while CTR dams received a ration that provided 1.8% M starting 10 days before artificial insemination.

4.2. GROWTH PERFORMANCES IN DAMS PREGNANT WITH A SINGLE FEMALE CALF

By experimental design, the mean Voluntary Dry Matter Intake (DMI) was lower in NR80 (n=8) compared to CTR (n=5) heifers from 10 days before conception to day 80 of gestation ($p<0.01$; Figure 13). Similarly, the mean DMI was lower in NR120 (n=10) compared to CTR heifers from 10 days before insemination to day 120 of pregnancy ($p<0.01$; Figure 13). Indeed, the average DMI in CTR heifers was approximately triple compared to both NR80 and NR120 dams on day 12 (CTR=12.9±0.54; NR80=4.4±0.08; NR120=4.4±0.09 kg/day) and day 71 of gestation (CTR=12.7±0.44; NR80=4.4±0.08; NR120=4.4±0.09 kg/day), whereas DMI was similar between NR80 and NR120 during the individual feeding regime (Figure 13). Following the end of the differential diet, DMI was estimated with prediction models. DMI was lower in NR80 and NR120 compared to CTR heifers on days 140 and 265 of gestation, whereas no difference was detected among the three groups at day 170 and 230 of gestation.

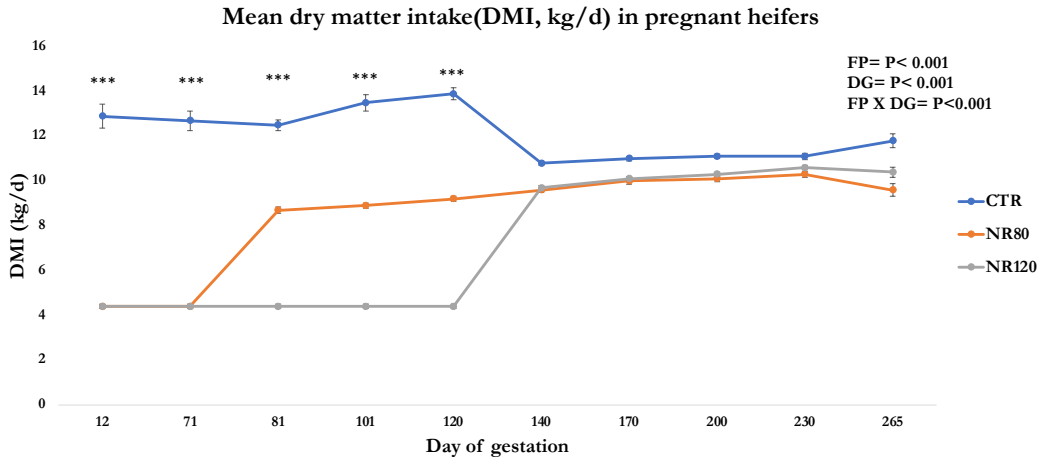

Figure 13. Voluntary Dry Matter Intake (DMI; mean ± SEM kg/day) in dairy heifers pregnant with a single female calf (CTR, n=5; NR120, n=10; NR80, n=8) during the entire gestation. Starting 10 days before artificial insemination, NR heifers were individually fed a ration providing 0.6% of their energy maintenance needs (M), while the CTR group was fed at 1.8%M. All heifers were inseminated with sex-sorted semen from a single sire and pregnancy was diagnosed at approximately 28 and 60 days post-AI. At 60 days of gestation, pregnant NR heifers were divided in two subgroups and individually fed at 0.6M until either day 80 (NR80) or 120 (NR120) of gestation. CTR group was individually at 1.8M from 10 days before AI until day 120 of gestation. From day 120 of pregnancy to calving all heifers were group fed ad libitum. FP=feeding program, DG=day of gestation, FPxDG= interaction of FP and DG. Asterisks indicate significant (***p<0.001) differences among groups at that day of gestation.

Heifers that would be subsequently pregnant in the three groups (NR120, n=10; NR80, n=8; CTR, n=5) were similar in weight before the start of the differential feeding regime (NR120=379.3±14.5; NR80=372.9±14.5; CTR=389.6±11.1; Figure 14) and body weight increased as pregnancy progressed in all groups ($p<0.0001$). CTR heifers were heavier than both NR80 and NR120 from day 12 of gestation to term, whereas no difference was detected between NR80 and NR120. Over the entire gestation period, heifers in NR120, NR80, and the CTR gained 32, 26, and 42% of their body weight, respectively.

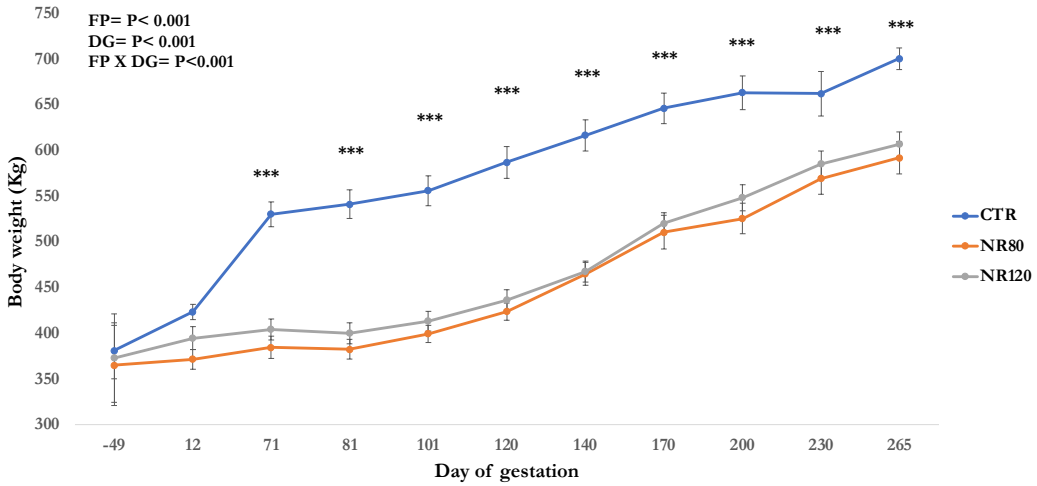

Figure 14. Body weight (mean ± SEM, Kg) in dairy heifers pregnant with a single female calf (NR120=10; NR80=8; CTR, n=5;) from day -49 to day 265 of pregnancy. ANOVA indicated that there were highly statistically differences in body weight between feeding programs (FP) and during pregnancy (day of gestation, DG). Asterisks indicate significant (***p<0.001) differences between groups at that day of gestation.

The second parameter used to evaluate the effect of the different experimental diets on maternal body growth was the assessment of Body condition score (BCS; Figure 15). Before the start of the experimental diet, BCS was similar among heifers that would subsequently be pregnant with a single female calf in the three groups. From day 10 to day 60-70 of gestation BCS was higher in CTR compared to both NR80 and NR120 heifers (p<0.0001). Prior to calving, BCS was similar among the three groups. Furthermore, 14 days after calving BCS decreased in all animals due to the physiological post-partum body reserve mobilization (CTR =2.90±0.06; NR80=2.75±0.06; NR120=2.89±0.04), but no difference was detected among the three groups at that timepoint.

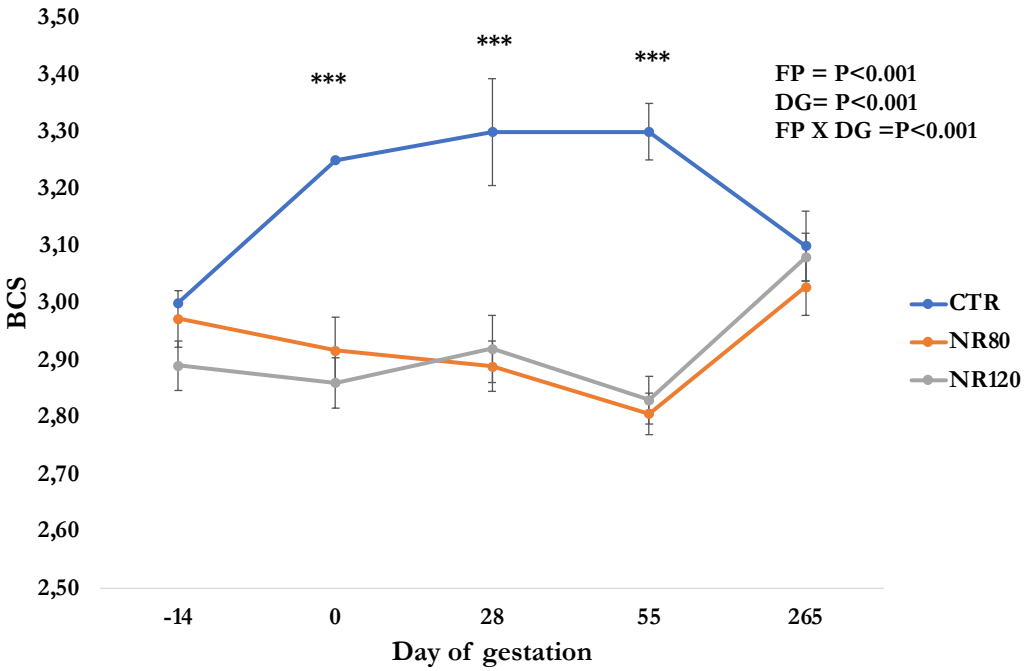

Figure 15. Body condition score (BCS) in the three experimental groups (CTR=5; NR120=10; NR80=8) from before conception to the third trimester of gestation. ANOVA indicated that there were highly statistically differences in BCS among feeding programs (FP) and during pregnancy (day of gestation, DG). Asterisks indicate significant (***p<0.001) differences between the CTR group and both NR80 and NR120 at that day of gestation.

4.3. GROWTH PERFORMANCES IN CALVES

4.3.1. CALVING AND POST-PARTUM MANAGEMENT

As by experimental design, calvings were concentrated in two periods: the first ranged from the 29th of January and the 3rd of February (CTR, n=3; NR80, n=5; NR120, n= 5) and the second group between 14th of February and 28th of February (CTR, n=3; NR80, n=6; NR120 n= 6). The mean length of gestation in all heifers (n=28) was 275.92±0.64 days. The gestation length was similar in heifers pregnant with a single female calf, among three experimental groups ($p>0.05$). Furthermore, deliveries were concentrated in the span of time between 8 pm and 8 am. Most of the dams did not require assistance at birth; only one heifer (NR 120) experienced dystocia, as the calf was in posterior presentation. A chain was used to facilitate calving and pull out the calf. A loop of the chain was placed above and a half-hitch below the fetlock joint, with the connecting chain on the top of the leg. Twenty-four single female calves were born (CTR=5; NR120=10; NR80=9). Unfortunately, one calf (NR120) died around 30 days of life due to omphalitis complications, but data from her dam were included in the study. Another calf (NR80) was excluded from the study (including data from her pregnant dam) because it was considered abnormal. When she was approximately one month old, the calf showed estrous behavior (mounting other calves and operators) and was heavier (44 kg at birth; 139 kg at slaughter) and taller compared to the other calves (38.5±0.72 kg at birth; 116.42±4.25 kg at slaughter). Furthermore, a pathological ovary was identified by transrectal ultrasound on three consecutive weekly exams starting at 35 days of age: the right ovary was enlarged, and two larger follicles (>3mm) were observed. Post-mortem, the right ovary weighed 20 g (vs. the weight of the left ovary, 0.7g) for 4 cm height, 4 cm length and 1.8 cm depth (vs. the dimensions of left one 1.6; 0.7; 0.4, respectively) and subsequently confirmed postmortem, larger than the left (6cm) and numerous follicles (>15).

4.3.2. CALVES BODY MEASUREMENTS

Healthy single female calves (CTR, n=5; NR80, n=8; NR120, n=9) born to mothers exposed to the differential diets in early pregnancy showed a difference in body weight at birth across experimental groups (p=0.024; Figure 16). Birth weight was lower in NR80 than CTR calves (p<0.05) and tended to be lower in NR120 compared to CTR individuals (p=0.107). NR80 and NR120 calves were similar in weight at birth (CTR=41.4±1.1; NR80=36.7±0.6; NR120=38.3±1.2 kg).

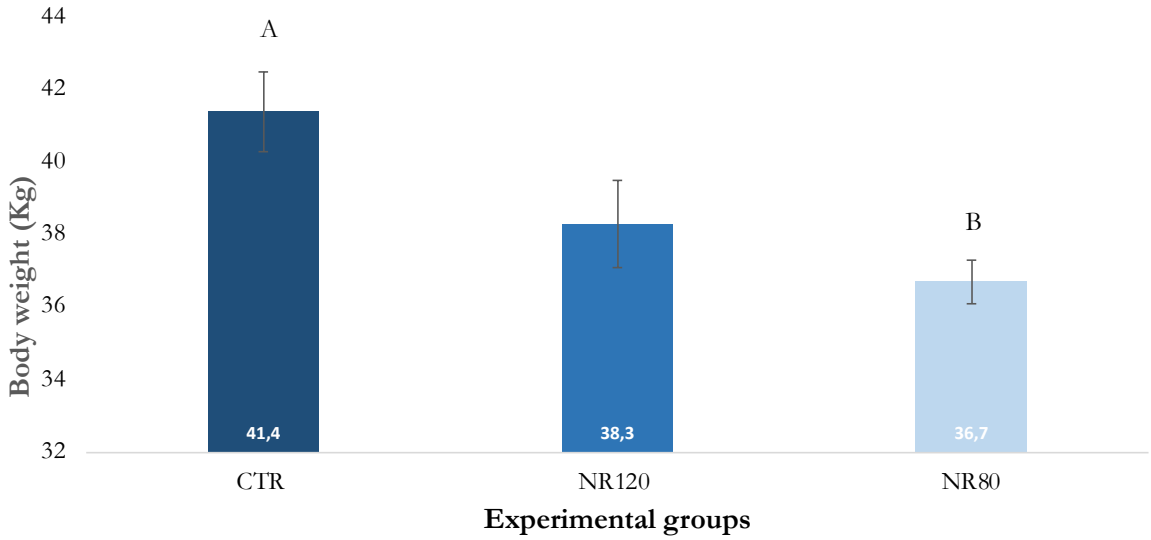

Figure 16. Body weight at birth in female calves born to mothers exposed to a different feeding program in early gestation (CTR, n=5; NR120, n=9; NR80, n=8). The Nutrient Restricted (NR) heifers were fed with ration at 0.6% of their maintenance requirement (M) until day 80 (NR80) or 120 (NR120) of gestation, whereas Control (CTR) dams received a ration that provided 1.8% M. Results are expressed as Mean± SEM. Different superscripts indicate statistical differences (p<0.05).
A vs. B =p<0.05.

Nonetheless, as calves grew older, body weight among the three experimental groups resulted linearly similar until slaughter at 135 days (Figure 17 and Table 7).

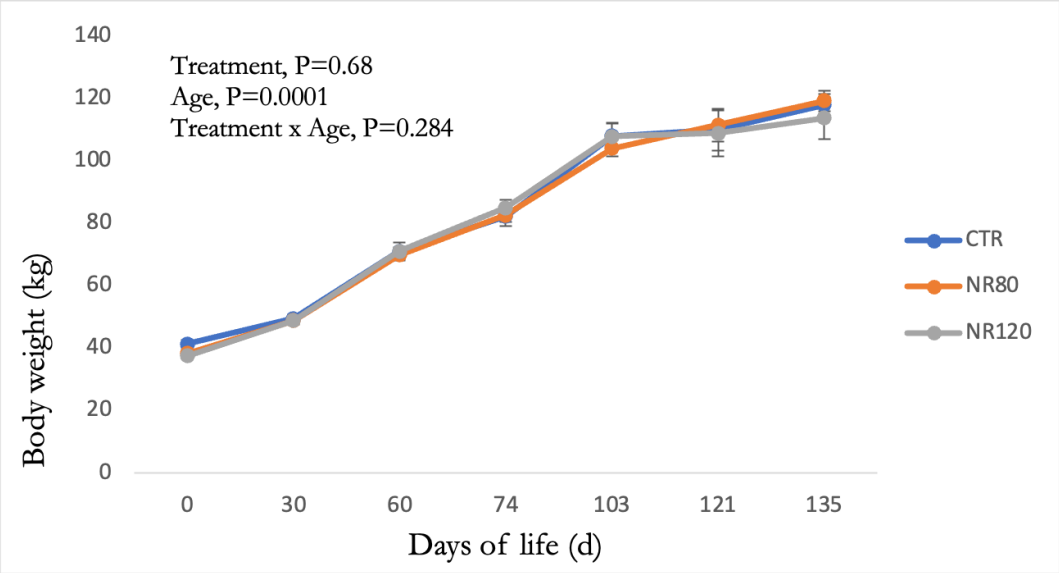

Figure 17. Body weight of female calves (n = 22) born to heifers exposed to a different feeding program in early gestation (CTR, n=5; NR120, n=9; NR80, n=8).
The Nutrient Restricted (NR) heifers were fed with ration at 0.6% of their maintenance requirement (M) until day 80 (NR80) or 120 (NR120) of gestation, whereas Control (CTR) dams received a ration that provided 1.8% M. Results are expressed as Mean± SEM.

Similarly, thoracic circumference was higher in the calves from control fed heifers than calves born to early gestation nutrient restricted heifers at birth (p=0.049). A tendency to differ for hip height between calves from control fed heifers and nutrient restricted fed heifers was observed (p=0.069). However, height at withers was similar among all calves at birth (P=0.677).

No difference was detected in height at withers, thoracic circumference and back length among calves of all experimental groups from birth to slaughter at 135 days of life (Table 7).

Table 7. Effects of maternal feeding program in early gestation on body growth in single female calves TC= Thoracic circumference, cm; HW= height at withers, cm; HH= Hip height, cm; BL= back length cm; FP= feeding program; Week= week of age. Data are expressed as mean ± SEM.

Parameter	Days post calving							P-value		
								FP	Week	FP x week
	0	30	60	74	103	121	135			
TC								0.27	<0.0001	0.875
CTR	77.4±1.2	82.4±0.9	93.2±1.5	100.6±1.3	106.6±1.3	106±2.2	110.4±1.8			
NR120	73.9±0.8	80.9±1.2	91.8±0.7	99±0.9	105.1±1.1	107.6±1.4	110.7±1.6			
NR80	74±0.9	80.6±0.5	93.2±0.6	99.6±1.1	106±1.4	109±3.3	108.6±2.1			
HW								0.229	<0.0001	0.641
Control	73.6±1.2	78.8±0.5	85.8±0.9	88±0.6	96.2±0.4	97.8±0.5	100.6±0.9			
NR120	72.4±0.8	76.6±0.7	84.8±0.5	87.9±0.7	93.3±0.6	96.2±0.5	99.7±1.1			
NR80	72.2±1.1	76.8±0.7	87.1±0.8	87.9±0.9	93.6±0.8	96.6±0.7	99.9±1.1			
HH								0.264	<0.0001	0.329
Control	79.2±0.9	82.8±0.4	90.6±0.7	93.2±0.4	100±0.7	101.2±0.7	105.4±0.5			
NR120	75.6±1.0	81.6±0.6	87.7±0.8	93.1±0.6	98.2±0.6	101±0.7	104.7±1.2			
NR80	75.5±1.1	80.5±1.2	87.9±1.2	92.4±0.9	98.1±0.6	101±0.6	104.4±1.3			
BL								0.279	<0.0001	0.687
Control	-	61.8±1	68.4±0.5	76.2±0.9	85±0.7	86±1.2	87.6±1.4			
NR120	-	63.6±0.9	68±0.7	74.9±0.5	85.7±0.5	87.2±1.2	90.2±0.8			
NR80	-	61.9±0.8	68.1±0.5	74.1±1.4	85.4±0.9	86.9±1.0	88.9±0.8			

4.4. ARTERIAL BLOOD PRESSURE AND ECHOCARDIOGRAPHY

One NR80 calf was excluded from the arterial blood pressure assessment from the echocardiography measurements and post-mortem analysis of the heart, because a ventricular septal defect was identified via echocardiographic examination. For example, at echocardiographic examination, LVIDs and LVIDd (Left ventricular internal diameter in systole and diastole, cm) were higher in pathological NR80 calf than the mean values of the other calves included in the study. At 30 days of life, LVIDs was 3.68 in NR calf vs. 2.87±0.09 cm in the other calves and LVIDd was 6.48 in NR calf vs.4.44±0.08 cm. The same situation was clear at 100 days of life where LVIDs was 4.43 in NR calf vs. 3.27±0.09cm in the other animals and LVIDd was 7.01 in NR calf vs.5.3±0.08cm. Despite the difference in this value, the other values were similar between the calf excluded and the mean value in other calves.

4.4.1. ARTERIAL BLOOD PRESSURE ASSESSMENT

Systolic, diastolic and mean arterial pressure and heart rate (measured during arterial blood pressure evaluation) were similar from one to 4 months of age. (Table 8 ; Figures 18, 19, 20, 21).

Table 8. Mean resting peripheral arterial blood pressure and heart rate analyzed every two weeks from day 30 of life (8 measurements) in female calves. SYST= systolic, DIAST=diastolic, MAP=mean arterial pressure, HR=heart rate, FP= feeding program

BP	Feeding program (FP)			p-value		
	Control (n= 5)	NR120 (n=9)	NR80 (n=7)	FP	Time	FP x Time
SYST	101.1±2.65	103.7±1.91	102.6±2.45	0.86	0.019	0.58
DIAST	48.8±1.67	51.6±1.31	49.7±1.68	0.432	0.055	0.76
MAP	69.4±1.97	72±1.46	70.4±1.75	0.546	0.056	0.44
HR	91.8±2.87	94.2±2.07	92±2.6	0.554	0.0001	0.436

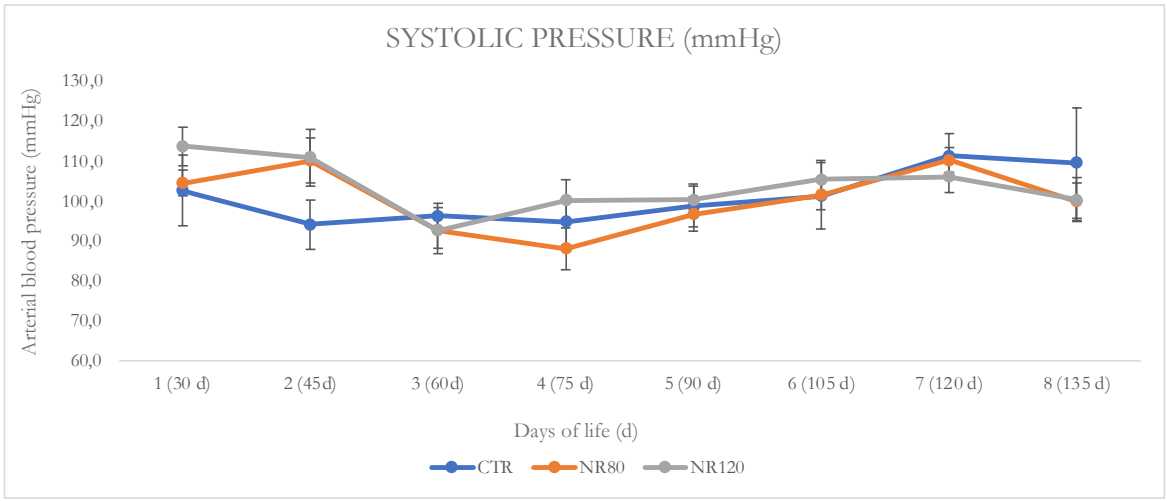

Figure 19. Systolic pressure measurement (mmHg) in three experimental groups (CTR=5; NR120=9; NR80=7) every two weeks from 35 days of life until slaughter, for a total of 8 measurements.

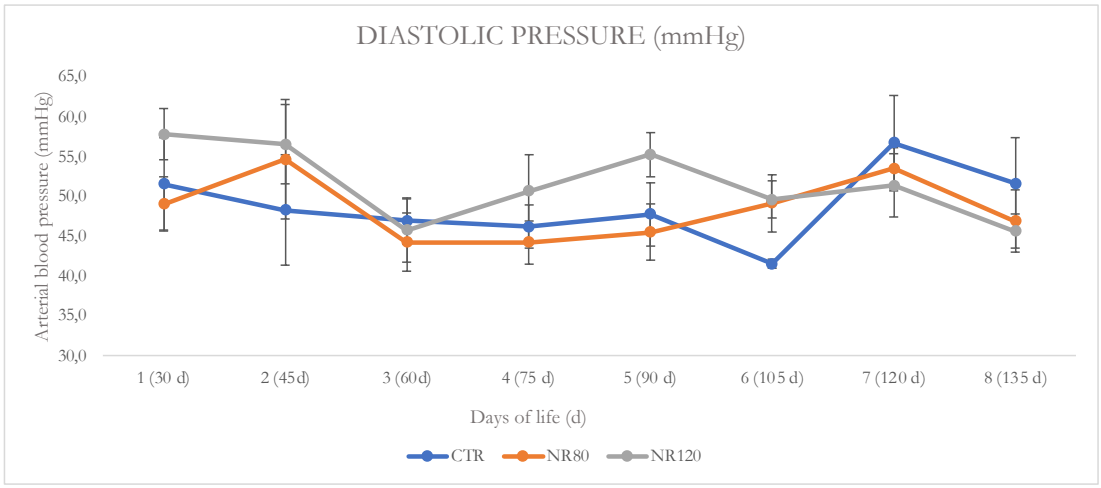

Figure 20. Diastolic pressure measurement (mmHg) in three experimental groups (CTR=5; NR120=9; NR80=7) every two weeks from 35 days of life until slaughter, for a total of 8 measurements.

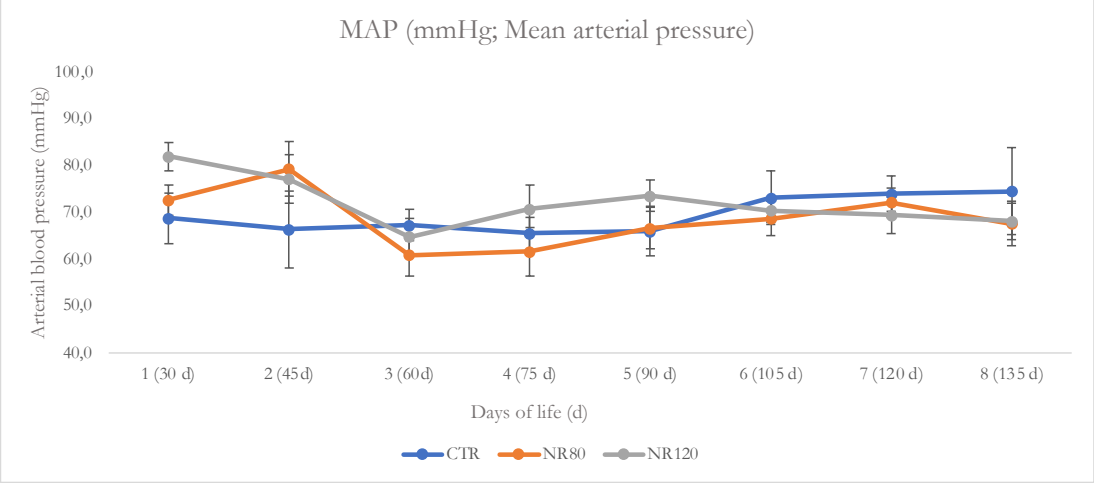

Figure 21. Mean arterial pressure measurement (average arterial pressure; mmHg) in three experimental groups (CTR=5; NR120=9; NR80=7) every two weeks from 35 days of life until slaughter, for a total of 8 measurements.

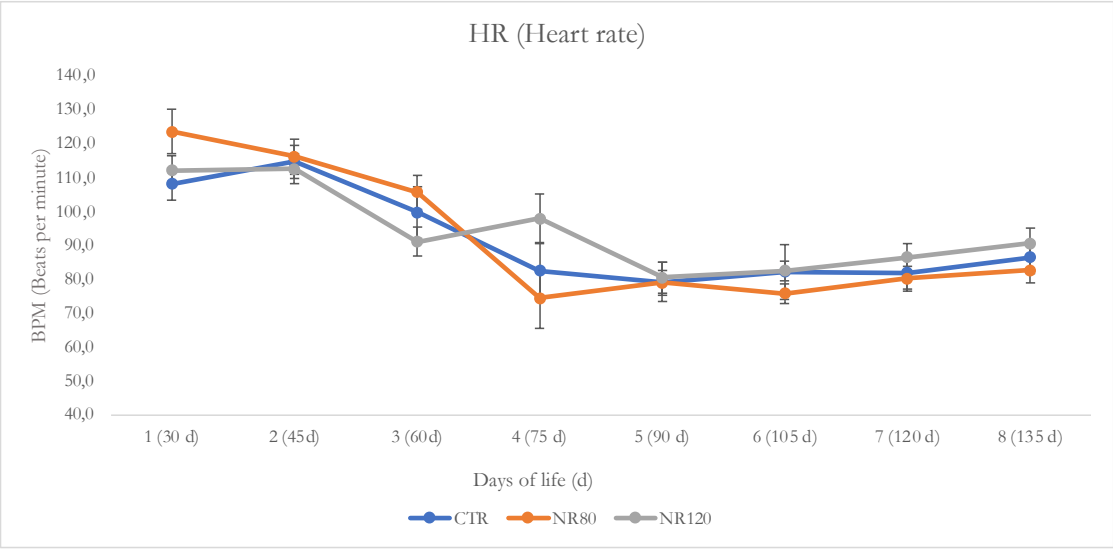

Figure 22. Heart rate (beats per minute) measured during evaluation of arterial blood pressure in three experimental groups (CTR=5; NR120=9; NR80=7) every two weeks from 35 days of life until slaughter, for a total of 8 measurements.

4.4.2. ECHOCARDIOGRAPHY PARAMETERS

Table 9 summarizes all parameters evaluated via echocardiography (2 measurement at 35 and 100 days). These values were not influenced by maternal feeding program.

Table 9. Different echocardiographic parameters evaluated around day 35 and 100 of life. IVSd: Interventricular septum in diastole; LVIDd: Left ventricular internal diameter in diastole; LVFPd: Left ventricular posterior wall in diastole; IVSs: Interventricular septum in systole; LVIDs: Left ventricular internal diameter in systole; LVFPs: Left ventricular posterior wall in systole; HR: Heart rate; FS: Fractional shortening (calculated by measuring the percentage change in left ventricular diameter during systole); Aot: Aortic root in transversal section (early diastole).

Parameter	Feeding program			p-value		
	Control (n= 5)	NR120 (n=9)	NR80 (n=7)	Time	FP	FP x Time
IVSd	1.1±0.04	1.1±0.03	1.1±0.03	0.458	0.968	0.717
LVIDd	5.1±0.178	4.7±0.137	4.9±0.174	0.001	0.149	0.647
LVPWd	1±0.01	1±0.02	1 ±0.02	0.602	0.441	0.741
IVSs	1.5±0.063	1.5±0.034	1.5±0.062	0.007	0.763	0.921
LVIDs	3.3±0.168	2.9±0.106	3.1±0.11	0.007	0.778	0.561
LVPWs	1.7±0.06	1.8±0.05	1.7±0.05	0.0002	0.192	0.541
AOT	3.4±0.13	3.2±0.10	3.2±0.10	0.0001	0.353	0.965
HR	99.2±6.92	96.8±5.9	93.1±7.15	0.000001	0.586	0.709
FS	34.8±2.18	38.4±1.42	35.7±1.48	0.085	0.412	0.465

4.5. POST-MORTEM RESULTS

4.5.1. OVARIES

Pairs of ovaries collected from NR120 calves were lighter compared to gonads retrieved from CTR individuals ($p<0.05$); also, pairs of ovaries in the NR80 group tended to be lighter than those from CTR animals ($p=0.07$). Ovarian weight was similar between NR120 and NR80 (NR80=7.4±0.9; NR120=6.7±0.5; CTR=10.4±1.3, Figure 22). No correlation was detected between weight of the ovaries and body weight at slaughter in female calves ($R=0.05$; $p<0.05$).

Albeit numerically greater in CTR than NR80 and NR120 calves, the volume of both ovaries was statistically similar among groups.

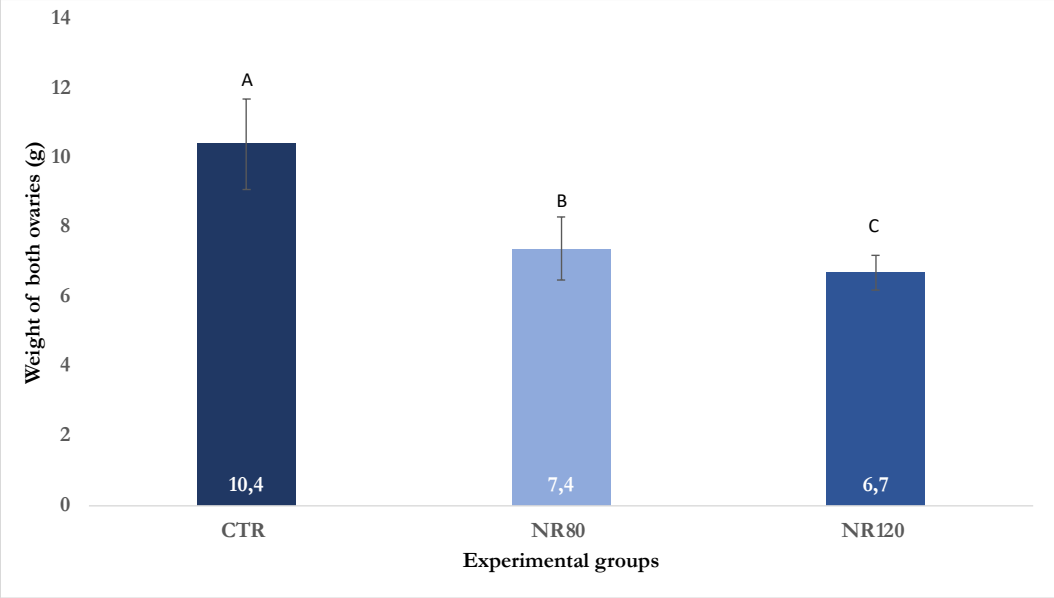

Figure 22. Ovarian weight in female calves born to mothers fed with individual diets during the first trimester of gestation (Control, CTR; Nutrient restricted until day 120 of gestation, NR120; Nutrient restricted until day 80 of gestation; NR80). Ovaries were weighted after slaughtering in the three different experimental groups (CTR=5; NR120=9; NR80= 8). Results are expressed as Mean±SD. Different superscripts

indicate statistical differences ($p<0.05$) or tendency ($p<0.07$). A vs. B = $P<0,07$; A vs. C = $P<0.05$

As reported in Figure 23 the total number of visible antral follicles, evaluated as the sum of follicles on the surface of right and left ovary, was different among groups ($p=0.037$). Calves born to NR120 mothers had less visible antral follicles (NR80=150.1±20.9; NR120=104.2±10.7; CTR=197.2±36.5; Figure 23), compared to CTR calves ($p<0.05$), whereas no difference was detected between NR80 and NR120 and between CTR and NR80 calves, respectively.

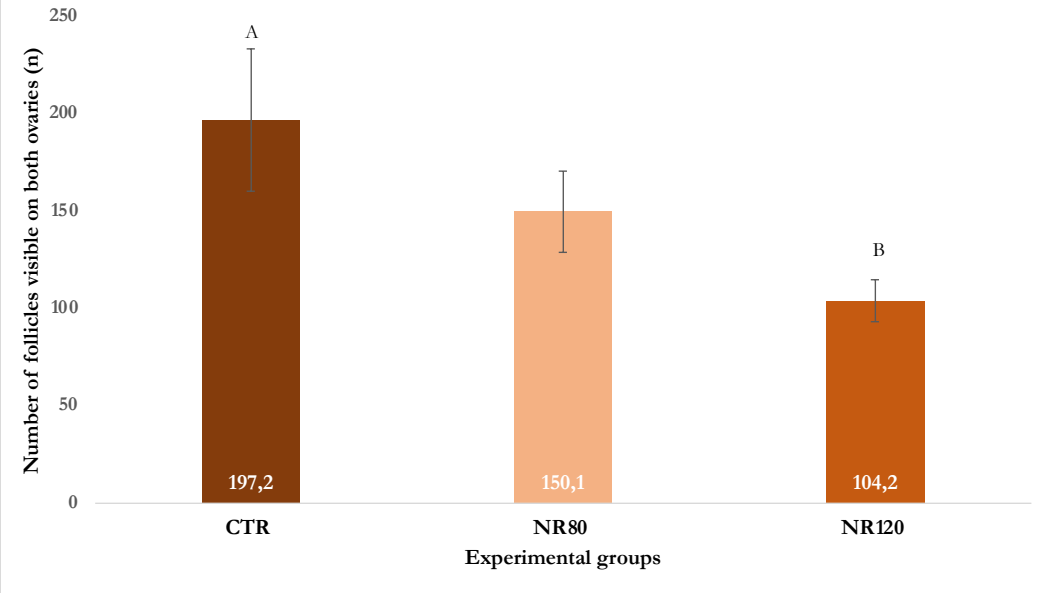

Figure 23. Total number of visible follicles (n) counted on the surface of both ovaries of female calves born to mothers fed with different diets during early gestation (CTR, n=5; NR120, n=9; NR80, n=8). The Nutrient Restricted (NR) heifers were fed with ration at 0.6% of their maintenance requirement (M) until day 80 (NR80) or 120 (NR120) of gestation, whereas Control (CTR) dams received a ration that provided 1.8%M. Results are expressed as Mean± SEM. A vs. B= $P<0.05$

The mean number of COCs (cumulus oocytes complexes) retrieved per animal was different among groups (p=0.029). Less COCs/individual were collected from NR80 and NR120 calves as compared to CTR individuals (p<0.05), but no difference was detected between NR80 and NR120 calves (NR80=48 ±3.5, NR120=48.2± 6.5, CTR=75.8±12.6; Figure 24).

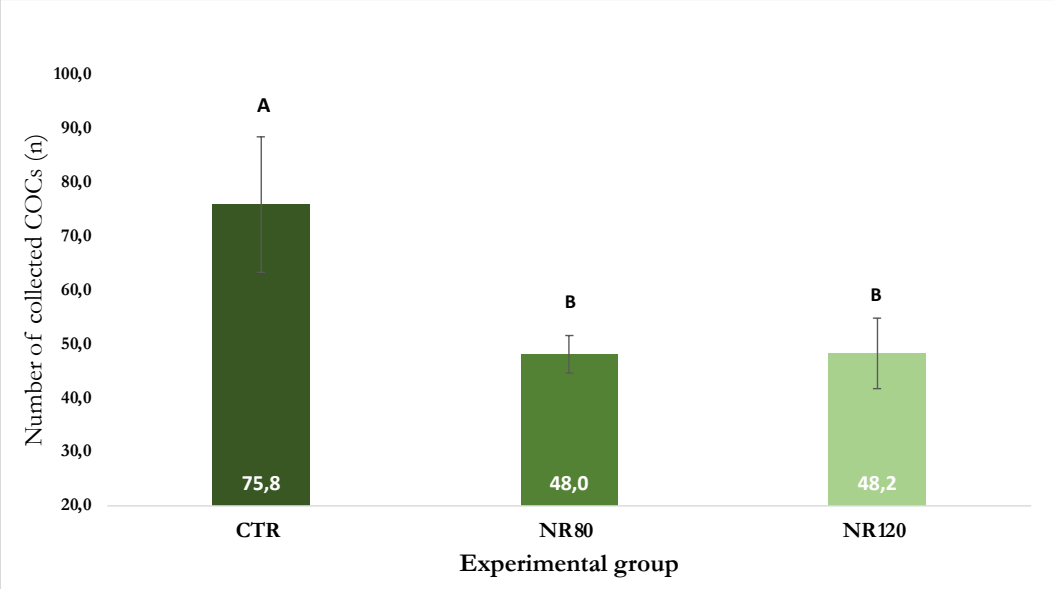

Figure 24. Total number of COCs (Cumulus oocytes complexes; n) retrieved per animal in female calves sacrificed at 135-days of age (CTR, n=5; NR120, n=9; NR80, n=8). Calves were born to mothers fed with different diets during early gestation. The Nutrient Restricted (NR) heifers were fed with ration at 0.6% of their maintenance requirement (M) until day 80 (NR80) or 120 (NR120) of gestation, whereas Control (CTR) dams received a ration that provided 1.8% M. Results are expressed as Mean± SEM. Different superscripts indicate statistical differences (p<0.05). A vs. B= p<0.05.

4.5.2. HEART AND KIDNEYS

No differences were detected among the three experimental groups in the heart parameters. In particular, the heart weight (g), internal diameter of the aorta and circumference of the aorta (centimeters) were similar among all calves (Table 10) . Heart weight and body weight at slaughter were positively correlated (R=0.77; p=0.0001).

Furthermore, the weight of both kidneys was similar between the three different experimental groups, and the weight of both kidneys and body weight at slaughter were not correlated (R=0.37; p=0.09).

Table 10. Effect of maternal nutritional restriction on female offspring sacrifices at approximately 4.5 months of age. The Nutrient Restricted (NR) dams were fed with ration at 0.6% of their maintenance requirement (M) until day 80 (NR80) or 120 (NR120) of gestation, whereas Control (CTR) dams received a ration that provided 1.8% M. Results are expressed as Mean±SEM. Different superscripts within the same row indicate statistical differences. a vs. b=p<0.05; a vs. c= p=0.07;

Post-mortem parameters	CTR	NR80	NR120
Female calves (n)	5	8*	9
Age (days)	133.4±3.6	136±2.3	136.6±3.3
Body weight (kg)	118±3.4	113.8±6.7	119.4±3.3
Ovaries			
Weight (g)	10.4±1.3[a]	7.4±0.9[b, c]	6.7±0.5[b]
Volume (cm^3)	51.2±9.16	45±3.16	37.17±6.60
Surface antral follicles (n)	197.2±36.5[a]	150.1±20.9	104.2±10.7[b]
Retrieved COCs (n)	75.8±12.6[a]	48±3.5[b]	48.2±6.5[b]
Heart			
Heart weight (g)	624±0.02	0.558±0.03	0.598±0.03
Circumference of aorta (cm)	9±0.8	9.4±0.4	9.6±0.5
Diameter of aorta (cm)	2.1±0.1	2.1±1.8	1.8±0.2
Kidney			
Total kidneys weight (g)	497.6±14.4	458.6±17.6	503.6±29.3

*7 calves NR80 were considered in heart evaluation

5. DISCUSSION

The main findings of the present study are:

1) Dietary restriction from 10 days prior to artificial insemination determined the development of a smaller preovulatory follicle in NR compared to CTR heifers, but it did not influence their pregnancy rates;

2) Maternal nutritional restriction in early gestation impaired body growth during the entire pregnancy, regardless of the duration of energy restriction (up to day 80 or 120 of gestation), but it did not influence AFC in the dams;

3) The maternal restricted diet impaired birth weight in N120 calves, but subsequent body growth until four months of age was similar among calves born to NR80, NR120 and CTR dams;

4) Daughters born to NR 120 had fewer visible antral follicles than CTR calves; fewer COCs/individual were recovered from NR80 and NR120 calves compared to CTR; ovarian weight was lower in NR120 and NR80 (tendency) vs CTR calves;

5) Maternal nutrient restriction did not impact the cardiovascular system of juvenile female offspring, as assessed by peripheral blood pressure, echocardiography and post-mortem evaluations (heart weight, aorta circumference).

When designing the experiment, conception and pregnancy rates were expected to be lower than in NR compared to CTR heifers, because energy restriction imposed in the preovulatory period (10 days prior to AI) may impair the growth of the ovulatory follicle and the developmental competence of its oocyte. Based on this notion, more heifers were initially assigned to the NR than CTR diet, to obtain a similar number of calves it the three experimental groups. On the contrary, the diet had no impact on conception (pregnancy per artificial

insemination at 28 days post AI), pregnancy (pregnancy per artificial insemination at 60 days post AI) and calving rates.

It is estimated that follicular development from primordial to ovulatory stage takes 4 to 6 months in cattle, and that follicular growth from the pre-antral to pre-ovulatory phase occurs in approximately 90 days (Fair, 2003). We can speculate that in the present study, preovulatory dietary restriction was acute and severe, but it did not impair the capacity of the oocyte to be fertilized and develop into a vital embryo that could effectively signal it presence to the mother.

The mean size of the preovulatory follicle (determined by reproductive ultrasonography when heifers were inseminated) was smaller in NR vs CTR heifers that would subsequently be pregnant. Furthermore, non-pregnant NR heifers had a smaller follicle compared to pregnant CTR heifers.

The diameter of the follicle has been positively associated with the acquisition of developmental competence by the oocyte in different species. Oocyte developmental competence can be defined as the ability of the female gamete to resume meiosis after gonadotropin stimulation, to meet mitotic divisions after fertilization, to develop an embryo to the blastocyst stage and finally to achieve birth of the offspring (Sirard et al., 2006). Oocytes retrieved from large follicles had greater developmental competence in sheep (Moor and Trounson, 1977), pig (Ito et al., 2008), mare (Goudet et al., 1997), and in cattle (Mermillod et al., 1999; Sirard et al., 2006) compared to oocytes obtained from smaller follicles.

The ovarian follicle has a primary role in controlling the estrous cycle, because it ensures competence in the development of the oocyte and the subsequent survival of the embryo; furthermore, the corpus luteum, responsible for the synthesis of progesterone, develops from the walls of the ovulated follicle. For these reasons, numerous studies analyzed the effect of malnutrition on the developmental dynamics of ovarian follicles in cattle. Scientific evidence shows

that long-term moderate dietary restriction can lead to a reduction in the growth rate of the dominant follicle, its maximum diameter and duration (Diskin et al., 2003). For example, beef cattle fed with diets at various levels of restriction (0.7; 1.1 or 1.8% of live weight as a daily dry matter) for 5 weeks, the maximum diameter and persistence of the dominant follicle were reduced even reaching ovulation (Murphy et al., 1991). In case of chronic malnutrition, the interval between the onset of food restriction and the onset of anestrus is variable (Diskin et al., 2003). This interval was 93 days for a loss of 0.8kg per day (Rhodes et al., 1995), 175 days for a loss of 0.5kg / day (Stagg, 2000) and 224 days for heifers who lost about 0.4kg per day (Bossis et al, 1999). From these results, in case of chronic malnutrition, the interval between the onset of food restriction and the onset of anestrus is inversely correlated with the rate of live weight loss. However, in case of acute malnutrition in beef heifers, an energy restriction of 1.2 to 0.4M starting the day before the end of the heat synchronization protocol based on the exogenous administration of progesterone, caused a significant reduction in the growth rate and the maximum diameter of the dominant follicle in the first follicular growth wave of the subsequent oestrus cycle (Mackey et al, 2000). This finding indicates that acute energy restriction reduces follicular growth faster than chronic malnutrition.

Results from our study indicate that in dairy nulliparous heifers acute malnutrition (0.6M) in the periovulatory period (10 days prior to AI) can limit the growth of the ovulatory follicle, without having a significant impact on the developmental competence of the ovulated oocyte.

As mentioned in the Introduction, the antral follicular count (AFC) is considered a reliable parameter indicative of the size of the ovarian reserve in cattle; AFC has been evaluated in numerous studies during different follicular waves of the same or different estrous cycles, and at various times of the productive life (prepuberty, pregnancy, postpartum, dry period) both in beef and dairy cows. Our study confirms the repeatability of AFC in the same

individual, including early gestation and suggests that AFC is not influenced by the energy restriction. During the bovine oestrus cycle, the development of the antral follicles occurs according to growth waves induced by FSH at intervals of about 10 days (Adams et al., 1992). Thanks to the use of ultrasonography, it was possible to determine that the number of antral follicles recruited per wave was highly variable among individuals (range 8-54 follicles), but highly repeatable (0.95, 1 = perfect) in the same cow, both in the same cycle and in subsequent cycles (Burns et al., 2005). These initial results were confirmed in other studies in a larger number of animals (188 follicular waves in 69 animals, Ireland et al., 2007).

For example, AFC was counted in 44 dairy cows and the ultrasound examination was repeated by the same operator after more than a year; the AFC per cow was similar from year to year and varied by about 2 follicles per cow (-1.65 ± 7.5; mean ± standard deviation; Mossa et al., 2012). Further tests have established that the repeatability of the AFC is not influenced by 1) the age of the cow or the breed; 2) the lactation season or phase and 3) the time lapse between AFC determinations in the same cow (Burns et al., 2005; Ireland et al., 2007; Ireland et al., 2008; Ireland et al., 2009; Mossa et al., 2012). The practical implication of these results is that a single accurate ultrasound examination of the ovaries, in any physiological phase of life, can allow a cattle to be phenotypically characterized. Results from the present study confirm such repeatability and provide evidence that the AFC does not diminish when a heifer is exposed to energy restriction. This observation further validates the potential use of AFC to estimate the size of the ovarian reserve in bovine.

However, it should be emphasized that to perform a correct estimation of the follicle count, the operator must be experienced and adequately trained. Indeed, in addition to the veterinarian ability to examine the entire ovarian layering and surface, good precision is necessary to count all follicles with a diameter greater than or equal to 2-3 mm. In the routine gynecological examination, the presence or absence of the corpus luteum is assessed, as well as the presence and

development of larger follicles, 5 mm in diameter and more (DesCoteaux et al., 2009). It is therefore clear that carrying out the follicular count extends the time of the gynecological visit and this can limit the use of this parameter on farm.

The constant monthly assessment throughout the entire gestation of body weight allowed us to establish that the experimental diets had a marked effect on growth performances in heifers. It should be noted that the TMR (total mixed ration) administered to the nutrient restricted dams was adequate in terms of nutrient composition (NRC, 2001), and larger in particle size (long fiber) than is often provided and recommended (peNDF of 21: Kmicikewycz et al., 2015). This level of dietary particle size minimized the risk of cows sorting out (Fustini et al., 2016).

Pregnant heifers in both NR80 and NR120 were constantly lighter than CTR dams, confirming that our experimental design was effective. It should also be noted that no differences were detected between NR80 and NR120 heifers in body weight and that heifers exposed to an early-gestation nutrient restriction compensated DMI/kg in the following trimesters of gestation regardless of the duration of restriction, for 80 days (NR80) and for 120 days (NR120).

All dependent maternal variables showed an interactive effect between the two predictors (feeding program and day of gestation) in the model, indicating that both day of pregnancy and feeding program had similar influence in body growth. The return to group feeding determined an increase in body weight, that was much greater in the nutrient-restricted heifers between the second and third trimesters of gestation than in the control group, indicating a compensatory growth after being exposed to refeeding. Despite this compensatory growth and even while gravid heifers in the NR80 and NR120 group heifers were exposed to ad libitum feeding, the difference in body weight persisted among experimental groups.

The effect of the maternal restricted diet on growth performance was evaluated not only through body weight measurement, but also with the BCS assessing. BCS evaluation provides a gross but accurate measure of energy reserves in cattle. It is considered an assessment of the proportion of body fat that cattle possess, and it is recognized by animal scientists and producers as being an important factor in dairy cattle management (Roche et al., 2009). At the beginning of the experimental trial the heifers had a homogeneous BCS among the experimental groups but after the start of the individual diet we assisted to a decrease in BCS in NR pregnant heifers, whereas BCS increased in CTR dams.

Taken together these findings indicate that nutrition in early gestation in heifers can influence maternal body growth for the entire pregnancy; energy restriction imposed from 10 day before conception to 80 days of gestation had the same impact on body weight and BCS as the same level of caloric restriction that lasted until 120 days of gestation. Fetal nutrient requirements increase quantitively in the last trimester of pregnancy, when more than 60% of fetal growth occurs. Thus, we can speculate that in our study, energy restriction was imposed in a window of gestation were fetal energy demands are minimal, and yet, it impaired body growth in NR80 and NR120 dams until term.
Nonetheless, heifers in the nutrient-restricted groups calved at > 80% of their adult weight, suggesting that adequate skeletal development was achieved regardless of the degree of nutrient restriction in early gestation. Also, cows usually experience an anticipation of calving when subjected to stressful events (Nagel et al., 2019), but in our study dietary regime did not affect gestation length. Finally, only one case of dystocia was recorded, thus, we can infer that NR heifers, albeit having a lower body weight, experienced a physiological gestation.

In female offspring, maternal diet influenced birth weight of the daughters. Calves in the NR80 were lighter than CTR ones and NR120 calves tended to be lighter than CTR individuals. Contrarily, during the entire duration of life, from

birth until slaughter (at 4.5 mo.), body weight and body measurements were linearly similar considering the interaction between age and maternal feeding program.

I contrast to our results, Barker and Clark (1997) in a study conducted in humans, showed that fetal undernutrition during early gestation had a different influence on birthweights and weight at one year at different stages in pregnancy. Indeed, maternal undernutrition during the first month of gestation determined a reduced birthweight and reduced weight at one year, while a maternal undernutrition during the third month of gestation had not an immediate effect on birthweight, but had long-term effects after one year of life with a reduced weight.

A similar study, conducted in beef cattle, showed that a maternal restriction during the first third of gestation (110 days) had no impact on prenatal growth, birth weights and postnatal growth rates compared to control group (Mossa et al, 2013).

In our study, the average daily gain of calves during postnatal life until slaughter was within the range reported in the literature (Bazeley et al., 2016), however, slightly below the target gains of > 0.75 kg/d required to achieve desired future calf production performances and minimize economic loss (MacDonald et al., 2005) particularly for the progenies whose mothers were nutrient restricted. Although BCS was not recorded in this study, the importance of ADG in the preweaning phase links to a reasonable extent, the maternal BCS gained during gestation and how it can impact calves growth in the preweaning stage (Moriel et al., 2021). In addition, calves ADG is one of the ways to measure the health status of these animals (Hyde et al., 2021) since a considerable percentage of calf mortality is recorded in the preweaning period (Hyde et al., 2022).

Contrarily to the growth trend until slaughter, during the entire postnatal life, maternal diet had a marked effect on development of gonads in their daughters. Female calves born to dams exposed to a restricted diet for 120 days of gestation

had less visible antral follicles on the surface of both ovaries. Less COCs/individual were collected from NR80 and NR120 calves as compared to CTR calves. Furthermore, ovaries recovered from NR120 calves were lighter than ovaries from CTR calves. Also, NR80 tended to be lighter than those from CTR animals. Taken together these data support the concept that energy restriction imposed during early fetal life can impair the establishment of the ovarian reserve in cattle.

These results are in accordance with the findings from the study conducted in beef cows (Mossa et al., 2013). Maternal nutritional restriction (NR, 0.6M vs Control, 1.2M) from shortly before conception to day 110 of gestation impaired the size of the ovarian reserve in the daughters, as assessed by reduced AFC, lower serum AMH and lower number of visible antral follicles post-mortem.
Different studies conducted in cattle aimed to establish which is the most critical period in development of ovaries and the establishment of the ovarian reserve, during primordial follicle formation (meiotic diplotene stage of prophase I) and follicular growth. All these studies revealed that this critical period correspond early gestation phase and the beginning of the mid gestation. Indeed, the primordial follicle formation in cattle was described from 74 days (Tanaka et al., 2001), 90 days (Russe et al.,1983; Yang and Fortune et al., 2008) and 110 days of gestation (Burkhart et al., 2010).
In support to our results, evidence revealed that the weight of an ovary reflects its potential to produce antral follicle. A study conducted on Holstein X Japanese Black (21-26 mo.) by Murasaka (et al., 2005) demonstrated the a positive correlation between ovarian weight and the number of ovarian follicles, both small ($r=0.64$; $P<0.0001$) and medium follicles ($r=0.46$; $P<=0.001$) in in cattle.
Interestingly, no difference was detected between NR80 and NR120 calves in the number of surface follicles, retrieved COCs and ovarian weight. This finding may indicate that the duration of energy restriction did not influence ovarian development in the fetus. On the other hand, these results provide evidence

that unbalanced nutrition for the first 2.5 months of gestation can negatively influence the creation of the pool of healthy oocytes and follicles in female cattle. Since female mammals are born with a finite number of oocytes, that progressively decreases with age, and is never replenished, the programming impact of maternal nutrition on the size of the ovarian reserve may be among the determinants of fertility in cattle.

According to the results obtained, maternal undernutrition had no effect on the peripheral arterial blood pressure of the daughters. These findings are in contrast with the similar study performed in beef cows (Mossa et al., 2013) where maternal undernutrition determined an increase in arterial blood pressure in the offspring. In the same study (Mossa et al., 2013) an enlargement of the aortic trunk in calves born to undernourished mothers was reported post-mortem. This result was not confirmed in our study, as no difference in the circumference and diameter of the aorta was detected post-mortem.
Such discrepancies between studies could be due to the difference in age at slaughter; in the present study calves were sacrificed at 4.5 months of age, whereas beef calves were slaughtered after puberty, at 22 months of age. It is plausible that a difference in peripheral arterial blood pressure could only be detectable with more BP measurements in a longer time-frame. Also, in humans, the impact of maternal nutrition on the cardiovascular system of the progeny is evident in adulthood. For example, Godfrey (et al., 1994) conducted a study on 10 years old Jamaican children and he demonstrated that an increased BP was correlated with a poor maternal nutrition status in programming cardiovascular function. Furthermore, another investigation conducted in Britain showed that forty-year-old men with a higher BP were born to mothers that consumed a low protein diet during their pregnancy. Fewer studies were conducted only on women population, as we purpose in our study. Chen (et al., 2014) investigated that prenatal famine had an impact on the health of adult women (46 years old) born during the Chinese famine, increasing the risk of developing hypertension

in adulthood. Moreover, early onset menopause increased the risk of heart disease and stroke in women and also associated to a low birth weight in childhood. The link between reproductive status and the risk in low birth weight women is still not know, thus further studies are necessary to clarify the mechanism underlying this condition (Stein et al., 2006; Tom et al., 2010).

This study for the first time attempted to investigate the effects of maternal undernutrition on cardiac conformation on young calves (at 30 and 100 days of life) by echocardiography. The evaluation of all the parameters taken into consideration (Interventricular septum in diastole and systole (IVSd-IVSs); Left ventricular internal diameter in diastole and systole (LVIDd-LVIDs); Left ventricular posterior wall in diastole and systole (LVPWd-LVPWs)) did not reveal any difference between the experimental groups considering the interaction between the age variables and the feeding program.

Cardiac function may be monitored by abovementioned echocardiographic parameters in humans (Zhang et al., 2017), dogs, cats and horses (Bonagura et al., 1995). For example, LVPW, LVID and IVS are important indicators of left ventricular hypertrophy in human (LVH; Browne et al., 1978) and cats (Payne et al., 2013). LVH is a compensatory process that develops in response to a tissue stress, volumetric or blood flow disfunction, thus related to hypertension (Bornstein et al., 2022). Recent studies confirmed also that IVS thickening is a predictor of systolic hypertension in human (Grossman et al., 2008).

Furthermore, another echocardiographic parameter considered in our study, the transverse Thoracic aorta (AoT), is an indicator of an enlargement of Ao dimensions. Ao dilation may be a predictor for aortopathy such as aneurysm formation, Ao dissection, and sudden cardiac death (Hiratzka et al., 2010). LVPW and IVS, are very useful for diagnosis of cardiac hypertrophy in cats (Bonagura, 2000).

Fewer studies have evaluated cardiac conformation through echocardiographic examination in subjects exposed to maternal undernutrition in fetal life.

A study conducted by Logan (et al., 2020) on adult mice, established that the offspring born to mothers undernourished during gestation have lower resistance to physical activity. This result was also confirmed at echocardiography where undernourished mice in fetal life had a reduced left ventricular mass compared to control diet mice. Furthermore, LVPWs wall of was thinner in undernourished mice than control diet mice.

Another study (Loche et al., 2018) instead investigated the effects of maternal overnutrition on the cardiac morphology of the progeny, not showing any difference in LVPW, and LVID compared to mice exposed to a control diet in the fetal life.

Our results are in contrast with those obtained in the previous study conducted in beef cattle (Mossa et al., 2013). This dissimilarity may be due to the fact that the echocardiographic assessment in beef was assessed from birth to adulthood (after puberty), while in our study these parameters were evaluated at 30 and 100-110 days, at an age comparable to infancy in humans. It would have been interesting to deepen this investigation in a longer time interval though more repeated evaluations, until the dairy cow at a closer age to puberty or adulthood.

6. CONCLUSIONS AND FUTURE DIRECTIONS

6.1. RESEARCH LIMITS

The experimental activities started one year later than expected because of the national rules imposed to limit the COVID pandemic. This has led to delays in the analysis of the histological samples and in the measurement of the hormonal circulating concentrations.

Also, ovarian ultrasonography in pre-pubertal calves had been programmed as in previous studies (Mossa et al., 2013), but ovaries were not reliably visible; this is probably due to the difference in body growth between beef and dairy calves.

6.2. CONCLUSIONS

Over the past decades fertility in dairy cows has declined, particularly where dairy production systems breed animals that have been selected for milk production (Walsh et al., 2010). As fertility is a multifactorial trait, this described decrease may be due to several contributing factors, including genetic, environmental and managerial causes.

In literature are described four primary mechanisms that depress fertility in lactating cows: anovulatory and behavioral anestrus (failure to cycle and to show estrus), suboptimal and irregular estrous cyclicity (for example ovarian disease and subnormal luteal function after breeding), abnormal preimplantation embryo development (poor oocyte quality), and uterine/placental incompetence (Lucy et al., 2007). One of the short-term solutions for improving fertility in high-producing dairy cows may include to provide a balanced diet, not only in quality but also in quantity. In this way dietary ingredients may invoke hormonal responses that regulate reproduction and improve reproductive performances in cattle.

This study indicates that an energetically insufficient diet in dairy heifers has deleterious effects on the development of the reproductive system of the daughters, similar to the findings in beef cattle, and potentially, on their future fertility. It should be noted that nutritional restriction resulted in an impairment of the ovarian reserve in the progeny, regardless of its duration (until day 80 or day 120).

It would be interesting to deepen a similar study and continue evaluating in vivo the reproductive and productive performances in the following generations. In addition, an epigenetic analysis could be performed to determine if a maternal nutrient restriction induce molecular changes from preconception to day 80 and 120 of gestation on the reproductive system of juvenile female offspring.

6.3. FUTURE OBJECTIVES

The in vivo trial has led to the creation of a tissue repository which could be analysed in follow-up projects (Summarized in Table 11), pursuing the following aims

• Evaluate the concentrations of circulating hormones such as testosterone, insulin and leptin in the blood samples collected during pregnandyfrom mothers exposed to the differential feeding regimes;
• Assess peripheral AMH concentrations in daughters as an indicator of the size of the ovarian reserve;
• Perform histological and stereological exams of the ovaries, heart and kidney to investigate the programming impact of maternal diet on the morphology of these tissues in the offspring;
• Evaluating the expression of candidate genes in the ovarian, cardiac and renal samples, to assess the programming impact of maternal diet on the function of these organs in the progeny

Table 11. Tissue repository created for potential subsequent analysis.

Organ	**Histological examination**	**Gene expression analysis**
Heart	Full tickness cardiac tissue	Full tickness cardiac tissue
Aorta	Base of the aorta	-
Ovary	Ovarian parenchima	Oocytes
Oviduct	Oviduct	Oviduct
Endometrium	Uterine horn- full-thickness tissue	Myometrium
Kidney	Renal cortex and medulla	Renal cortex and medulla

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