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**Plan of nutrition in dairy goats diets on mammary
gland development**

Dissertação apresentada ao Programa de Pós-Graduação em Zootecnia da Escola de Veterinária da Universidade Federal de Minas Gerais como requisito parcial para obtenção do grau de Mestre em Zootecnia
Área de concentração: Produção Animal
Orientador: Prof. Dr. Iran Borges

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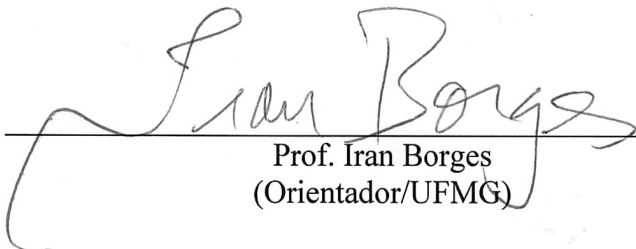
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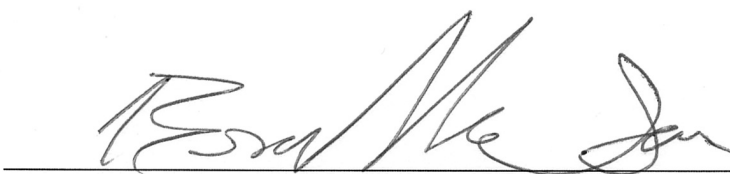
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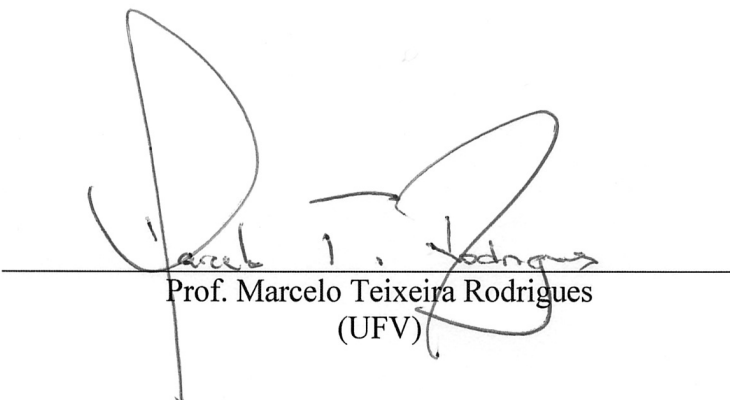
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Aos meus pais. Meus maiores
mentores, meus maiores mestres.

Ostra feliz não faz pérola

“... Pois havia num fundo do mar uma colônia de ostras. Eram ostras felizes. Sabia-se que eram felizes porque dentro de suas conchas saía uma delicada melodia, música aquática, como se fosse um canto gregoriano, todas cantando a mesma música. Com um exceção: de uma ostra solitária que fazia um solo solitário. Diferente da alegre música aquática ela cantava um canto muito triste. As ostras felizes riam dela e diziam: Ela não sai da sua depressão... Não era depressão. Era dor. Pois um grão de areia havia entrado dentro de sua carne e doía, doía, doía. E ela não tinha jeito de se livrar dele, do grão de areia. Mas era possível livrar-se da dor. O seu corpo sabia que, para se livrar da dor que o grão de areia lhe provocava, em virtude de suas asperezas, arestas e pontas, bastava envolvê-lo com uma substância lisa, brilhante e redonda. Assim, enquanto cantava seu canto tristem o seu corpo fazia o trabalho – por causa da dor que o grão de areia lhe causava. Um dia, passou ali um pescador com seu barco. Lançou a rede e toda a colônia de ostras, inclusive a sofredora, foi pescada. O pescador se alegrou, levou-as para casa e sua mulher fez uma deliciosa sopa de ostras. Deliciando-se com as ostras, de repente seus dentes batem num objeto duro que estava dentro de uma ostra. Ele o tomou nos dedos e sorriu de felicidade: era uma pérola, uma linda pérola. Apenas a ostra sofredora fizera uma pérola. Ele a tomou e deu-a de presente para sua esposa. Isso é verdade para as ostras. E é verdade para os seres humanos. No seu ensaio sobre *O nascimento da tragédia grega a partir do espírito da música*, Nietzsche observou que os gregos, por oposição aos cristãos, levavam a tragédia a sério. Tragédia era tragédia. Não existia para eles, como existia para os cristãos, um céu onde a tragédia seria transformada em comédia. Ele se perguntou então das razões por que os gregos, sendo dominados por esse sentimento trágico da vida, não sucumbiram ao pessimismo. A resposta que encontrou foi a mesma da ostra que faz uma pérola: eles não se entregaram ao pessimismo porque foram capazes de transformar a tragédia em beleza. A beleza não elimina a tragédia, mas a torna suportável. A felicidade é um dom que deve ser simplesmente gozado. Ela se basta. Mas ela não cria. Não produz pérolas. São os que sofrem que produzem beleza, para parar de sofrer. Esses são os artistas. Bethoven – como é possível que um homem completamente surdo, no fim da vida, tenha produzido uma obra que canta a alegria? Van Gogh, Cecília Meireles, Fernando Pessoa...”

Rubem Alves

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LIST OF ABBREVIATIONS

2D: Two Dimensional
ADF: Acid Detergent Fiber
ADG: Average Daily Gain
BG: Body girth
BL: Body length
BMP: Bitmap
BST: Bovine somatotropin
BW: Body Weight
CP: Crude Protein
CPI: Crude Protein intake
CW: Chest width
d: day
DFFT: Dried-fat-free tissue
DMI: Dry matter intake
DNA: Deoxyribonucleic acid
E₂: Estradiol
g: gram
GH: Growth Hormone
h: hour
HG: Heart girth
HG: High Gain
Hz: Hertz
IGF-1: Insulin-like growth factor 1
Kg: Kilogram
LG: Low Gain
LL: Leg length
LP: Leg perimeter
M: Million
ME: Metabolizable Energy
MEI: Metabolizable Energy intake
MFP: Mammary fat pad
MG: Mammary Gland
mg: Milligrams
MHG: Medium-high Gain
MHz: Megahertz
mL: Milliliters
MLG: Medium-low Gain
MRI: Magnetic Resonance Imaging
mRNA: messenger Ribonuclec acid
NDF: Neutral Detergent Fiber
NEFA: Non esterified fatty acids
NSC: Nonstructural Carbohydrates
PCA: Principal Component Analyses
P₄: Progesterone
PAR: Parenchyma
Prl: Prolactin
PUF: Polyunsaturated Fats
REML: Restricted Maximum likelihood

RH: Rump height
RL: Rump length
RNA: Ribonucleic acid
RW: Rump width
SH: Sternum height (SH),
SL: Shoulder length
SW: Shoulder width
T₃: Triiodothyronine
T₄: Thyroxine
TD: Teat distance
TL: Teat lengths
TW: Teat widths
US: Ultrasound
WH: Withers height

ABSTRACT

Rearing replacement dairy animals is one of the most costly phases on a dairy farm, for being it an unproductive period. For that reason, high plane of nutrition is given to provide high gains and decrease age at sexual maturity. However, this high energy and protein consumption, during the prepubertal period, may affect body growth and mammary secretory tissue development. The aim of this experiment was to evaluate the effect of different average daily gains (ADG) and dry matter (DMI), crude protein (CPI) and metabolizable energy intake (MEI) on body and mammary development. Forty Saanen kids, 109.1 ± 21.4 days old and 12.9 ± 2.9 kg initial body weight, had their body and gland morphometry, and mammary parenchyma (PAR) measured until 30 days of pregnancy. They were separated in four diets with ten animals each, where each group received a different protein-rate diet – 15, 17, 18 and 19% –, allowing different ADG (90, 130, 170 and 210 g/day) and nutrient consumption. Mammary morphometry was obtained weekly, whereas body morphometry and mammary PAR at every fourteen days. For PAR access, a B-mode ultrasound with a 5.0 MHz convex probe was used to examine in a 2-dimension PAR area, perimeter and grey scale, of both glands, throughout the trial. Data was analyzed with a multiple regression using RStudio software. Throughout the trial, all variables augmented. ADG had the major impact, influencing positively all body measurements ($p < 0.01$) and mammary teat growth ($p < 0.01$), PAR area ($p < 0.01$), PAR perimeter ($p < 0.001$). MEI influenced only rump length and chest width ($p < 0.05$) on body growth, right PAR area and perimeter ($p < 0.05$). CPI and DMI did not influence body growth; however, protein affected considerably PAR development, increasing area, perimeter, and maximum grey value. Allometry coefficients of PAR area were low when compared to other body coefficients. However, it was possible to visualize a change of proportion in fifteen times for this variable, which means that PAR area increased more proportionally with time. Increasing levels of CPI, conversely from MEI, had an important and positive impact on body and mammary growth. Mammary growth could not be associated with any body growth pattern.

Key-words: dairy goat, mammary gland, morphometry, parenchyma, ultrasound

RESUMO

A recria é uma das fases mais caras em uma fazenda leiteira, já que é um período improdutivo. Por essa razão, dietas com altos planos nutricionais são dadas para possibilitar altos ganhos e diminuir a idade de maturidade sexual. No entanto, este alto consumo de energia e proteína, durante o período de pré-púbere, pode afetar o crescimento do corpo e desenvolvimento do tecido secretor mamário. O objetivo deste trabalho foi avaliar o efeito de diferentes ganhos de peso diário (GPD) e matéria seca (CMS), proteína bruta (CPB) e consumo de energia metabolizável (CEM) no corpo e desenvolvimento mamário. Quarenta cabritas Saanen, com $109,1 \pm 21,4$ dias de idade e $12,9 \pm 2,9$ kg de peso corporal inicial, tiveram seu corpo e morfometria da glândula, e parênquima mamário (PAR) medidos atingirem 30 dias de gestação. Elas foram separadas em quatro grupos com dez animais cada, onde cada grupo recebeu uma dieta com diferentes taxas de proteína - 15, 17, 18 e 19% -, permitindo diferentes GPD (90, 130, 170 e 210 g/dia) e consumo de nutrientes. A morfometria mamária foi obtida semanalmente, e a morfometria corporal e PAR mamário, a cada quatorze dias. Para a mensuração interna da glândula, um ultra-som modo B com uma probe convexa de 5,0 MHz foi usado para examinar, em duas dimensões, a área de PAR, o perímetro e a escala de cinza, de ambas as glândulas, por todo o período experimental. Os dados foram analisados com regressões múltiplas usando *software* Rstudio. Durante o experimento, todas as variáveis aumentaram. GPD teve o maior impacto, influenciando positivamente em todas as medidas do corpo ($p < 0,01$) e no crescimento das tetas mamárias ($p < 0,01$), área de PAR ($p < 0,01$), o perímetro de PAR ($p < 0,001$). CEM influenciou apenas no comprimento garupa e largura no peito ($p < 0,05$), na área da glândula direita do PAR e perímetro ($p < 0,05$). CPB e CMS não influenciaram no crescimento do corpo, no entanto, CPB afetou o desenvolvimento consideravelmente do PAR, aumentando área, perímetro e valor máximo de cinza. Os coeficientes alométricos de área do PAR foram baixos em comparação com outros coeficientes corpo. No entanto, é possível visualizar uma mudança da proporção em quinze vezes para esta variável, o que significa que a área de PAR cresceu mais proporcionalmente ao longo do tempo. Níveis crescentes de CPB tem um importante e positivo impacto no crescimento corporal e da glândula mamária. O crescimento mamário não pode ser associado a qualquer padrão de crescimento do corpo.

Palavras-chave: biometria, glândula mamária, parênquima, ultra-som

CHAPTER 1. Introduction

Goats have been domesticated since ancient times, to provide milk, meat, skin and hair. They are highly adaptable to harsh conditions such as different climates, regions and altitudes, have the ability to graze bad quality forages and still produce milk and meat, and have high body resistance to draughts and long distance walks.

According to FAOSTAT (2015), there are one billion goats worldwide. Asia and Africa are the continents with the biggest flocks, with respectively 586 and 364 M goats, contributing to associating goat production with underdevelopment and poverty, The latter is not entirely true. It could more likely be justified by historical connections, easy management, food for rural populations and as well as providing work to women and children, low investments, and consequently low risks. On the other hand, about 150 years ago, more exactly after the two world wars, Mediterranean countries like France, Spain, Greece and Italy, as well as the USA, invested in dairy goat to produce milk and top quality cheese. These countries started to make genetic improvements, allied with superior nutritional management, thus, increasing milk production. They also developed a highly organized chain with the creation of social bonds of goat breeders and other classes (Boyazoglu et al., 2005).

Brazil, currently with 8,9 M goats, occupies the 22nd position in number of goats and the 18th position in goat milk with production of 153 thousand tons of milk a year (FAOSTAT, 2015). Most of the herd is concentrated in the Northeast of the country, spread in small native breed flocks and raised in extensive management. Nevertheless, it is in the southeast of Brazil where most of the specialized dairy herd is situated, with approximately 25% of national goat milk production (IBGE, 2013). Constituted mostly by European dairy breeds – Saanen, Alpine and Toggenburg – milk production is now driven by gastronomy and powdered milk.

However, despite its large milk production, Brazil does not have a structured and organized chain. Fonseca and Bruschi (2008) express that productivity is achieved by genetics and environment, i.e., management, sanity and nutrition. Production is low when both, genetic value of the herd and environment, are unsatisfactory, or, if there is some genetic value but environmental conditions are inappropriate. For that reason, investing in the three pillars of the environment (management, sanity and nutrition) is so important to obtain good milk production and top quality products. Although sanity and management are reasonably good

on most Brazilian farms, it is nutrition that is not always adequate and the ratio formulation is mostly unbalanced.

Between birth and the beginning of milk production, raising female kids is extremely arduous because the nanny goat does not provide any benefits until giving birth and the onset of milk production; therefore, it is a long term breeder investment. That is the reason why a rapid development from neonate to puberty, together with fast growth and elevated daily body gain is desirable. To achieve high gains, dairymen give high protein and energy concentration ratio, to make the animals develop fast. However, this rush could compromise body growth, in particular mammary gland growth, and consequently future milk production.

The mammary gland is one of the organs that suffers most alterations during all animal life, contemplating size, structure, composition and activity (Akers and Denbow, 2013). Its growth takes over complexity factors involving animal, environment and nutrition. Anyway, despite a large number of studies on mammary gland development in mice, rats, sows, cows and sheep, few works were found on goat mammary gland peculiarities.

Many techniques have been developed to accompany organ growth such as comparative slaughter, biopsy, metric measurements, nuclear magnetic resonance imaging (MRI) and ultrasound. This last technique permits to see in real time, in two dimensions and in 256 gray scale, the complexity of the analyzed body part with little invasion. There is no need to slaughter or remove part of the organ, which allows following the changes in the evaluated period.

The scarcity of research on dairy goat mammary gland development is a motivation for investigating the implication of different nutritional planes, leading to divergent average daily gains, in female kids from weaning to pregnancy, and their consequences on body and mammary gland growth.

In this project, mammary parenchyma and body growth of 40 female goats, with $109,1 \pm 21,4$ days of age and weighing $12,9 \pm 2,9$ kilos, were monitored respectively, from weaning to 30 days of pregnancy, by ultrasound technique and an hypometer. The kids were divided into four average daily gain diets – 90, 130, 170 and 210 grams a day – with ten animals each. Biweekly ultrasound images were obtained from each mammary gland, to follow its development over time. Teat length, width, and distance between teats were weekly measured as a tool for comparison of parenchyma development with the external parts of the mammary gland. Body parts, such as withers, rump and sternum height; body, shoulder, leg and rump length; shoulder, rump and chest width; leg perimeter; and chest and body girth

were measured every two weeks to compare the different growth of body parts with mammary gland parenchyma.

Comparison between mammary gland, body growth, nutrient intake, as also comprehension of goat mammary parenchyma growth, is a step for understanding the importance of nutrition on gland establishment and possibly, on future lactations.

CHAPTER 2. Literature Revision

The mammary gland produces “nature’s most perfect food”. Understanding its anatomy, histology, ontology, physiology and biochemistry allows manipulation of animal production and helps preventing maladies that affect not just milk production but human and animal health (Schmidt, 1971). A better knowledge of these characteristics drives researchers to work with the mammary gland in all its aspects, helping increasing efficiency not only for dairy cows, but also for sheep, goats, water buffalos and camels.

For this study, goats were chosen not only because of their socio and economic importance, but mainly due to the lack of works on nutrition and mammary gland development in this domestic ruminant. Since goats are not taken as an experimental model, literature revision will be made comparing different mammals, but preferably does used on dairy farms.

2.1 Anatomy of the mammary gland

The mammary gland is an anatomical structure characteristic of mammals, whose primary function is transferring food from parent to offspring (Larson, 1985; Ferreira et al., 2013). It also has thermoregulatory, immunological, antibiotic and behavioral functions (Akers, 2002), important to seal bonds between mother and progeny, and helping offspring survival under harsh conditions. Because soft tissues are not well preserved as fossils, evolutionary origins of the mammary gland are unclear (Akers and Denbow, 2013). It is suggested that this organ arose from the combination of sweat and sebaceous glands, since it follows apocrine and meocrine modes of secretion (Kon and Cowie, 1961; Akers, 2002; Capuco and Akers, 2010).

The mammary gland is composed of an epithelial of secretory cells, supported by a connective tissue and nourished by nerves, blood and lymphatic vessels, associated with ducts that allow the passage of milk from inside to outside through a channel that can be supported by a teat or not (Akers, 2002). Owing to the fact that mammals adapt to different environments, its location will depend from specie to specie, such as the number of glands, openings per teat, and milk composition (Schmidt, 1971; Akers, 2002). Rodents, lagomorphs and carnivores have glands in thoracic, abdominal and inguinal position, varying in numbers from eight to twelve, with only one opening per teat for rodents, and a large number for lagomorphs and carnivores. Artiodactyls, excluding swine, have only inguinal glands, varying

from two for sheep and goats, to four for cows and buffalos, with only one opening per teat. Sows have mammary glands distributed all over the ventral part of the body, with two openings per teat. Primates, like humans, have only two mammary glands, located in thoracic position, but a substantial number of openings per teat (Larson, 1985) (Table 2.1).

Table 2.1. Position, number and openings per teat in some species

Order	Common Name	Position of the gland			Total Glands	Opening per teat
		<i>thoracic</i>	<i>Abdominal</i>	<i>inguinal</i>		
Marsupilia	Red Kangaroo		4		4	15
Carnivora	House cat	2	6		8	3-7
Carnivora	Domestic dog	2	6	2	10	8-14
Rodentia	House mouse	4	2	4	10	1
Lagomorpha	Rabbit	4	4	2	10	8-10
Cetacea	Whale			2	2	1
Proboscidea	Elephant	2			2	10-11
Perissodactyla	Horse			2	2	2
Artiodactyla	Cattle			4	4	1
Artiodactyla	Sheep			2	2	1
Artiodactyla	Goat			2	2	1
Artiodactyla	Pig	4	6	2	12	2-3
Primate	Human	2			2	15-25

Source: Adapted from Larson (1985).

In rodents and lagomorphs, the mammary glands do not develop very much beyond the primary ducts, having a flat aspect form, thus permitting a more accurate study of the whole organ. On the contrary, in ungulates, the highly branched ducts show a more developed mammary gland (Larson, 1985).

2.1.1 Connective tissues

Patterns of *mammogenesis* in bovine, ovine and caprine are similar, even when ruminants have a different number of glands with size variation among them. Located in the udder, the mammary gland is protected by skin and a thin layer of connective tissue below, uniting the four or two mammary quarters. The skin and the connective tissue provide protection but little sustentation and stabilization, although the main support is provided by a thick, elastic and prominent ligament attached to the pelvic bone, known as the median suspensory ligament. Composed of mesodermic elastic and fibrous connective tissues, especially elastin, this ligament divides the udder in two halves, allowing the animal to absorb shocks when walking and lying down, due to its great strength (Turner, 1952; Schmidt, 1971; Larson, 1985).

Other supportive tissues, besides the skin and the suspensory ligament, that help udder support in ungulate mammals, are the subcutaneous tissue attached to the skin, the cordlike

tissue that bonds the dorsal surface of the udder with the abdominal wall, the lateral suspensory ligaments, the lamellar plates that extend from the ligaments to the mammary parenchyma and the subpubic and prepubic tendons (Turner, 1952). The strong tendons of the abdominal muscles also help udder support. Weight and udder capacity increase until a determined time, with their maximum enlargement between the first and second lactation (Schmidt, 1971). With aging, these connective tissues lose their support capacity and the udder becomes pendulous. Because of this reason, for dairy animal longevity, it is recommended that the udder should extend well forward, be strongly attached, be balanced and symmetrical, with its floor above the animal's knuckle and have a soft, pliable and elastic texture (Turner, 1952). Unfortunately, despite their importance, connective tissues are not among the main objectives of studies and, instead, neglected and left aside.

2.1.2 Blood supply

In the mammary gland, blood supply it is extremely important since it carries essential components needed for milk production. In virtue of that, studies on the concentration of blood components in mammary vessels, and its influence on milk composition are common, although animal mammary *angiogenesis* does not receive much attention. In humans, breast vascularization and factors that stimulate it, are largely investigated because of their relation with breast cancer (Akers, 2002).

Blood supply in mares and ruminants comes from the posterior aorta that branches in the internal and external iliac arteries. The internal iliac arteries supply primarily the reproductive tract, and to a lesser extent, the udder, by its branches called perineal arteries. The external iliac arteries become femoral arteries as long as they proceed caudally. The femoral arteries branches in prepubic arteries before passing through the inguinal ring, where they become posterior abdominal and the external pudendal arteries entering the right and left halves of the udder (Turner, 1952; Schmidt, 1971; Larson, 1985, Akers, 2002). In the cow, the pudendal artery penetrates the udder from the anterior border, whereas in the sheep and goat, it penetrates from the posterior border (Luiz and Miglino, 2000). Once inside the mammary gland, the vessels are named mammary arteries going all through the parenchyma and the teats, known as papillary arteries (Turner, 1952; Schmidt, 1971; Larson, 1985). In the cow, the mammary artery is divided in cranial and caudal. The differences concerning mammary arteries among ruminants are only about position, caliber and arrangement (Luiz and Miglino, 2000).

For rabbits, rats, mice, sows, bitches and queens, since their glands are located on each side of the midline extending from the thoracic to the abdominal region, blood supply comes from several vessels of the external pudendal arteries and their branches, and from vessels that supply the body and abdominal and thoracic walls (Schmidt, 1971).

Blood draining, different from the arterial route, leaves the udder by two divergent paths in most animals with inguinal glands. The first path is a combination of the cranial and caudal branches of the mammary vein, forming the venous circle. Then, this venous circle becomes the external pudendal vein, going all the way back paralleled to the arteries with the same name, until it reaches the vena cava and the right atrium of the heart. The second route, despite its massive size, is less important and physiologically minor. It is composed by the subcutaneous abdominal vein, or simply milk vein, and unites with the internal thoracic vein in the xiphoid process to enter the anterior vena cava (Turner, 1952; Schmidt, 1971; Larson, 1985, Akers, 2002). In virgin ruminants, the first path drains practically all mammary blood back to the heart. That is the reason why, as well as the fact that the blood from the second route mixes with that coming from the reproductive tract, the milk vein is not recommended for mammary gland metabolite measurements (Akers, 2002). For animals with glands spread all through the thoracic and abdominal area, the veins follow the opposite way back to the parallel arteries (Schmidt, 1971).

2.1.3 Lymphatic system

The lymphatic system complements the cardiovascular system, collecting interstitial fluids that bathe the cells, and move them in one direction through the lymph vessels, towards the lymph nodes. In the mammary gland there are innumerable lymph vessels, scattered all through the gland, moving fluid to the supramammary lymph node located dorsally and posteriorly on the gland fat pad. The lymph, with the help of on-way valves, is pumped out carrying lipids, waste products, immune bodies and fibrinogen. Once the lymph gets to the supramammary lymph nodes, it proceeds through large lymph vessels across the inguinal ring towards other lymph nodes – inguinal, external iliac, prefemoral, receiving the draining from the abdominal and inguinal glands, and mediastin cranial, ventral superficial, from the thoracic glands – connected to a lymphatic trunk that becomes the thoracic lymph duct, converging in the vena cava and mixing with venous blood (Larson, 1985, Akers, 2002). Because it is a slow flow, especially around parturition, interstitial fluid can accumulate causing udder edema. The latter may cause mammary secretion increase, occluding stromal tissues and minimizing passive flow, and inadequate occlusion of immature tight junctions

allowing flows from alveolar to interstitial spaces, altering osmolarity and hydrostatic pressure (Schmidt, 1971; Akers, 2002). Udder massaging towards the lymph node and onset of frequent milking helps the draining system (Akers, 2002).

2.1.4 Nervous system

Mammary gland development and milk synthesis and secretion have small influence on the nervous system. Denervated animals continue milk production for a period of time, as isolated cells are capable of continued producing (Akers, 2002). However, interactions between the nervous and endocrine system on milk ejection and removal are essential. Signals from the external environment such as sounds, eye contact with the offspring and tactile contact on the mammary gland stimulate oxytocin release and, consequently, milk release. However, since sheep and goats have large gland cisterns, milk ejection is not as essential for milk removal, as is for the cow (Marnet et. al., 1998; Lérias et. al., 2014). Denervated ewes had only 20% retention of the total milk volume (Marnet and McKusick, 2001).

The inguinal mammary gland is innervated by four pairs of lumbar nerves that communicate directly with the spinal cord. The first lumbar nerve does not reach the parenchyma, only the cranial part of the udder and abdominal wall. The second lumbar never innervates the anterior parts of the udder. Branches from the second, third and fourth nerves, anastomose forming the inguinal nerve composed by afferent and efferent fibers. This nerve enters the inguinal ring and branches in the anterior and posterior inguinal nerve, innervating the gland parenchyma, udder skin and teat. The perineal nerves that arise from the sacral nerves innervate the posterior part of the udder (Turner, 1952; Larson, 1985). Animals with thoracic and abdominal glands have their innervation coming from the subcutaneous nerve arising from the fourth cervical nerve (only for rabbits), the lateral cutaneous nerves from the third lumbar nerve to the third thoracic nerve, the external spermatic nerve, and the ventral cutaneous branches of intercostal nerves (Schmidt, 1971).

2.1.5 Secretory tissue

Responsible for milk synthesis and secretion, the secretory tissue, or parenchyma, completes the mammary gland structure. The parenchyma is composed by secretory epithelial cells, bound by tight gap and cell junction complexes, arranged in a single layer that surrounds a central lumen where they eject the synthesized milk. When these junctions are not occluding the passage between cells, material transit, between the interstitial space and lumen, occurs. Before parturition, this is extremely important for milk enrichment with immune globulins and blood constituents. However, in sick glands, modified cells bonds can also

change milk composition and osmolarity. The single circular cuboidal thick cell layer arrangement of the epithelial cells forms the alveolus, or acinus. The alveolus is surrounded by myoepithelial cells and intertwined blood vessels that provide nutrition, support and contraction. A group of alveoli joined by a common duct and surrounded by connective tissue forms a lobule, and many lobules bound also by connective tissue forms a unit called lobo (Turner, 1952; Schmidt, 1971; Larson, 1985; Akers, 2002).

An udder considered hard at palpation, indicates large amount of connective tissue probably due to mastitis, although it also can be an inherited characteristic (Schmidt, 1971). All these units are connected by two cell layer ducts, followed by bigger ducts, that run towards the gland cistern, forming a dense and compact tissue. This cistern does not produce milk, but works as a storage compartment where the tortuous system of ducts empty. There is a continuous connection with the gland cistern, only separated by an annular fold, with the teat cistern and canal, where the milk is secreted through. The milk is stored inside alveoli, luminal spaces of ducts, and in the gland cistern, even though storage quantities will vary among animal species (Akers, 2002). It is known that small ruminants have proportionally bigger gland cisterns than cows (40 to 80% of the total volume), providing bigger space for outside alveoli storage (Marnet and McKusick, 2001).

The teat is a stromal tissue, containing nerves, blood and lymphatic vessels, nerves and smooth muscles, protected by the keratin barrier. Present in most mammals, especially those with udders, it is hairless in the cow and sow, but not in small ruminants and mares. Devoid of sweat or sebaceous glands only in ruminants and sows, it varies in shape and size, and it is where the milk is ejected out. It can have a single or more openings per teat, depending on the specie, called streak canal that leads directly to the teat cistern (Schmidt, 1971). The streak canal is a physic barrier, minimizing milk leakage or contamination. Its diameter is related to the milk flow. Above the streak canal, there is an area called Furstenberg's rosette made of connective tissue that also helps as a physic barrier and secretes bactericidal proteins (Turner, 1952; Akers, 2002). Nevertheless, cell renovation, keratin and bactericidal components production are constant in the streak canal, retrograde milk movement due to vacuum fluctuations or air entrance in teat mechanical machine cups, can allow bacteria to pass the thick layer of cells and keratin that protect teat entrance, increasing mastitis susceptibility (Akers, 2002).

2.2 Ontogeny of the mammary gland

Ontogeny refers to the development of an individual organism, anatomic structure or behavioral feature from its earliest stage, until maturity, and in this case, mammary gland growth. In the female, although the mammary gland changes through all animal life, it can be divided into five distinct phases: prenatal, prepubertal, postpubertal, pregnancy and lactation (Schmidt, 1971). This classification could also be given as *mammogenesis*, *lactogenesis*, *galactopoiesis* and involution.

The prenatal phase goes from the early stages of embryo development until parturition. Hormones are not required for normal development, although it is because of sexual hormones that males have undeveloped mammary glands (Akers, 2002). It is in this phase that all basic structures are formed. Prepubertal development occurs between birth and puberty, and it is then when large gland growth and development occurs (Sinha and Tucker, 1969). After reaching puberty, the gland continues developing, almost entirely under hormonal influence, but not as fast as in the prepubertal period. It is during pregnancy, that a resumption of gland development takes place, especially during the last third of gestation, when major growth occurs. With the advent of lactation, the gland is all developed and producing milk. Through all this period it will suffer transformations, restructuring, and will start its regression after the lactation peak (Schmidt, 1971; Akers, 2002). It will be fully-grown after the second lactation, inasmuch the cycle of growing and involution will be repeated at each pregnancy and lactation, reaching its maximum weight and production around the sixth lactation (Turner, 1952)

Mammary development of the placental mammal fetus is comparable in all species (Schmidt, 1971; Akers, 2002). Mammary growth starts in an ectoderm area on both sides of the midline on inguinal, thoracic or the entire abdominal surface depending on the specie. It is referred to as mammary band and occurs on day 32 of embryo development in the cow, 21 in the sow and 35 in humans (Akers, 2002). One or two days after this development begins, the mammary band narrows to form a mammary streak, preceded by a mammary line (Larson, 1985).

The mammary line continues its rapid proliferation forming a mammary crest from where the teat and gland will eventuate, and then form a mammary hillock (Akers, 2002). In one pair of gland species, the mammary line diminishes gradually in length from the caudal extremity, thickening the cranial extremity forming an epithelial node. In multiple mammary gland species, the milk line fragments give rise to separated epithelial nodes (Kon and Cowie,

1961). Complete cell proliferation, turning into a hemispherical or convex shape, will form the primitive mammary bud, symbolizing an important differentiation and transition stage of mammary development, since it is from the mammary bud that all individual epithelial gland structures arise. Until then, male and female embryos show the same evolution in mammary growth. After, differences between sexes will occur and the embryo will become a fetus (Schmidt, 1971; Akers, 2002).

The mammary buds turn to a spherical shape and sink entirely into the mesenchyme leaving a small opening causing a depression called mammary pit in bovines, or just a depression in rodents (Akers, 2002). The tissue around the mammary bud forces it to rise above the surrounding epithelium, and at the same time, an opening on the distal ends of the bud occurs leading to teat formation (Larson, 1985). In humans, the first sign of nipple prominence appears only with four months, but occurs earlier in fetal life in other species (Kon and Cowie, 1961). Inasmuch, in some animals, males do not present teats, so it can be concluded that male gland development, at this point, is slower than in females (Schmidt, 1971). In male mice, rats, horses and beavers, the mammary apparatus grows only to the primary sprout stage and, therefore, do not develop teats or nipples (Akers, 2002).

The invagination of the mammary bud, pushing the mesenchyme cells aside and arranging them give rise to the primary sprout, starting the formation of the teat and the gland cisterns, and the major ducts of the udder. The center cells of the primary sprout are separated and the teat and cistern lumen are formed. The teat opening is known as galactophore, and the number of openings will vary between animals. Ruminants and rodents have just one opening per teat, but humans and carnivores have multiples (Larson, 1985).

The cistern lumen continues growing and enlarging, and on its distal end, it narrows to form the keratinized streak canal. Secondary sprouts, or secondary buds in rodents, arise from the end of the first sprout in various angles to give form to the duct system. In ruminants, only primary ducts are seen on male mammary apparatus (Larson, 1985). Tertiary sprouts develop from the secondary ones, and form other sprouts until all mammary duct system is arranged, even if this growth is small in the fetus (Schmidt, 1971). This duct system grows in coordination and organization, not allowing duct overlapping.

Mesenchymal tissue forms the skin, and starts to differentiate in fibrous tissue, blood and lymphatic vessels. Median suspensory ligament formation, as other connective tissues that support the udder, is completed before total growth of the fetus. A layer of adipose cells around the mammary bud forms the mammary fat pad with 14 days in mice, 18 in rats and 80 in cows (Sheffield, 1988; Larson, 1985). In humans, the mammary fat pad does not appear

until puberty, so the mammary ducts do not grow over an adipose tissue like in other mammals, but over a fibrous stromal tissue (Sheffield, 1988; Akers, 2002). In females, the mammary fat pad is considerably bigger than in males and in case of major mammals is extremely necessary for *mammogenesis*. This fat pad is very important for duct network formation in puberty and maintenance of ductal and alveolar architecture in the adult mammary gland as a structural support. It is also responsible for setting the hormonal milieu, helping mammary morphogenesis regulation (Landskroner-Eiger et. al., 2010). Connective tissue cells form whorls will be further replaced by lipids and secretory tissue when the alveoli appear.

The development aforementioned occurs in the first half of fetal growth and non-secretory tissues are well formed at time of birth, even if the mammary gland is configured by rudimentary ducts, and the udder volume is mainly due to the fat pad (Table 2.2).

Table 2.2. Embryonic and fetal *mammogenesis* in cattle, pigs and humans

Stages of development	Cattle		Pigs		Human		Goat	
	Age of embryo days	Crown-rump of embryo, mm	Age of embryo days	Crown-rump of embryo, mm	Age of embryo days	Crown-rump of embryo, mm	Age of embryo days	Crown-rump of embryo, mm
Mammary band	32	14	21	10	35	6		
Mammary streak	34	16	22	12	36	8	25	4-5
Mammary line	35	17	23	15	37	10		
Mammary crest	37	19	25	18	40	13		
Mammary hillock	40	21	26	20	42	15		28
Mammary bud	43	25	28	22	49	20	37	30
Early teat formation	65	80	40	50	120			
Primary sprout	80	120	50-70	75-120	150	120-150		113
Secondary sprouts	90	160	77	130				
Canalization of primary sprout	100	190				180-200	88	226
Development of gland and teat cisterns	110-130	230-300						

Source: Adapted from Turner (1952), Kon and Cowie (1961) and Larson (1985).

From birth to conception, despite small alveolar development, the duct system proliferates through the adipose and connective tissues. In rodents and ruminants, this happens in a more extensive way than in carnivores and rabbits (Akers, 2002). However,

rodents have limited and sparse collagenous tissues with a unilocular adipose tissue. It is from these tissue that mammary parenchyma arises in an actively duct growth characterized by the presence of bulbous expansions, called terminal end buds (Capuco and Ellis, 2013). Before puberty, there is no ductal branching, for that and other reasons, mice and rats are not very good models for peripubertal mammary growth (Knight and Sorensen 2001). Differently from rodents, ruminants, humans and other animals provided with an udder, have a more inter and intralobular parenchyma with an arborescent shape, called terminal duct unit, that penetrates deeper into the mammary fat pad while the parenchyma grows (Capuco and Ellis, 2013).

Variables such as mammary gland weight or area, DNA content and parenchyma mass are used to compare mammary development with body grow. To know if the gland fills in allometric law $y = ax^b$, where y can be referred as mammary size, a as the intercept, x as the body weight and b as the power function on this equation. When $b = 1$, it means that the gland is growing at the same speed as the body, i.e., it has an isometric growth. When $b > 1$, the gland is growing faster than the body with a positive allometric growth. When $b < 1$, it is an enantiometric growth, the organ growth is slower when compared with the body, having a negative allometric growth (Anderson, 1976; Akers, 2002).

The length of the prepubertal period varies from specie to specie. In dairy cattle, onset of puberty occurs between six and eight months, whereas in beef and zebu cattle, this happens later. For small animals like mice, rats and dogs, it takes place after 28, 40 days and five to eight months, respectively, and for humans, it occurs after several years. An allometric growth occurs soon after birth (Capuco and Ellis, 2013), preceded by an isometric growth, returning to allometric before and through the first estrous cycles. In dairy heifers, the second allometric period appears after three months (Akers, 2002), and, in mice, at 22 days (Schmidt, 1971). The mammary gland increases in the connective tissue and fat deposition, but especially in the mazy ducts. According to many authors, this is a highly critical phase during which alterations in mammary growth could dramatically affect milk production (Schmidt, 1971; Larson, 1985; Akers, 2002).

These alterations could be caused by overnutrition (Knight and Sorensen 2001) and will be approached in another section, diseases, or even ovary secretions. Yart et al. (2012) in an experiment with 30 alpine goats, reported that ovariectomy affected negatively mammary gland parenchyma, suggesting that the ovary and its hormones are important to epithelial cell proliferation. However, ovariectomy did not stop the onset of mammary growth in rodents and heifers (Akers, 2002), but had reduced it (Akers et al., 2005).

After reaching puberty, the period that animals reach maturity and are capable of reproducing, some mammary development occurs, especially under hormone influence. Alveolar development begins, especially controlled by progesterone. Estrogen and Insulin-like growth factor I help ductular growth, elongation branching and thickening. The influence of these three hormones leads to an expansion and establishment of the milk secretory tissue (Knight and Sorensen 2001; Capuco and Ellis, 2013), since in every cycle a hormonal surge stimulates parenchyma proliferation to prepare the gland for future milk production. Prolactin and somatotropin are also important to mammary development. However, as some species have short cycles without a luteal phase, such as rodents, estrogen works in synergism with prolactin and somatotropin, and duct development begins only at conception (Larson, 1985).

Although mammary development begins in the uterus and an expressive growth occurs before puberty, it is only during pregnancy that its major maturation eventuates, when more than 60 percent of development happens, reaching up to 90 in some species (Akers, 2002). This growth can be described by the equation $Y = a^{bt}$, where Y = mammary size, t = days of gestation, a and b are the constants (Sheffield, 1988).

Duct length and cistern size increase, vascular and lymphatic systems take a definitive form, as the amount of secretory tissue, which replaces the fat pad, form the alveoli and lobules. This lobule-alveolar tissue starts to be formed, as the alveoli start to increase remarkably in size and number during the second half of gestation. All this happens in a more languid way in the beginning of pregnancy but increases absurdly in the last third, when the alveoli also start to show secretory activity (Schmidt, 1971).

Giving birth, the female starts the lactation period. Although *mammogenesis* occurs in large proportions by the end of pregnancy, it continues during the first part of lactation where a “wave” of mitosis occurs shortly around parturition (Schmidt, 1971). In rats, more than 20 percent of mammary growth occurs during lactation, as in other rodents and sows, the epithelium doubles between the last days of pregnancy and the first days of lactation (Akers, 2002; Capuco and Ellis, 2013). Parenchyma growth continues until the peak of lactation, but after that, there is little cell proliferation. From birth to lactation, mammary parenchyma increases 11.000 times in cows (Capuco and Akers, 2010) and recurring pregnancies increase mammary growth and production capacity, reaching maximum yield during fourth lactation (Schmidt, 1971). Since suckling and milking are important for growth in early lactation because they reduce cell apoptosis, it has already been proven that “extra” milking increases milk yield (Svennersten-Sjaunja and Olsson, 2005; Capuco and Ellis, 2013).

With the progression of lactation, cells that are destroyed and eliminated are not replaced in the same amount, thus, beginning tissue remodeling and giving start to the involution phase. If milk removal is stopped, intramammary pressure increases, preventing precursors to move towards the secretory cells and blood to flow adequately, accelerating the involution phase (Akers, 2002).

Dairy animals such as cows and goats lose their secretory capacity with the lactation progress (Elsayed et al. 2009), but maintain the alveolar structure and renew progenitor cells and epithelial cells during the period that they are not producing milk, known as the dry period. Differences in milk production between beef and dairy animals are due to increased parenchymal mass and enhanced activity of the secretory cells in dairy animals (Akers, Capuco and Keys, 2006). Rodents, on the contrary, lose their alveolar structure but have their progenitor cells also renewed (Akers, 2002). Stress factors, mastitis, decreased milk frequency, as well a new pregnancy are also responsible for a negative milk production yield since they are related to apoptotic cell death and consequently, epithelium reduction. In absence of gestation, secretion also continues diminishing with the gradual loss of epithelial cells and cell activity (Capuco and Ellis, 2013). This loss has close relation with lactation persistency, varying between species and breeds, milking frequency, nutritional requirements coping, number of parturitions, hormonal influences, photoperiod, bovine somatotropin supplementation, prolactin levels, antioxidants and local mechanisms (Capuco et al., 2003; Svennersten-Sjaunja and Olsson, 2005, Capuco and Ellis, 2013). At remodeling, cell increase and mammary gland growth will take place in the subsequent lactations.

2.3 Hormonal regulation of mammary development

Hormones are regulatory organic chemical products, synthesized in small concentrations by endocrine glands and secreted into the blood in response to internal or external stimuli. They are transported to their target cells where they bind specifically with receptor molecules. Some hormones bind with specific protein receptors on the cell surface membrane, while others enter the target cells and bind to specific cytoplasmic receptors. Thus, each particular hormone-target cell interaction results in a variety of biological responses (Larson, 1985; Rodwell, 2015) that play a central role in *mammogenesis*, *lactogenesis* and *galactopoiesis*. Interaction between systemic hormones and local growth factors regulate mammary development (Akers, 2005).

The interaction between cell and hormone can occur in three different ways: endocrine action, where the hormone is secreted by specialized cells into the blood to travel to the target cells; paracrine action, which acts locally by diffusing from its source to the neighboring target cells; and autocrine action where the hormone acts in the same cell that produced it. All these three interactions may act agonistically or antagonistically with target cell enzymes and molecules, or gene expression modulation (Rodwell, 2015).

2.3.1 Estrogen

Estrogen is a steroid hormone, synthesized mainly in the ovaries, that has a direct effect on *mammogenesis* and induces secretions of growth factors from the pituitary, kidney and mammary gland (Rodwell, 2015) – IGF-1, epidermal growth factor (not in cattle), fibroblast growth factor, transforming growth factor- α , hepatocyte growth factor and macrophage colony stimulating – help to mediate its effects (Tucker, 2000). Estrogen receptors appear initially with onset of puberty, and the number of receptors increases with increasing tissue weight and estrogen concentrations (Larson, 1985).

Because of its mitogenic effect, in rodents, estrogen induces stroma proliferation, followed by ductular epithelium proliferation. In cattle, neither adipocytes nor fibroblasts of the mammary stroma proliferate in estrogen response (Tucker, 2000), but there is an intense duct growth (Schmidt, 1971). Estrogen increases number of progesterone receptors and, during pregnancy, plus progesterone to induce lobule-alveoli formation (Larson, 1985). Estrogen is involved in the beginning of lactation in the periparturient period causing prolactin release and increase of prolactin receptors on the mammary gland. In humans, its administration may suppress lactation by interference on milk-ejection reflex (Tucker, 2000).

During late gestation, estrogen and progesterone concentrations are elevated, what may lead to believe that both are responsible for mammary allometric growth (Larson, 1985; Akers, 2002). They accelerate cell division rate, especially in the terminal end buds, since high affinity binding receptors are located in this epithelial cell, as well as in the adipose and connective tissues of the mammary gland. In prepubertal ovariectomized animals, mammary development is impaired. However, ovary transplantation to other parts, as well as hormone supplementation, restores normal mammary growth (Larson, 1985).

2.3.2 Progesterone

Like estrogen, progesterone is a steroid hormone, produced by the corpus luteum, ovaries, adrenal glands and, during pregnancy, by the placenta. It induces DNA synthesis and cell division at the end buds and mammary duct walls where its receptors are located (Tucker, 2000). Estrogen- progesterone synergism induces lobule-alveolar growth and, consequently, mammary development. It is an important hormone to suppress lactation during pregnancy because it prevents prolactin from increasing its receptors in the mammary gland, blocks glucocorticoid receptors on the mammary tissue, reduces estrogen-prolactin synergism, and inhibits synthesis initiation of milk components such as lactose, α -lactalbumin and casein. However, after parturition, the concentration decreases and at the onset of lactation, progesterone has no more effect on milk yield, possibly due to its higher affinity for milk fat than for its own receptor (Tucker, 2000).

2.3.3 Prolactin

Prolactin (Prl) is a peptide hormone, produced by the pituitary gland, known as the primary lactogen hormone. It has the ability to regulate its own receptors, in an “up-down regulation” system, where a gradual increase of the hormone concentration augments its number of receptors (Larson, 1985). Besides inducing mammary gland lobule-alveolar growth, it also regulates gonadal function and influences maternal behavior and the immune system (Hennighausen et al., 1997). Prolactin is essential for lactation onset, due to its indirect effect on ductal side branching and terminal end bud regression, and as its direct effect increasing lobule-alveolar development during pregnancy by epithelial cell metabolism stimulation (Tucker, 2000). In sheep, pigs, rodents and primates, Prl depletion during lactation can limit milk secretion and lactation persistency, conversely what happens in goats and cows, where its concentration does not interfere after lactation onset. Nevertheless, it is possible that dairy ruminant mammary tissue developed a high avidity for blood circulating Prl, permitting adequate responses even with very low levels of the hormone (Knight, 2001).

Prl is responsible for maintaining mRNA concentrations for the synthesis of milk proteins (Svennersten-Sjaunja and Olsson, 2005). There is also some evidence that Prl itself, in the absence of steroid hormones, has a modest induction on mammary growth (Knight, 2001). Furthermore, without Prl, steroid hormones fail to stimulate *mammogenesis*. However, P₄ antagonizes its effect on the prolactin receptor and that is the reason why pre-partum P₄ concentration decrease is needed to unlock Prl receptors and onset lactation. Prl is also inhibited in cold weather and during short photoperiods, but stimulated by milking and suckling association or even teat palpation (Akers, 2002), showing a small effect on lactation

persistence (Knight, 2001). Prl increases intestinal calcium absorption and facilitates uptake of long-chain fatty acids for milk fat synthesis (Svennersten-Sjaunja and Olsson, 2005).

2.3.4 Growth Hormone

The growth hormone (GH), or somatotropin, is a peptide hormone produced by the pituitary gland and responsible for growth stimulation, cell reproduction and regeneration (Rodwell, 2015). In ruminants, GH, replacing Prl, binds to receptors on hepatocytes and stimulates IGF-1 production, causing a mitogenic effect on the mammary gland, helping milk secretion persistence. Inasmuch, it influences fat synthesis and, together with Prl, has an impact on milk production (Svennersten-Sjaunja and Olsson, 2005), despite little change in blood concentration during all lactation period. Apparently, it is the primary galactopoietic hormone in most mammals, with exception of rodents (Akers, 2002).

Bovine somatotropin (bst) is an identical synthetic growth hormone, widely used on dairy farms to increase milk production and lactation persistence without gross changes in milk composition. Bst increases the parenchymal mass and mammary cell numbers, without health implications (Tucker, 2000). It causes corporal store mobilization and leads to increasing mammary gland ability to synthesize milk components by nutrient and energy partitioning from the body and blood flow. It has a dose dependent response and lasts just a few days. Therefore, constant injections are needed to increase lactation efficiency, causing insulin action inhibition, lipolysis, hepatic glucose increase, mammary gland glucose uptake and lactose synthesis, NEFA utilization for fat synthesis and amino acids for milk protein synthesis (Akers, 2002).

2.3.5 Insulin

Insulin is a peptide hormone produced in the pancreas and is responsible for regulating the metabolism of carbohydrates and fats by the absorption of glucose, and is involved in the nutrient partitioning to the mammary gland during lactation. Despite its little effect on *mammogenesis in vivo*, in *in vitro* experiments it has demonstrated to be essential because of the IGF-1 mimic effect. However, in cattle, insulin serum concentrations decrease during pregnancy, as well as the mammary gland uptake of glucose, acetate, β -hydroxybutyrate, triglycerides and amino acids, independent of insulin, transpiring that insulin concentration does not limit *mammogenesis* in this specie (Tucker, 2000), despite exogenous insulin inhibition on milk production (Akers, 2002).

2.3.6 IGF-1

Insulin-like growth factor 1 (IGF-1) is a peptide hormone with similar molecular structure of insulin, produced by a wide spectrum of tissues, but synthesized primarily in the liver and locally in the stromal mammary gland cells under GH control (Zhou et al., 2008). It is essential for normal mammary development due to its ability to stimulate epithelial cell development and, consequently, rapid mammary growth (Cannata et al., 2010). It mediates, in association with GH and estrogen, cell growth, differentiation, development, maintenance and apoptosis prevention (Akers, 2002; Akers et al., 2005). Long-day photoperiod can increase IGF-1 secretion, positively influencing lactation persistency (Svennersten-Sjaunja and Olsson, 2005). Additionally, the feeding level can also affect IGF-1, since it is deeply related with mammary growth and feed nutrition level. In heifers on high feeding level, IGF-1 has a reduced sensitivity due to an increasing production of local binding proteins that inhibit its effects (Sejrsen et al., 2000). Inasmuch, in ovariectomized heifers, there is less local IGF-1 and also fewer receptors and IGF-1 mRNA, reducing epithelial cell proliferation rates (Akers et al., 2005).

2.3.7 Placental Lactogen

Placental lactogen is a placental peptide hormone, with similar structure and function to GH or PRL in most species, binding in a common receptor site of these two hormones in the epithelial membrane. Its secretion increases at mid pregnancy until parturition. In rodents, its concentration is correlated with the number of fetuses, and consequently, with mammary growth stimulation. Similarly, in small ruminants, the greatest secretion of placental lactogen coincides with the most intense growth of the lobule-alveolar system (Tucker, 2000). It is also involved in the preparation of the mammary gland for lactation, fetal growth and maternal metabolism alterations. However, despite all its influence in many species, the role of placental lactogen in cattle physiology is still unclear (Akers, 2002).

2.3.8 Glucocorticoids

Cortisol is the predominant glucocorticoid, a steroid hormone, produced in the adrenal cortex of the adrenal gland, responsible for lobule-alveolar differentiation (Tucker, 2000). Larson (1985) reported that glucocorticoid administration to pre-pubescent animals induces ductular and lobule-alveolar development; adrenalectomy reduced mammary development; and, in pregnant animals, inhibits subsequent lactogenesis and lactation.

In general, its concentrations remain low throughout gestation, increasing in the last portion, just before delivery of the offspring, when it competes with progesterone for the binding receptor in the epithelial cell, reducing progesterone block for milk production, regulating α -lactalbumin and β -casein secretions. Glucocorticoids binding is also positively correlated with uptake into mammary tissue, amplification of prolactin effects on lactogenesis (Tucker, 2000), and it is important for the maintenance of milk production in dairy species (Akers, 2002). Cortisol has an effect on the lactating animal metabolism, helping maintain the epithelial secretory activity (Svennersten-Sjaunja and Olsson, 2005), as well as, together with Prl, regulate secretory cell differentiation (Akers, 2002).

2.3.9 Thyroid Hormones

Thyroxine (T_4) and triiodothyronine (T_3) are thyroid hormones, produced by the thyroid gland, partially composed by iodine. T_4 stimulates oxygen consumption, protein synthesis, milk yield and T_3 , Ca and P metabolism. Although thyroid hormones are not essential for *mammogenesis*, hypothyroidism retards ductal and lobule-alveolar development. T_4 given in low doses enhances estrogen and progesterone effects on mammary growth during pregnancy, as well as mammary growth itself, and increases milk production. T_3 enhances Prl ability to stimulate lactose, as well as GH synthesis regulator and alteration of GH hepatic receptor enhancing IGF-1 synthesis. It is also known that surgical removal of the thyroid gland reduces milk yield (Larson, 1985; Akers, 2002), noting that parathyroid removal also occurs in this type of surgery. Thus, thyroid hormones are not essential for mammary development, since thyroidectomized animals conceive and lactate (Akers, 2002); however they are needed for maximal mammary growth and strongly influence milk synthesis (Larson, 1985).

2.3.10 Leptin

Discovered just in 1994, leptin is a protein hormone, synthesized primarily by adipose cells, but also by a variety of tissues like stomach, skeletal muscle, pituitary, mammary tissue and placenta. Its secretion follows the circadian rhythm, nutritional and physiological status (Zieba et al., 2005), with potential effects on many parts of the body, activity behavior, thermoregulation and stress (Friedman, 2014), presenting a link between metabolic status and neuroendocrine axis (Barb And Kraeling, 2004; Svennersten-Sjaunja and Olsson, 2005). It is also associated to reproduction and reproductive behaviors acting as a metabolic gate, inducing luteinizing hormone release to promote puberty onset, if there is no nutritional perturbation.

On the mammary gland it is a powerful Prl release stimulator, possibly interacting with this hormone to regulate metabolism, growth, osmotic homeostasis or cell maturation (Tipsmark et al., 2008). Its presence was found in mammary adipocytes, epithelial and myoepithelial cells during pregnancy and lactation, since its gene is expressed in mammary tissue of dairy ruminants. Leptin concentrations increase over pubertal development and postpartum (Barb and Kraeling, 2004). As discussed in a review (Chilliard et al., 2001), leptin synthesis by the mammary adipocytes may be due to a paracrine role in epithelial cell growth by the hormone itself, or indirectly, by mediated growth factors. It has also been also suggested that leptin has a role in duct and alveoli formation, and that elevated nutritional planes increase leptin production by the mammary stroma, nevertheless, no direct action on epithelial cells was found (Thorn et al., 2006).

2.4 Nutrition impact on mammary development

On dairy farms, nutrition is responsible for a large amount of the total costs and, although cows, goats and ewes pay these expenses with milk production, the young dairy replacement animals do not provide any revenue during the rearing period, considered one of the most costly phases in the dairy business (Whitlock et al., 2002).

The rearing period can be divided in four phases: from birth to weaning (lactating), from weaning to puberty (prepubertal), from puberty to conception (postpubertal) and pregnancy period (Sejrsen et al., 2000). For that reason, attempts to provide a decrease of this rearing phase length are made by providing high plane nutrition with the purpose of achieving high gains, decrease sexual maturity age and, consequently, breeding and conceiving earlier leading to advanced milk production. This high feeding level has been studied in sheep and bovines for the past 50 years in an attempt to suggest the best rearing management without affecting mammary secretory tissue development and, consequently, changes on total milk amount, as well as development of techniques that allow selecting good replacement animals at young age.

In this aspect, many studies have suggested that high gains during prepubertal mammary gland growth phase may impair development and, consequently, future milk production. Sejrsen et al. (2000) concluded that the relationship between growth rate, mammary growth and milk yield leads to three aspects: prepubertal high feeding level can reduce mammary growth and milk yield potential, increased gains in postpubertal and pregnancy period due to high feeding level have no effect on mammary growth and milk yield

and higher body weight gain due to genetic potential for growth is positively correlated to milk yield.

The negative impact of high-gain diets in the development of the ductal epithelium would influence secretory epithelium (alveoli) development and, consequently, milk production (Sejrsen et al., 2000). Since duct developments occur at early stages of the animal's life, the peripubertal period has often been characterized as the most critical and coincides with one of the allometric mammary growth phases.

However, despite the large number of studies in bovines, the consequences of high-gain diet on mammary gland development and reduction of lifetime milk production still remains controversial (Capuco et al., 2003; Meyer et al.; 2006a, b), mainly because of interacting factors (Daniels et al., 2009). Some studies compare mammary growth of animals at equivalent body weight, but disregard their age. So, individuals reared with greater rate gains will achieve target body weight at a younger age, ignoring their physiological development for further comparisons. Conversely, other studies compare equivalent ages, but not the animal's body weight, and few studies make interactions between weight and age. Additionally, most analyses use parenchyma and fat pad weight or size, DNA and RNA amount, and protein and ether extract mass only in the studied phase, but only a small number verified mammary subsequent lactation performances to prove negative consequences of high-gain diets.

Despite the consistent data that high-gain induces fattening and impairs mammary growth, information relating these prepubertal impacts and ensuing lactations are needed (Capuco et al., 2003). The mammary gland high plasticity might allow a compensatory growth during other development stages, providing suitable development of the parenchyma that was impaired previously (Capuco et al. 1995).

To better illustrate, a summary of the top studies on the influence of nutrition on the domestic animal mammary gland through the past years is represented in Table 2.3.

Table 2.3. Top findings on the domestic animals mammary gland nutrition impact

Year	Authors	Studied animal	Findings
1966	Srivastava and Turner	Rats	- MG DNA decrease on the restricted feeding group - Feed restriction up to 50% during 19 days, under E ₂ and P ₄ stimuli, has a less depressive effect on MG growth, than endocrine gland removal

Continues on next page

Year	Authors	Studied animal	Findings
1982	Serjrsen et al.	Heifers	<ul style="list-style-type: none"> - Ad libitum feeding during prepuberty lowered mammary secretory tissue and DNA content - No difference in postpubertal growth between restricted and ad libitum feeding. - Mammary parenchyma was not affected by plane nutrition in pre and postpubertal heifers.
1990	Stelwagen and Grieve	Heifers	<ul style="list-style-type: none"> - Increasing plane of nutrition resulted in fatter mammary gland, but did not affect total mammary DNA.
1995	Bowden et al.	Goats	<ul style="list-style-type: none"> - Kids fed with <i>ad libitum</i> intake had greater ADG, larger mammary glands, increased adipose deposition and connective tissue. - Kids on the restricted diet had more DNA and protein per gram of mammary gland, indicating greater secretory development.
1995	Capuco et al.	Heifers	<ul style="list-style-type: none"> - Heifers reared at silage corn high rate gain had reduced parenchymal DNA and RNA, greater volume of adipocytes and lower volume of epithelial cells. - Milk production did not differ among treatments.
1997	Kim et al.	Rats	<ul style="list-style-type: none"> - Compensatory nutritional regimen during peripubertal growth phase stimulated mammary epithelial cell proliferation and improved lactation performance.
1999	Lammers, Heinrichs and Kensinger	Heifers	<ul style="list-style-type: none"> - Accelerated prepubertal growth regimen decreased first lactation fat-corrected milk yield in 7,1%, but increased udder size, probably because of increased fat deposition.
2001	Lyvers-Peffer and Rozeboom	Gilts	<ul style="list-style-type: none"> - Restricted energy regimen during prepubertal growth may limit mammary development but enhance lactation performance. - Restricted regimen had less parenchymal tissue, but during lactation consumed more feed and tended to wean heavier litters.
2002	Whitlock et al.	Heifers	<ul style="list-style-type: none"> - Dietary protein did not affect puberty age, body weight and withers height gain, average mammary parenchyma, teat length, mammary development. - Feeding low-protein diets increases risk of impaired mammary development when heifers are fed for rapid growth
2005	Guerreiro et al.	Ewes	<ul style="list-style-type: none"> - Nutritional planes based on grazing with constant supplementation did no influence mammary development. - Group with high ADG had more fat on the mammary gland. - Milk production did not differ among diets.
2006	Meyer et al.	Heifers	<ul style="list-style-type: none"> - Mammary fat pad weight, lipid and DNA content were greater in heifers with elevated gains, but their mammary parenchyma weight and DNA were lower when compared to restricted heifers. - However, when age was analyzed as a covariant term, level of nutrient intake had no effect on parenchyma composition. - Dynamics of allometric and isometric mammary growth were also unaffected by the level of nutrient intake. - Treatment did not negatively influence epithelial cell proliferation or the parenchyma DNA accretion rate.

Continues on next page

Year	Authors	Studied animal	Findings
2006	Sørensen et al.	Gilts	- High feeding level from 3 months of age to puberty has a stimulatory effect on mammary development in prepubertal gilts. - <i>Ad libitum</i> group had higher mammary tissue, RNA and DNA content compared to restricted feeding.
2015	Albino et al.	Heifers	- Mammary lipid content was influenced by metabolizable protein: metabolizable energy ratios in diets of heifers subjected to accelerated growth rates. - Diets with MP:ME ratios less than 38g/Mcal might induce fat accumulation in mammary parenchyma.
2015	Esselburn et al.	Heifers	- Diet did not affect mass, protein or DNA content of mammary parenchyma in young heifers.

A classical study carried out by Sejrsen et al. (1982), investigating the influence of plane of nutrition on mammary development in heifers, found that *ad libitum* feeding during prepuberty lowered mammary secretory tissue in 23%, connective tissue in 32%, increased the amount of adipose tissue and mammary total weight when compared to restricted feeding. However, no differences were found in the postpubertal phase, concluding that heifers raised with high-gains during gland allometric growth might have less secretory tissue.

Using the same animals, Sejrsen et al. (1983) measured GH, Prl, insulin and glucocorticoids hormone concentration to correlate their levels with mammary development. GH was elevated in restricted heifers, whereas prolactin, insulin and glucocorticoids were lower when compared with *ad libitum* animals, leading to the conclusion that mammary secretory tissue was positively correlated with GH concentration and extra-parenchymal adipose tissue was negatively correlated. However, when adipose tissue, parenchymal DNA and hydroxyproline were compared postpuberty, no differences were found. The variations obtained earlier in the experiment, when the animals did not onset puberty, were probably due to age differences at slaughter time and not because of feeding level, since age and weight interaction was not statically made, mammary development might be underestimated.

Contrary from what is currently found in heifers, Lyvers-Peffer and Rozeboom (2001) and Sørensen et al. (2006) testing prepubertal feeding levels in gilts, concluded that prepubertal restricted regimen might limit mammary development but, due to a compensatory effect, enhance sow lactation performance, and that high feeding level from three month of age to puberty had a stimulatory effect on mammary development, increasing mammary tissue as well as RNA and DNA contents.

Furthermore, diet composition may also have an effect on mammary development because of the degradability of the nutrients and the interaction between them, causing key hormones, local growth factors, tissue responsiveness alterations and, consequently, changes on mammary and adipose tissues development (Bowden et al., 1995; Sejrsen et al., 2000; Capuco et al., 2003; Meyer et al., 2006b). Excessive energy intake in the prepubertal period could induce permanent modifications in endocrine and adipose tissues, inhibiting lactogenesis and mammary gland homeostatic control, influencing future propensity of local fat deposition. However, compensatory growth might occur during subsequent period of high nutrition to overcome decreasing mammary growth (Capuco et al. 1995).

To test the effect of a different ingredient, as well as hormone injections, McFadden et al. (1990), in an experiment with ewe lambs divided into feed restriction ration, high energy ration, high energy ration injected with bovine growth hormone (bgh) and a ration including formaldehyde-protected sunflower seed supplement (PUF) groups were slaughtered and mammary gland composition compared, concluding that supplemental dietary lipids promoted parenchyma growth, increasing amount of fat pad and bigger total gland size than in other treatments. Bgh increased parenchyma weight but decreased entire gland size, possibly due to a reduction of the fat pad weight. The lack of differences in parenchymal development between high and restricted plane nutrition contradicts with the general perception that high nutrition impairs prepubertal mammary growth. The only difference found was in fat pad and total gland amount.

Testing the same proposal, Stelwagen and Grieve (1990) and Bowden et al. (1995) also noticed increased fat deposition in Holstein heifers and French Alpine Goats, respectively, reared with higher gains treatment, corroborating with the previous findings. However, the goats presented also increased connective tissue and larger mammary glands, but the heifers' other mammary components, besides fat pad, did not change among treatments.

Also working with dietary oil, Thibault et al. (2003), in an experiment with 116 Holstein heifers divided in conventionally concentrated feeding and high soybean oil concentrate feeding, discovered that high oil feeding increased mammary fat pad, corroborating with the previous findings in sheep by McFadden et al. (1990). However, both treatments had similar mammary development; and subsequent milk production and composition were not affected.

On the other hand, using different ingredients besides divergent average daily gains and dietary oil, Capuco et al. (1995) found that high feed level of corn based diet had much

more severe effects on the mammary gland than high feed level of alfalfa based diet; increasing fat deposition and decreasing epithelium and stroma growth. In the same study, heifers on high corn diet presented lowest GH and higher IGF-1 concentration levels than the other cohorts. Both GH and IGF-1 are deeply related with mammary growth and feed nutrition level (Sejrsen et al., 2000), since GH is required for duct growth and it has been proven that its exogenous administration enhances mammary growth (McFadden et al., 1990). IGF-1, instead, has a reduced sensitivity in heifers on high feeding level, due to an increasing production of local binding proteins that inhibit the effects of IGF-1 (Sejrsen et al., 2000). Conversely, Whitlock et al. (2002) did not find alterations in the somatotropin blood concentration profile in heifers raised on low, standard and high protein rations, and concluded that dietary protein does not have a major effect on mammary development of prepubertal heifers when it contains an adequate protein level for normal body growth. Meanwhile, low-protein diet must be used with attention because of the increased risk of impaired mammary development.

Nevertheless, not only different ingredients and nutrient levels were tested as influential factors on *mammogenesis*, but the length of the dietary treatment as well. The time length during which high energy and protein intake is provided may influence cell development and reprogram changing cellular responses, as well as permit a compensatory growth after the submitted treatment.

To determine whether a compensatory regimen during pregnancy improves lactation performance, affecting proliferation and apoptosis of mammary epithelial cells, female rats were used, and it was concluded that a compensatory regimen during the peripubertal phase stimulated mammary epithelial proliferation and improved lactation performance (Kim et al., 1998).

In a similar experiment with beef heifers, Yambayamba and Price (1997) measured the effect of feed restriction followed by realimentation on mammary development. The heifers in the restricted group had lighter mammary glands with lower lipid content, suggesting that feed restriction followed by realimentation could enhance mammary development. Nevertheless, like in many researches, no data interpolation considering age and weight covariates was made, changing the data interpretation. It must also be added that no further production measurements such as milk yield or calf daily gain were taken.

In the same line of thought, Davis Rincker et al. (2008), to establish the effects of prepubertal high-energy diet feeding, made a different trial, giving the same high energy diet to all heifers, but during different time lengths. In case of a long energy diet treatment, a

linear decrease of mammary epithelial cell percentage and mass of fat-free mammary parenchyma per unit of carcass was found. On the other hand, mammary total mass, extraparenchymal and intraparenchymal fat increased, suggesting that feeding heifers with a high-energy diet reduced mammary parenchyma mass, impairing mammary gland development.

In a different experiment to test mammary development of sheep under feeding restriction during late pregnancy, Nørgaard et al. (2008) found that although colostrum yield was reduced in restricted feeding animals, milk yield was not. No significant changes in the parenchyma or tissue type distribution were found, or mammary gene expression for leptin and IGF-1. Nevertheless, body condition score changes were evident, suggesting extensive mobilization of body tissues giving priority to fetus growth and mammary gland development, compensating the diminished offered nutrient. IGF-1 and the leptin plasma concentration was also lower in feed-restricted animals.

Corroborating with the previous findings, Abeni et al. (2000) testing the effect of moderate and accelerated average daily gain of Holstein-Friesian pre and postpubertal heifers, found that milk production and protein were not affected by average daily gain. Even so, postpubertal high average daily gain decreased milk fat percentage, early calving influenced negatively milk production, whereas it influenced positively protein.

Contrary to Nørgaard et al. (2008) and Abeni et al. (2000), McCann et al. (1989), rearing ewes on high and low concentrated diet found that high weight gains limited milk production ($P < 0.10$) and increased fat pad area, suggesting that factors associated with fat deposition might be responsible for the reduction of milk yield. In contrast, Capuco et al. (1995) observed that heifers raised with a high gain diet had a greater volume of adipocytes and lower volume of epithelial cells, and, although numerically milk yield was reduced by 1.35 kg/d, there was no decline in subsequent milk production of these animals and production did not differ among all treatments groups. They propose that a compensatory growth might have occurred, or, the treatment might have been initiated too late for lactational effect, suggesting that high-energy intake is less deleterious in the late prepubertal period. Conversely, Lammers et al. (1999), in an experiment with standard and accelerated growth rates, and estrogen implant or not, found that prepubertal accelerated growth decreased first lactation fat-corrected milk yield (7,1%), and estrogen implants reduced 5,2%. Udder size increased in the high gain group, also probably because of fat deposition.

Differently, an unprecedented trial conducted by Villeneuve et al. (2010a, b), using Dorset ewe lambs, determined the effects of prepubertal feed restriction on first and second

lactation performances. Although stroma and fat pad weight were greater for *ad libitum* group, as dry fat-free tissue was greater for the control and restricted groups; no differences between groups were found at the first lactation. However, during the second lactation, the group reared under restricted feeding presented higher milk yield, with higher fat and protein yield. Since, after the restriction phase, the lambs in the lowest gain groups achieved near full compensatory growth, and consumed less feed than the unrestricted cohort over the entire period, the results suggest that restricted feeding before puberty improves mammary development. Still, it must be considered, somehow, that the animals slaughtered for mammary gland comparison had the same age, but not the same weight. Owing to that fact, it must be added that data interpolation was not made considering weight as a covariate on mammary gland composition of ewe lambs slaughtered at the same age.

Through improved measurement techniques and statistics, a huge step towards solving doubt regarding the impact of nutrition on mammary gland development, and permitted Meyer et al. (2006 a, b) to make an impressive counterpoint when analyzing age as a covariant term for heifer weight gains, observing that the level of nutrition intake had no great effect on DNA, lipid, protein and connective tissue composition of mammary gland parenchyma, mammary epithelial proliferation and total parenchyma mass. Inasmuch, it was observed that high gains still enhanced mammary fat pad. Daniels et al. (2009), using the same heifers, found no histological differences in mammary parenchymal composition when using body weight and age to determine the nutritional impact on mammary development, and did not support the hypothesis that rate gain negatively impacts ductal development.

In 2011, scientific animal researchers started using ultrasound as an effective noninvasive tool for measuring *in vivo* changes in mammary gland parenchyma, discarding the comparative slaughter method and allowing to follow heifer development to reveal milk production capacity. Esselburn et al. (2015) found that diet composition did not affect mass or parenchyma composition of young heifers, corroborating with the previous findings by Meyer et al. (2006a, b) .

It has been postulated that high levels of feeding in the prepubertal period impair mammary development. Nevertheless, most data point out that excessive body and mammary fattening mechanisms in rapid rearing programs remain unclear, possibly leading to reduction of secretory cells, nutrient reprogramming and negative body size. However, many experiments demonstrated that the gland capacity to produce milk is related to the number of epithelial cells, and these, to genetics, and proliferation and apoptosis rates (Capuco et al., 2003),

meaning that mammary size as parenchyma dimension is not the only aspect to be considered on milk secretion.

Sejrsen and Purup (1997) hypothesize the lack of effect of those trials is because of short treatment periods, high pretreatment growth rates, small growth rate differences between treatments, variations within treatment groups and treatments conducted outside the critical period, but, they do not explain the absence of the treatment effect.

Analyzing some experiments, it is possible to verify that the number of animals was small. Dairy heifer trials are mostly carried out with Holstein heifers and only the first lactation is followed, statistic analyzes are rudimentary and most of the time no interactions between covariates are made. Additionally, most researches are conducted in calves and heifers, however, ewe lambs and kids should also be used as a model. Thus, less milk yield in the first lactation was not due to impaired development of the mammary gland, but because of nutrient division between the mammary gland and the continued body growth changing metabolic status. Since the mother gives birth at a younger age, she could not be as physiologically developed as animals reared more slowly.

Another doubt on mammary gland development evaluation is that it seems that animals with high genetic potential for milk production tend to lean fat deposition, so effects of dietary regimen will be hidden (Lohakare et al., 2012). Silva et al. (2002) concluded that there was no relation between individual body weight gain and mammary development. However, since body fat concentration was negatively related to body weight gain, heifers that gained more proportion of fat grew slower and had less mammary parenchyma at puberty, showing that body fatness was a better predictor of impaired *mammogenesis* than daily gain itself. Inasmuch, animals with higher genetic potential for milk might not have higher propensity for fat body accretion. This work reinforces the last theory that high average daily gains do not impair mammary gland development.

Furthermore, animals fed on higher nutritional planes may have a cellular reprogramming, thus, permitting a more intense use of nutrients, as well as more productive cells due to organism adaptation. Since leptin is related with glucose metabolism and the closeness of the mammary adipose tissue from the mammary parenchyma provides a rapid energy source and hormone control of epithelial cell demands, adipose deposition on the mammary gland might be a positive consequence rather than negative. Veerkamp et al. (2003) concluded that the selection for higher milk yield would cause changes on energy portioning and hormone and metabolites profiles, influencing GH, IGF-1, insulin and, consequently, glucose utilization towards milk synthesis and mammary gland.

With the advance of new measurement techniques also for statistics, data is starting to be used differently, leading to new results. If accelerated body growth occurs in absence of excessive fattening, with appropriate nutritional management, protein and energy balance, mammary development could not be impaired, but, on the contrary, benefited.

2.5 Techniques for studying mammary growth

There are many ways to evaluate mammary tissues, not only to follow its growth but also to detect alterations and diseases. These evaluations can be divided in two categories: *In vitro* and *in vivo*.

In vitro evaluations consist of growing mammary tissue in specialized cultures using it as a model to analyze its physiological, biochemical and immunological functions, avoiding *in vivo* experiments that can result in systemic effects on animals (Fu et al., 2014). However, since epithelial cells die quickly and connective tissue persists for a reasonable time, maintaining the environment of the mammary gland proves to be difficult (Larson, 1985; Fu et al., 2014) and sometimes the response on the tissue is different from *in vivo* studies. Mammary tissues are obtained by healthy mammary glands or fetal tissue, and grown and purified in special cultures to select normal morphology and growth characteristics. Several bioactive factors are used to help cell growth, influencing their metabolism (Hu et al., 2009), which allows observing the individual effects of each one. *In vitro* studies also permit transgenic animal development, consequently, transgenic milk production.

In vivo evaluations allow quantitative and qualitative measures to evaluate the mammary gland, with invasive and non-invasive methods, most commonly used to observe size, gross appearance or organ growth. The methods consist of using biopsy, comparative slaughter, water displacement, palpation, gland and teat measurements, magnetic resonance imaging (MRI), ultrasound (US) and others. These techniques are normally correlated with body growth measurements – body weight and withers height – in classical allometry studies, to verify if there is an isometric, allometric or enantiometric growth of the organ when compared to the entire body.

2.5.1 Biopsy

Biopsy is used for both research and clinical diagnosis, and permits to obtain *in vivo* tissue samples for histological and biochemical evaluation, without necropsy procedures. There are two types of biopsy used especially in large animals: surgically large tissue fragment removal or needles and biopsy pistols to extract small fragments. The first one

demands animal sedation, causes large tissue damage and induces further complications. The second method may cause inflammation and hemorrhage, but in a very small proportion, especially when compared with surgically biopsy, reducing complications after the procedure (Lima et al., 2015). The tissue fragment taken by biopsy is then sliced and studied for its DNA and RNA content, or cell number and structure for histology evaluation.

Knight et al., 1992, in an experiment with lactation cows, suggest that mammary biopsy can be performed successfully, with rapid recovery, with no adverse effect on secretory tissue when the procedure is conducted with care. Milking at 12 h intervals and antibiotic therapy are essential for this recovery.

2.5.2 Comparative slaughter

Comparative slaughter enables correlations of organ development at different stages of life and milk production. In counterpart, it demands a large number of animals and their sacrifice, not allowing observations of the same specimen performance through time. It consists in gland removal after slaughter. The organ is weighed to register the wet weight and, in large animals, udder is trimmed to remove skin, teats, lymph nodes, extra fat, large blood vessels and milk, if any. Water and lipids are extracted by hot ethanol and ether leaving proteins and nucleic acids (DNA and RNA). After drying completely, the remaining tissue is known as dried-fat-free tissue (DFFT) and used to verify the number of epithelial cells (DNA measurement), cell activity (RNA measure) and quantity of connective tissue (hydroxyproline measurement – amino acid component of complex of protein collagen). After the extractions, the remaining tissue is known as the parenchymal tissue. In addition to these measurements, the whole mount organ can be also used for histological study as subjective value for mammary growth, growth normality and total alveolar surface area determination, by measuring the number of alveoli in histological sections per length of line correlating them with milk production (Schmidt, 1971).

2.5.3 Water displacement and plaster cast

Water displacement or plaster cast impressions are used to evaluate gland volume and weight. The displacement of water uses the Archimedes' principle and consists in submerging a complete udder in a container full of water. The residual liquid subtracted from the initial volume gives the gland cubic measurement. This technique is used to study mainly goat udders, since they have a more pendulous shape than those of other mammals, however, it could be also used to estimate sheep, cow, sow and dog udders (Linzell, 1966).

The plaster cast is used to verify udder shape and volume by making an impression of the glands, applying the same technique used in dentistry, in a mold and next, a cast of the gland. This method is more precise than water displacement (Linzell, 1966). However, both are not commonly used any more, since they determine only external gross dimensions and do not measure individual glands independently (Fowler et al., 1990).

2.5.4 Palpation and udder measurements.

Palpation is a technique used by dairymen to identify and select prepubertal animals by touching the mammary gland as a tool for predicting future milk production. Although studies and bulletin have been published on palpation scores, it is a very subjective evaluation and less accurate as a method of mammary gland development (Esselburn, 2012). Udder morphology, on the contrary, is a moderately heritable characteristic, easier to measure and correlate with milk yield. Using a tape, caliper, t-square and a plumb line, it is possible to measure udder circumference; width, length and height; cistern height; teat angle, length and width. These quantitative measures are correlated to milk yield, milking time, percentage of milk fat and somatic cell count (McKusick et al., 1999), being used by genetic improvement programs as an important tool for animal selection. Although Capuco et al. (2012) suggest that teat length is correlated with estrogen levels and, consequently, with mammary stem cell growth, indicating indirect mammary epithelial cell development, Whitlock et al. (2002) do not recommend using teat length and elongation as external indicator of mammary development.

2.5.5 Magnetic resonance imaging

MRI is also a non-invasive technique for monitoring secretory tissue mass *in vivo*. Electromagnetic radiation excites hydrogen nuclei of water and lipid molecules, resulting in a detectable signal that varies its magnitude between tissues producing interpretable images of body slices. Although MRI is accurate, reliable, reproducible, with only 2% of error, and used for a variety of applications including human breast cancer (Fowler et al., 1990), it is very expensive, difficult to be conducted in large animals and demands animal restraint with sedation because of the long procedure time.

2.5.6 Ultrasound

US was first used for mammary gland evaluation in farm animal examination in 1967 using A-mode ultrasonography, an amplitude modulation that displays amplitude spikes at different heights. In 1986, B-mode ultrasonography, or brightness modulation in 2D map,

was used for mammary papilla evaluation (Fasulkov et al., 2010). However, it was only in the 1990s that B-mode ultrasound started to be commonly used as a veterinary diagnostic tool for evaluating and investigating many conditions, because of its easy and quick management, accuracy and good internal view of the examined area (Fasulkov, 2012).

This method allows, without causing any harm, evaluation of the mammary gland parenchyma health and congenital defects, teat abnormalities such as teat stenosis, teat wall proliferation and fibrosis, foreign bodies, milk stones, teat diameter and wall thickness, cistern measurements and pathological changes such as mastitis, hematomas, abscesses and tumor diagnostic (Fasulkov, 2012; Fasulkov et al., 2014).

It consists of a probe, connected by a cable to a console that emits and captures specific sound wave frequencies, converting them in electronic signals that are amplified and digitized as images in pixels, at a gray scale with 16, 64 or 256 shades of gray. The probes can vary in convex, sector or linear shape, differing in image shape, tissue depth visualization and image resolution (DesCôteaux, et al., 2010). Convex and sector probes provide a broader view of the scanned organ, showing a more favorable angle of beam incidence. However, extra efforts are needed to keep the US beam perpendicular, thus, giving a less precise structure depth. Linear probes, on the other hand, produce a straight US beam and an equal width to the image from the transducer size, to the deeper structures (Boezaart and Ihnatsenka, 2010).

The image frequency differs from 2 to 10 MHz, and is represented by the number of crystals oscillating per second – number of hertz, Hz, – and the crystal characteristics – type and thickness. Tissue penetration is inversely proportional to frequency, meaning that high ultrasound frequency reduces the scanned field depth but gives a better image resolution. At a lower frequency, best field depth can be achieved but with lower image resolution. The ultrasound machine calculates how long it takes for the sound to travel back to the probe, and this amplitude of the wave return provides different images, with different echogenicity because of the different densities of the body tissues. The image resolution also depends on physical pixel size on the display screen. So, the bigger the pixel size, the lower the resolution (DesCôteaux, et al., 2010).

Echogenicity is the ability to transmit US waves from the surrounding tissues. Dense tissues, like tendons and ligaments, have high reflection power, reflecting the ultrasound beam more intensely and producing brighter images with higher pixel values, known as hyperechoic images. Bones, besides their density, appear as an anechoic image with a bright hyperechoic rim because the US wave cannot penetrate them, making an acoustic shadow beyond them. Tissues with small reflection capacity and greater wave penetration, such as

cartilages, blood vessels and muscles, produce darker images with fewer pixels, known as hypoechoic images. Inasmuch, tissues or components that do not reflect any wave at all but absorb all, like air, water and most fat tissues, produce black images called anechoic images. Some tissues such as lymph nodes can appear with both image types, as anechoic or hypoechoic (Boezaart and Ihnatsenka, 2010).

The use of US for mammary internal structure examination was first reported in sheep by Ruberte et al. (1994). Next, it was used to examine heifer mammary glands at different life stages (Nishimura et al., 2010), and now it is being used to describe and examine quantity of parenchyma tissue *in vivo* in calves (Esselburn et al., 2015) and heifers (Albino et al., 2015), as a tool to predict future lactation performance potential based on size and growth characteristics of the mammary secretor tissue. The US images are compared with post-mortem sections of the mammary gland, describing the parenchyma structure as a hypo to anechoic image, surrounded by a hyperechoic mammary fat pad, providing scientific validation for US image as a viable tool for monitoring mammary growth. This could enable dairy producers to accurately select replacement dairy heifers, sheep or goats using a tool that is, on most farms, already being used for other diagnostics, reflecting future milk potential and positive return of the rearing investment phase.

2.6 Hypostasis

Distinguished daily weight gains affect mammary growth and goat body.

2.7 Main Objective

Study the implications of dairy goat nutrition on mammary gland development.

2.8 Specific objectives

Analyze the effect of different nutritional managements that provide average dairy gain of 90, 130, 170 and 210 grams on mammary gland and body morphometry.

Compare mammary gland morphometric measurements with teat and body biometry of female kids subjected to different nutritional planes.

Compare body allometry coefficients with mammary parenchyma area coefficient.

2.9 References

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CHAPTER 3. Effect of average daily gain, dry matter, crude protein and metabolizable energy intake on mammary gland development and body growth in Saanen kids

(Will be submitted to Animal Production Science)

3.1 Abstract

The aim of this experiment was to evaluate the effect of different average daily gains (ADG) and dry matter (DMI), crude protein (CPI) and metabolizable energy intake (MEI) on body and mammary development. Forty Saanen kids, 109.1 ± 21.4 days old and 12.9 ± 2.9 kg initial body weight, had their body and gland morphometry, and mammary parenchyma (PAR) measured until 30 days of pregnancy. They were separated in four groups with ten animals each, where each group received a different protein rate diet - 15, 17, 18 and 19% -, allowing different ADG (90, 130, 170 and 210 g day^{-1}) and nutrient consumption. Mammary morphometry was obtained weekly, and body morphometry and mammary PAR each fourteen days. For PAR access, B-mode ultrasound with a 5.0 MHz convex probe was used to examine in 2-dimension PAR area, perimeter and grey scale, of both glands, throughout the trial. During the trial, all variables increased. ADG had the major impact, influencing positively all body measurements ($p < 0.01$) and mammary teat growth ($p < 0.01$), PAR area ($p < 0.01$), PAR perimeter ($p < 0.001$). MEI influenced only rump length and chest width ($p < 0.05$) body growth, right PAR area and perimeter ($p < 0.05$). CPI and DMI did not influence body growth; however, protein affected considerably PAR development, increasing area, perimeter, and maximum grey value. Allometric coefficients of PAR area were low when compared to other body coefficients. However, it was possible to visualize a change of proportion in fifteen times for this variable, which means that PAR area grew more proportionally through time. Increasing levels of CPI, conversely from MEI, had an important and positive impact on body and mammary growth. Mammary growth could not be associated with any body growth patterns.

Key words: dairy goat, mammary gland, morphometry, ultrasound.¹

¹ **Abbreviation key:** AVG= Average Daily Gain, CPI= Crude Protein Intake, MEI= Metabolizable Energy Intake, DMI=Dry Matter Intake, WH=Withers Height, CP=Corporal Depth, RH=Rump Height, SH=Sternum Height, BL=Body Length, SL=Shoulder Length, LL=Leg Length, RL=Rump Length, LP=Leg Perimeter, SW=Shoulder Width, RW=Rump Width, CW=Chest Width, HG=Heart Girth, BG=Body Girth, BCS=Body Condition Score, RTL=Right Teat length, LTL=Left Teat Length, TD=Teat Distance, RTW=Right Teat Width, LTW=Left Teat Width, **Area R**= Mammary Gland ultrasound area right side, **Mean R**=Mean Grey value right side, **Mode R**=Most Frequently Occurring grey value right side, **Min R**= Minimum grey value right side, **Max R**= Maximum grey value right side, **Area L**= Mammary Gland ultrasound area left side, **Mean L**=Mean Grey value left side, **Mode L**=Most Frequently Occurring grey value left side, **Min L**= Minimum grey value left side, **Max L**= Maximum grey value left side, **PAR**= Mammary Parenchyma, **MFP**= Mammary fat pad

3.2 Introduction

The goat has been one of the first animals to be domesticated to provide milk, meat and hair. Nowadays, they are being used not only for these purpose, but also as a genetic engineering tool for the production of transgenic animals and, thus, contribute to human health. However, besides its importance in world history and transgenic milk production, few works have been carried out to study the impact of nutrition on mammary gland (MG) growth and milk performance in goats.

The MG suffers alterations in size, structure, composition and activity during all animal life (Akers and Denbow 2013). Its growth starts in the uterus, where all main structures are formed. After birth, the MG undergoes two periods of expressive growth: before puberty and during gestation (Sinha and Tucker 1969; Sheffield 1988). Although the main development occurs during pregnancy (Larson 1985; Akers 2002), studies have shown that nutrition changes at early age can affect mammary parenchyma (PAR), mammary fat pad (MFP) and future milk production.

Rearing replacement dairy animals is one of the most costly phases on a dairy farm, due to it being an unproductive period, taking up 15 to 20 percent of the overall expenses of milk production (Whitlock *et al.* 2002). For that reason, high plane nutrition is given to provide high gains and decrease age at sexual maturity. Calving earlier, the female starts to produce milk at a younger age, thus, shortening rearing time. However, this high energy and protein consumption, during the prepubertal period, may affect mammary secretory tissue development (Capuco *et al.* 1995), cause hormone changes and local grow factors (Bowden *et al.* 1995) and ,consequently, modify total milk production yield.

Some authors suggest that increasing weight gains impair MG development (Sejrsen *et al.* 1982; Bowden *et al.* 1995; Capuco *et al.* 1995; Davis Rincker *et al.* 2008), while others concluded that, despite growth differences, MG and milk production were not only not compromised (McFadden *et al.* 1990; Stelwagen and Grieve 1990; Whitlock *et al.* 2002; Meyer *et al.* 2006; Albino *et al.* 2015; Esselburn *et al.* 2015), but even increased (Brow *et al.* 2005). It is certain that high gains enhance MFP size (Capuco *et al.* 1995), increasing total gland dimensions (Bowden *et al.* 1995).

Since forty to seventy percent of total costs of rearing is due to feeding, achieving a balance between expenses – investment on rearing – and profits – milk production –, could positively impact the farm cash flow (Stelwagen and Grieve 1990), especially, if it is known whether high average daily gains (ADG) affect negatively, positively or do not influence MG

development at all. If it is established that the goat's best calving time is around one year of age, the nanny goat must gain more than 170 grams a day to achieve the minimum weight for breeding. For small ruminants, this is considered a high gain, impacting directly on body and mammary growth, as well as on rearing investments.

Since goats reach puberty three to five months earlier than European dairy cow breeds (Bowden *et al.* 1995), studies that measure rearing period and milk production can be carried out in a more reasonable time frame. Also, there are increasing demands to analyze the nutrition impact on goat's MG growth and its consequences, as is already known in case of dairy heifers and ewes. So, in this study, MG development was evaluated by ultrasound (US) technique, a low cost and non-invasive procedure that provides a real time visualization at a 2-dimensional gray scale (Nishimura *et al.* 2011), making it possible to follow organ development over time without tissue damage. Through US evaluation and body morphometry, we aimed to compare body and MG measurements with average daily gain (ADG), dry matter intake (DMI), crude protein intake (CPI) and metabolizable energy intake (MEI) of 40 dairy kids, submitted to four diets, with the aim of causing different gains. With allometric coefficients predicted by body measurements and PAR area, we also aimed to correlate the PAR growth pattern with a body pattern so as to help dairymen select the more productive animals at early age.

3.3 Material and methods

The Committee of Ethics and Animal Welfare of the Universidade Federal de Minas Gerais, under protocol no. 265/2013, previously approved all procedures on this experiment in accordance with animal welfare and ethical guidelines.

3.3.1 Animals, design and management

Forty Saanen female kids (109.1±21.4 days old; 12.9±2.9 kilos initial BW), borrowed from a single farm, were monitored until 30 days of pregnancy between February 2015 and December 2015, at the University experimental farm, in Igarapé, Minas Gerais, Brazil. The farm is located between the 20° south and 43° west parallel, in a savanna/Atlantic forest transition zone, with a tropical climate (Cfa according to Köppen and Geiger), and annual pluviometric precipitation of 1362 mm.

At the beginning of the trial, groups of ten kids were fed four diets, with the aim of obtaining different gains, according to the animals' initial BW, age and type of gestation. The diets predicted average daily gains (ADG) of 90, 130, 170 and 210 g/d, described as: low rate

gain (LG), medium-low rate gain (MLG), medium-high rate gain (MHG) and high rate gain (HG), respectively. Kids were housed and fed in pairs, in 2.0 x 3.8 m indoor pens, bedded with sawdust and provided with a wooden pallet of 1.2 x 1.1 m for environmental enrichment.

Fecal samples of all animals were collected to determine the number of oocysts per gram of feces at the beginning of the trial, revealing high *Eimeria* spp. infection. All kids were treated with 20mg/BW of toltrazuril (Baycox®, Bayer, Brazil) one week after their arrival and fifteen days after the first dosage. Clostridiosis and leptospirosis vaccination was also carried out in all animals and repeated after 30 days. Fecal samples were collected again revealing that treatment was inefficient. As an alternative treatment and parasite control, 1.5 mg/BW of monensin (Rumefort® 20%, Vallée SA, Brazil) was added to the diet seven weeks after the beginning of the trial until the end of the experiment. No problems were encountered subsequently after monensin addition.

Dietary treatment was mainly based on *cynodon* hay, soybean meal, corn meal and mineral salt in 30:70 roughage:concentrate proportion, allowing 10% of remnant. Diet composition is summarized in Table 3.1. Diets were formulated to meet nutrient requirements for the expected gains (NRC, 2007). Crude protein predicted was of 15, 17, 18 and 19% for LG, MLG, MHG and HG diets, respectively.

Table 3.1. Formulation and diet nutrient composition

	Diets ¹			
	LG	MLG	MHG	HG
Ingredients (%DM)				
Cynodon Hay	28.78	28.95	29.40	28.25
Corn Meal	53.90	47.60	43.64	42.17
Soybean Meal	13.90	19.54	22.49	25.05
Limestone	0.95	1.17	1.75	1.69
Sodium Bicarbonate	1.00	0.99	1.00	0.99
Dicalcium Phosphate	0.46	0.61	0.70	0.80
Minerals and vitamins premix ²	0.48	0.50	0.49	0.50
NaCl	0.48	0.50	0.49	0.50
Nutrients composition (DM basis)				
CP, %	14.51	17.53	18.15	18.75
ME, Mcal	1713	1974	1994	1686
NDF, %	17.68	19.52	18.44	16.84
ADF, %	5.89	6.78	6.30	5.80
NSC, % ³	55.62	49.45	49.45	49.85

¹Diets: LG= Low Gain, MLG= Medium Low Gain, MHG= Medium High Gain, HG= High Gain ²One Kilogram contained 135.000,00 IU of Vitamin A, 68.000,00 UI of Vitamin D3, 450,00 UI of Vitamin E, 240,00 g of Ca, 71,00 g of P, 28,20 g of K, 20,00 g of S, 20,00 g of Mg, 400,00 mg of Cu, 30,00 mg of Co, 10,00 mg of Cr, 250,00 mg of Fe, 40,00 mg of I, 1.350,00 mg of Mn, 15,00 mg of Se, 1.700,00 mg of Zn, 710,00 mg of Fl (max.).

³Non-Structural carbohydrates.

Animals were fed twice a day, in equal meals, at 0800 and 1600 h, and had *ad libitum* access to water. Feed intake was recorded daily and adjusted weekly according animal

weight. To calculate individual feed intake, the pen dry matter intake was divided by the weight sum of the animals in that enclosure, and then multiplied by each animal's weight. The result was transformed in digestible crude protein and metabolizable energy intake obtained by apparent digestibility trial (Bastos *et al.* 2016 – unpublished data).

3.3.2 Reproduction

When animals reached 32 kg BW, a nine-day estrous induction and synchronization hormonal protocol was carried out. The protocol consisted in administrating 5 mg of dinoprost tromethamine (Lutalyse®, Pfizer, Guarulhos, Brazil) in the latero-vulvar area, and placing an intravaginal sponge impregnated with 60 mg of medroxyprogesterone (Progespon®, Zoetis/Syntex, Buenos Aires, Argentina). An antibiotic with corticoid (Terracortril®, Zoetis/Pfizer, Guarulhos, SP, Brazil) was sprayed on the sponge before placing it into the animal to avoid vaginitis. Eight days after the beginning of the protocol, another dose of 5mg of dinoprost tromethamine (Lutalyse®, Pfizer, Guarulhos, SP, Brazil) was injected in the latero-vulvar area, and 200 IU of equine chorionic gonadotropin (Novormon®, Zoetis/Syntex, Buenos Aires, Argentina) in the hind muscle. Twenty-four hours after the injections, the sponge was removed and the goats were brought to another shed where two selected fertile bucks were housed. They were placed in a pen in front of the bucks when estrus was observed. Females were considered in estrus when they allowed to be mounted. Mating occurred every 12 hours after the onset of estrus to the end, always at 0700 and 1900 h. No more than three goats were synchronized to mate with the same buck on the same day, as there was a three-day interval between one mating group and the other. After 24 days since the last mating, a B-mode transrectal ultrasonographic scanner with a 5 MHz linear transducer (Chison D600 Vet®, Chison, Jiang Su Province, China) was performed to verify pregnancy. A PVC tube was taped to the transducer to help with its manipulation. Goats were maintained in standing position, fecal pellets were removed from the rectum and 10 mL of ultrasound gel (Renygel®, RenyLab, Barbacena, Brazil) was placed into the anus with a syringe. When a vesicle and a heart beating were detected, pregnancy was confirmed, if not, ultrasound was repeated after seven days. When the goat did not become pregnant, the doe was submitted to the same hormonal protocol again. If after three attempts the doe did not become pregnant, her data was used until that date.

3.3.3 Body measurements

Animals were weighed weekly, in the morning before feeding. Morphometric measurements and body condition score (BCS) were taken each fourteen days. Withers height

(WH), rump height (RH), sternum height (SH), body length (BL), shoulder length (SL), shoulder width (SW), leg length (LL), rump length (RL), rump width (RW), chest width (CW), were measured with an hypometer, and leg perimeter (LP), heart girth (HG) and body girth (BG) were measured with a tape. For BCS was used a scale of 0 (very thin) to 5 (very fat).

WH evaluated the distance between the highest part of the back, from the base of the neck above the shoulders to the ground. RH was evaluated as the distance between the ilium and the ground, while SH was considered the distance between the sternum and the ground. BL evaluated the distance between the cranial border of the scapula and the femoral head. SL and SW were the distances between the dorsal and ventral extremities, and cranial and caudal middle borders of the scapula. LL and LP were the distances measured between the cranial and caudal extremities of the femur, and the middle of leg width. RL and RW were the distances between ilium and ischium, and between ilia. CW was measured between the cranial borders of the scapula. HG and BW were measured around the thorax behind the scapula, and behind the ribs on the umbilicus. All measurements were taken by the same researcher, with the animal in standing position, on a straight floor.

3.3.4 Mammary gland ultrasound and measurements

Goats were evaluated, always by the same researcher, for mammary growth by weekly measuring teat length (TL), teat width (TW) and teat distance (TD) and biweekly mammary gland ultrasound. Mammary secretion was also inspected since some animals started producing a whitish substance.

Teat measurements were taken with a millimeter ruler. TL was measured from the teat insertion into the udder to its distal part. TW was determined by the placement of the ruler on the middle of the teat making a 90° angle. TD considered the length between teats insertion.

Goat's PAR and MFP of both glands were examined with a real-time B-mode ultrasound (Chison D600 Vet®, Chison, Jiang Su Province, China) connected to a 5.0 MHz probe, until 30 days of pregnancy. Two images of each gland were saved in bitmap (BMP) format and captured in a pen drive for further analysis. Udders were not clipped of excess hair, but ultrasound gel (Renygel®, RenyLab, Barbacena, Brazil) was placed in the analyzed area to enhance contact with the probe and help image quality. At each exam, four animals were placed in a locking yoke so they could be restrained and put in standing position without sedation, not altering the gland format.

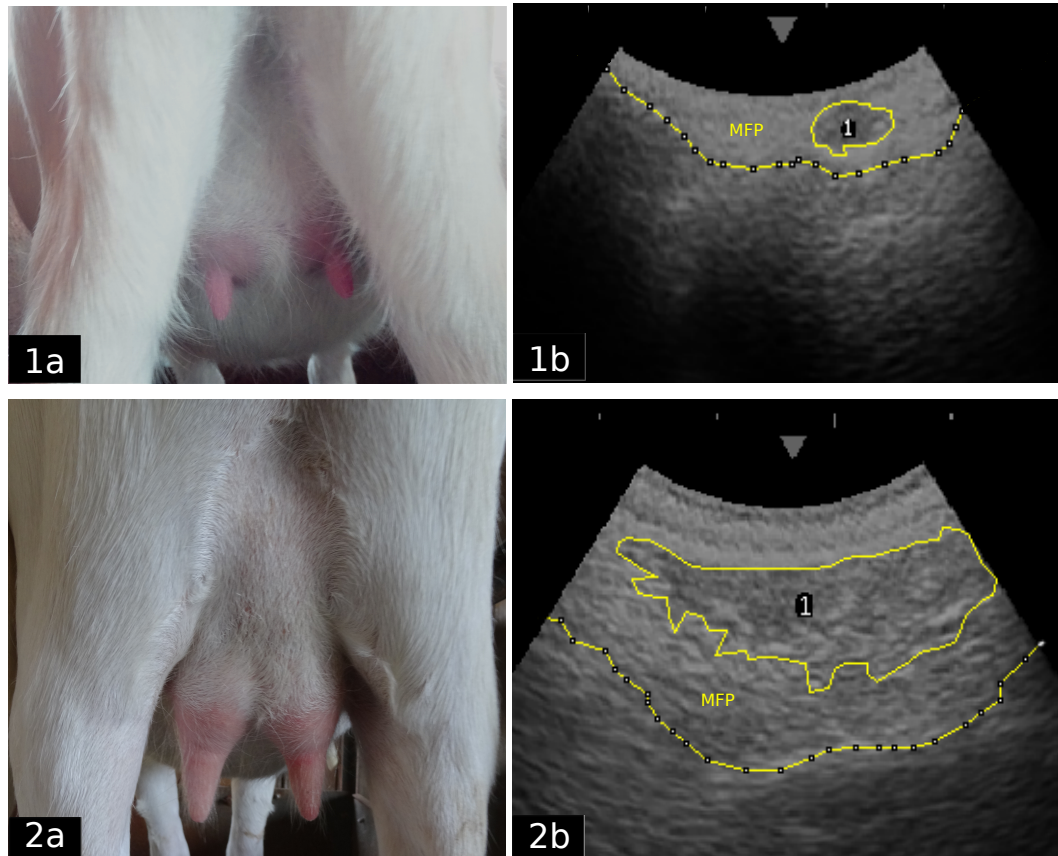


Figure 3.1. Appearance of udders at the beginning and end of the trial (1a-2a), ultrasound images (1b-2b) showing the parenchyma area (1), the mammary fat pad (MFP) separated from the body wall with a dashed line. Grey tick marks on the top of the ultrasound screen are spaced 1 cm apart.

For mammary gland analysis, studies carried out by Ruberte *et al.* (1994); Nishimura *et al.* (2011) and Esselburn *et al.* (2015) were taken as reference. The probe was placed on the caudal side of teat insertion, making a 45° angle to visualize PAR and MFP. Minimum pressure was applied to avoid organ shape and size variation. For image capture, PAR structure and echogenicity were considered. When animals were young, PAR appeared as a hypoechoic feature shown as a round or oval structure surrounded by a hyperechoic area, the MFP. With their growth, the hypoechoic area lost its clear definitions and spread extensively and in an irregular shape from a medium echogenicity to a hypoechoic, area (Figure 3.1). PAR and MFP could be distinguished on the body wall, gastrointestinal tract and other components throughout the trial. The choice was made to evaluate until 30 days of pregnancy since PAR and MFP could be visualized in the same ultrasound image, so the number of fetuses would not significantly interfere on mammary growth.

3.3.5 Digital Image Processing

The saved mammary gland image was digitalized and transformed in pixels. The value of these pixels varied in a 256 grey scale from 0, black, to 255, white. Dense structures that

better reflect the ultrasound wave have greater pixel values as well as more white pictures, called hyperechoic images. These structures could be bones, ligaments or fat tissues. When there is no or little reflection, the pixel values are low and the images darker, producing a hypoechoic picture, such as muscles and mammary gland parenchyma. Pixels were converted into centimeters using the ultrasound marks to define how many pixels there were in one centimeter. The scale was established to have 53 pixels in one centimeter. That made it possible to convert the square pixel area into square centimeter area, permitting to verify mammary gland size. Because fat is visualized as a hyperechoic area and parenchyma as a hypoechoic area, it was supposed that a whiter contrast image, contained more fat, and a darker contrast image, more parenchyma. Images were analyzed on ImageJ (National Institutes of Health; National Research Council (U.S.) 2007; <http://imagej.nih.gov/ij/index.html>) to determine the PAR area, in cm², and the perimeter, in cm, using the cursor to demark the studied area as well as the grey intensity value. Mean, mode and median grey value were used to verify whether there was more parenchyma or fat in the analyzed section.

3.3.6 Statistical Analyses

Three animals, on the LG, LMG and HG diets, died early in the study. Their data was disregarded for statistical analyses. Autopsy revealed pneumonia, dehydration, anemia, diarrhea and intestinal infection. Animal death was probably caused by coccidiosis associated with pneumonia. Four animals started to produce a whitish substance in large amounts before their pregnancy, interfering in the image analyses. Therefore, their ultrasound data was considered as long as image quality or mammary size were not compromised, the other measurements were not discharged. Five goats did not become pregnant after three attempts, so data was evaluated 30 days after the last hormonal protocol.

A simple linear model was fit for each animal, regressing their MG and body measurements on days of the trial to obtain individual rates and slopes. These linear mixed effect models were then adjusted to the data, estimated by restricted maximum likelihood (REML). The rates obtained with the linear model were the dependent variables, analyzed by multiple regressions. ADG, DMI, CPI and MEI were the independent predictors following the model:

$$Y = \beta_1X_1 + \beta_2X_2 + \beta_3X_3 + \beta_4X_4$$

Where Y is the dependent variable; $\beta_1, \beta_2, \beta_3$ and β_4 are the predicted regression coefficients; X_1 is the ADG value, X_2 the DMI value, X_3 the CPI value and X_4 the MEI

value. Model adjustment was verified by examining the coefficient of determination.

Allometric studies were also conducted in this paper by performing multivariate analyses for each body and mammary gland measurements. A principal component analysis (PCA) based on the covariance matrix was computed using the natural logarithm of each variable, such as the standard errors. To evaluate the hypothesis of isometry, when all body and mammary glands have similar allometric coefficients, a chi-square test with degree of freedom equal to the number of the parts being tested (p) minus one was performed (*i.e.*, $df = p - 1$). The trial was also divided in six phases, where for each one, a multivariate analysis was carried out, to compare whether the coefficients were stable among all phases or not as well as body and mammary growth. Analyses were performed using RStudio environment (R Development Core Team 2015).

3.4. Results

3.4.1 Body Growth

All body measurements were altered by the ADG ($p < 0.002$), except for BCS ($p > 0.06$). DMI did not influence any variable, but had a tendency on CD and RL ($p < 0.09$). MEI altered RL and LP ($p < 0.05$), and had a tendency on WH, LL and BCS ($p < 0.09$). Conversely, CPI did not impact any body measurements at all, but had a tendency on BG ($p < 0.07$; Table 3.3).

The average DMI throughout the trial was of 168 ± 39.53 kg, with a MEI and CPI of 311.54 ± 80.57 Mcal and 28.8 ± 6.26 kg respectively, allowing an ADG of 0.14 ± 0.03 Kg (Table 3.2). Body parts growth varied at an average rate of 0.02 cm/day to 0.13 ± 0.02 cm day⁻¹, with minimum and maximum growth of 0.01 and 0.19 cm day⁻¹ (Table 3.6). WH, RH, BL, PP, HG and BG had the biggest growth rates, with meant daily growth above 0.08 ± 0.2 cm a day, reaching more than 0.12 cm/day. CD and SL had an intermediate growth with 0.05 ± 0.01 and 0.04 ± 0.01 cm day⁻¹ rate respectively, and LL, RL, SW, RW and CW had the smallest rates, with growth levels below 0.03 ± 0.01 cm/day. BCS remained stable throughout the trial (Table 3.6).

Table 3.2. Average daily gain, Total Dry Matter intake, Total Metabolizable Energy intake and Total Crude protein intake of Saanen goats

Variables	Mean	Standard deviation	Median	Min	Max	se
ADG (kg/day)	0.14	0.03	0.13	0.09	0.20	0.00
DMI (Kg)	168.31	39.53	152.52	113.33	265.86	6.50
MEI (Mcal)	311.54	80.57	285.33	191.08	522.94	13.24
CPI (Kg)	28.80	6.26	27.46	19.61	45.97	1.03

Table 3.3. Dependent variables and standard deviation values of body morphometric measures of Saanen goats

	ADG	MEI	CPI	DMI
WH	0.6282273±0.07254767***	-0.0002077±0.00011716 [§]	0.0005978±0.00117775	0.0002742±0.00018369
RH	0.6699580±0.08146660***	-0.0000523±0.00013157	-0.0000122±0.00132255	0.0000625±0.00020627
CD	0.3559391±0.04361330***	-0.0001137±0.00007044	0.0000625±0.00070803	0.0001941±0.00011043\$
BL	0.5910626±0.07324019***	-0.0001751±0.00011828	0.0009852±0.00118900	0.0001604±0.00018544
SL	0.29029692±0.03453827***	0.00001514±0.00005578	-0.00014673±0.00056070	0.00001193±0.00008745
LL	0.18487363±0.028587545***	-0.00008153±0.000046169 [§]	0.00074400±0.000464096	0.00005132±0.000072383
RL	0.23822101±0.03638043***	-0.00014733±0.00005875*	0.00056082±0.00059061	0.00017452±0.00009211 [§]
LP	0.7760157±0.08077887***	-0.0001786±0.00013046	0.0007390±0.00131138	0.0001352±0.00020453
SW	0.09018147±0.027439105**	-0.00001521±0.000044314	0.00010542±0.000445452	0.00006149±0.000069475
RW	0.25091915±0.022238236***	0.00000640±0.000035915	-0.00020885±0.000361020	0.00000253±0.000056307
CW	0.26472782±0.024835700***	-0.00008180±0.000040110*	0.00042626±0.000403188	0.00006500±0.000062883
HG	0.9760473±0.07113903***	-0.0000161±0.00011489	-0.0004158±0.00115489	0.0000659±0.00018012
BG	0.8453309±0.11944883***	0.0002859±0.00019291	-0.0036371±0.00193916\$	0.0001285±0.00030244
BCS	0.026936619±0.013951784 [§]	-0.000041434±0.00002253 [§]	0.000322475±0.000226496	0.000020934±0.000035325

AVG= Average Daily Gain, CPI= Crude Protein Intake, MEI= Metabolizable Energy Intake, DMI=Dry Matter Intake, WH=Withers Height, CP=Corporal Depth, RH=Rump Height, SH=Sternum Height, BL=Body Length, SL=Shoulder Length, LL=Leg Length, RL=Rump Length, LP=Leg Perimeter, SW=Shoulder Width, RW=Rump Width, CW=Chest Width, HG=Heart Girth, BG=Body Girth, BCS=Body Condition Score, \$P<0.10 * P<0.05 **P<0.01 ***P<0.001

3.4.2 Mammary biometry

Mammary exterior measurements increased over time and had a growth rate about 0.01 cm/day for RTL, LTL and TD, with maximum growth of teat length of 0.02 cm (Table 3.6). RTW and LTW growth rates were very small throughout the trial and were not affected by any fixed effect. Only DMI had a tendency to affect teat width (p<0.07). Similarly, TD was also unaffected by the gain and diet intake, but had a tendency for ADG (p<0.10). RTL and LTL were affected by ADG (p<0.003), but not by the intake (Table 3.4).

Table 3.4. Dependent variables and standard deviation values of mammary gland morphometric measurements of Saanen goats

	ADG	MEI	CPI	DMI
RTL	0.08633408±0.018160643***	-0.00001838±0.000029329	0.00001899±0.000294824	0.00003277±0.000045982
LTL	0.07166630±0.022074084**	-0.00003256±0.000035650	0.00010747±0.000358355	0.00005508±0.000055891
TD	0.027340668±0.015925912 [§]	0.000031299±0.000025720	-0.000389862±0.000258545	0.000030869±0.000040324
RTW	0.009497842±0.005938507	-0.000011792±0.000009591	0.000006219±0.000096407	0.000029788±0.000015036 [§]
LTW	0.0019617516±0.007392652	-0.0000067761±0.00001194	-0.0000420723±0.00012001	0.0000351353±0.000018718 [§]

AVG= Average Daily Gain, CPI= Crude Protein Intake, MEI= Metabolizable Energy Intake, DMI=Dry Matter Intake, RTL=Right Teat length, LTL=Left Teat Length, TD=Teat Distance, RTW=Right Teat Width, LTW=Left Teat Width. \$ P<0.10 * P<0.05 **P<0.01 ***P<0.001

3.4.3 Mammary ultrasound

Ultrasound showed to be a useful and reliable tool for mammary PAR external monitoring in dairy kids, as has been shown previously for dairy heifers (Nishimura *et al.* 2011; Esselburn *et al.* 2015). Differences in MFP and PAR bright and dark contrasts allowed image evaluation throughout the experiment. PAR area was affected, in both glands, by ADG ($p < 0.004$) and CPI ($p < 0.008$, Table 3.5) with a growth rate of $0.03 \pm 0.01 \text{ cm}^2 \text{ day}^{-1}$ and $0.04 \pm 0.01 \text{ cm}^2 \text{ day}^{-1}$ for right and left glands, respectively (Table 3.6). Perimeter was similarly impacted by the same effects ($p < 0.003$, Table 3.5), with a growth rate of $0.05 \pm 0.01 \text{ cm day}^{-1}$ (Table 3.6). Conversely, MEI affected only the right parenchyma area and perimeter ($p < 0.05$).

Since the PAR grows over the fat pad, and it is presumed that excess fattening can impair mammary growth, grey value measurements were used in both glands as supposed values of predicted quantity of fat in the parenchyma. When grey values were closer to 255, it was believed that PAR had higher fat content; when values were closer to 0, the opposite, less fat content and more PAR was observed. MEAN corresponded for the mean grey value in the evaluated area, MODE for the most frequently occurring grey value and MAX and MIN for the maximum and minimum grey values. None of these values were affected by DMI. MEI affected only the MAX values ($p < 0.01$). CPI, on the contrary, affected more variables such as MAX on both glands ($p < 0.02$), and MEAN and MODE for the left gland ($p < 0.008$ and $p < 0.02$, respectively). The MODE value decreased throughout the experiment evidencing that most frequently the grey value diminished on the mammary gland, showing a darker area. ADG affected MIN on the right side ($p < 0.03$) and left side ($p < 0.007$, Table 3.5).

Table 3.5. Dependent variables and standard deviation values of mammary gland ultrasound analyses of Saanen goats

	ADG	MEI	CPI	DMI
RTL	0.08633408±0.018160643***	-0.00001838±0.000029329	0.00001899±0.000294824	0.00003277±0.000045982
LTL	0.07166630±0.022074084**	-0.00003256±0.000035650	0.00010747±0.000358355	0.00005508±0.000055891
TD	0.027340668±0.015925912 ^S	0.000031299±0.000025720	-0.000389862±0.000258545	0.000030869±0.000040324
RTW	0.009497842±0.005938507	-0.000011792±0.000009591	0.000006219±0.000096407	0.000029788±0.000015036 ^S
LTW	0.0019617516±0.007392652	-0.0000067761±0.00001194	-0.0000420723±0.00012001	0.0000351353±0.000018718 ^S
Area R	0.17865849±0.05752269**	-0.00019343±0.00009290*	0.00299736±0.00093384**	-0.00009266±0.00014565
Mean R	-0.13123771±0.3184630	0.00008140±0.0005143	0.00254901±0.0051700	-0.00079166±0.0008063
Mode R	0.19128377±0.4759070	0.00073865±0.0007686	-0.00051485±0.0077260	-0.00177435±0.0012050
Min R	-1.0365951±0.4684869*	0.0008546±0.0007566	-0.0111452±0.0076055	-0.0002765±0.0011862
Max R	0.3222579±0.19637124	-0.0008765±0.00031714**	0.0077329±0.00318793*	0.0003328±0.00049721
Perim R	0.22866925±0.04974787***	-0.00018456±0.00008034*	0.00291258±0.00080762***	-0.00006819±0.00012596
Area L	0.24259932±0.06326557***	-0.00008505±0.00010217	0.00186734±0.00102707 ^S	-0.00014262±0.00016019
Mean L	-0.5985611±0.3525375 ^S	-0.0007549±0.0005693	0.0160990±0.0057232**	-0.0011852±0.0008926
Mode L	-1.0674531±0.6099840 ^S	-0.0008501±0.0009851	0.0247377±0.0099026*	-0.0022316±0.0015445
Min L	-1.4941820±0.5165069**	0.0001800±0.0008342	-0.0008078±0.0083851	-0.0004288±0.0013078
Max L	0.17945936±0.18218305	-0.00082621±0.00029423**	0.01162833±0.00295760***	-0.00034032±0.00046128
Perim L	0.26624313±0.05474199***	-0.00012541±0.00008841	0.00207207±0.00088869*	-0.00006149±0.00013861

AVG= Average Daily Gain, CPI= Crude Protein Intake, MEI= Metabolizable Energy Intake, DMI=Dry Matter Intake, Area R= Mammary Gland ultrasound area right side, Mean R=Mean Grey value right side, Mode R=Most Frequently Occurring grey value right side, Min R= Minimum grey value right side, Max R= Maximum grey value right side, Perim R = Mammary Gland ultrasound perimeter right side, Area L= Mammary Gland ultrasound area left side, Mean L=Mean Grey value left side, Mode L=Most Frequently Occurring grey value left side, Min L= Minimum grey value left side, Max L= Maximum grey value left side, Perim L = Mammary Gland ultrasound perimeter left side. S P<0.10 * P<0.05 **P<0.01 ***P<0.001

Table 3.6. Daily growth rates of body and mammary gland morphometry and mammary parenchyma of 37 Saanen goats

Variables	Mean	Standard deviation	Median	Min	Max	Se
WH (cm day ⁻¹)	9x10 ⁻²	0.02	0.08	0.04	0.12	0.00
HI (cm day ⁻¹)	9x10 ⁻²	0.02	0.09	0.04	0.12	0.00
CD (cm day ⁻¹)	5x10 ⁻²	0.01	0.05	0.03	0.08	0.00
BL (cm day ⁻¹)	8x10 ⁻²	0.02	0.08	0.05	0.13	0.00
SL (cm day ⁻¹)	4x10 ⁻²	0.01	0.04	0.03	0.06	0.00
LL (cm day ⁻¹)	3x10 ⁻²	0.01	0.03	0.02	0.05	0.00
RL (cm day ⁻¹)	3x10 ⁻²	0.01	0.03	0.02	0.05	0.00
LP (cm day ⁻¹)	1x10 ⁻²	0.02	0.10	0.06	0.13	0.00
SW (cm day ⁻¹)	2x10 ⁻²	0.00	0.02	0.01	0.03	0.00
RW (cm day ⁻¹)	3x10 ⁻²	0.01	0.03	0.02	0.04	0.00
CW (cm/day)	3x10 ⁻²	0.01	0.03	0.02	0.06	0.00
HG (cm day ⁻¹)	1.3x10 ⁻¹	0.02	0.13	0.08	0.18	0.00
BG(cm day ⁻¹)	1.2x10 ⁻¹	0.03	0.12	0.08	0.19	0.00
BCS (cm day ⁻¹)	0	0.00	0.00	0.00	0.01	0.00
RTL (cm day ⁻¹)	1x10 ⁻²	0.00	0.01	0.00	0.02	0.00
LTL (cm day ⁻¹)	1x10 ⁻²	0.00	0.01	0.00	0.02	0.00
TD (cm day ⁻¹)	1x10 ⁻²	0.00	0.01	0.00	0.01	0.00
RTW (cm day ⁻¹)	0	0.00	0.00	0.00	0.00	0.00
LTW (cm day ⁻¹)	0	0.00	0.00	0.00	0.01	0.00
Area R (cm ² day ⁻¹)	3x10 ⁻²	0.01	0.03	0.01	0.07	0.00
Mean R (grey value)	-5x10 ⁻²	0.06	-0.05	-0.22	0.06	1x10 ⁻²
Mode R (grey value)	-6x10 ⁻²	0.09	-0.03	-0.27	0.10	1x10 ⁻²
Min R (grey value)	-2.4x10 ⁻¹	0.09	-0.23	-0.52	-0.09	1x10 ⁻²
Max R (grey value)	5x10 ⁻²	0.04	0.04	-0.03	0.14	1x10 ⁻²
Perim R (cm day ⁻¹)	5x10 ⁻²	0.01	0.05	0.03	0.07	0.00
Area L (cm ² day ⁻¹)	4x10 ⁻²	0.01	0.03	0.02	0.08	0.00
Mean L (grey value)	-5x10 ⁻²	0.07	-0.05	-0.19	0.11	1x10 ⁻²
Mode L (grey value)	-7x10 ⁻²	0.12	-0.06	-0.34	0.16	2x10 ⁻²
Min L (grey value)	-2.4x10 ⁻¹	0.10	-0.26	-0.45	-0.01	2x10 ⁻²
Max L (grey value)	-4x10 ⁻²	0.04	0.05	-0.04	0.16	1x10 ⁻²
Perim L (cm day ⁻¹)	5x10 ⁻²	0.01	0.05	0.03	0.07	0.00

AVG= Average Daily Gain, CPI= Crude Protein Intake, MEI= Metabolizable Energy Intake, DMI=Dry Matter Intake, WH=Withers Height, CP=Corporal Depth, RH=Rump Height, SH=Sternum Height, BL=Body Length, SL=Shoulder Length, LL=Leg Length, RL=Rump Length, LP=Leg Perimeter, SW=Shoulder Width, RW=Rump Width, CW=Chest Width, HG=Heart Girth, BG=Body Girth, BCS=Body Condition Score, RTL=Right Teat length, LTL=Left Teat Length, TD=Teat Distance, RTW=Right Teat Width, LTW=Left Teat Width, Area R= Mammary Gland ultrasound area right side, Mean R=Mean Grey value right side, Mode R=Most Frequently Occurring grey value right side, Min R= Minimum grey value right side, Max R= Maximum grey value right side, Area L= Mammary Gland ultrasound area left side, Mean L=Mean Grey value left side, Mode L=Most Frequently Occurring grey value left side, Min L= Minimum grey value left side, Max L= Maximum grey value left side.

3.4.4 Allometric Growth

The trial days were divided into six equal phases to compare body and mammary tissue growth through time, and these values were also used in one only period that included the whole trial. The coefficients show how much that variable changed, taking the whole body as a proportion. So, as all measurements increased over time, the coefficients showed how much that variable grew. In this way, correlations will be high since all body and mammary parts grow. The allometric test demonstrated that body and mammary parts do not have an isometric growth, which means that they do grow but not at the same intensity. If they did, all coefficients should be equal to 0.2234. This value can be found by the square root of p , i.e., $p^{-0.5}$. Since 20 variables were taken in this study, the square root of p is $20^{-0.5} = 0.2234$.

Observing only the last column of Table 3.5 that considers growth during the whole trial period, Area R and Area L had very similar coefficients which meant that they grew in similar proportions (0.1094 and 0.1139). Similarly to PAR area, SL, LP, RL and LL also showed an intermediate growth rate. WH, RH, BL, LP, HG and BG, on the other hand, had the highest growth indices (>0.30), showing more intense growth during the trial. SW and all udders morphometric measurements – RTL, LTL, TD, RTW and LTW – presented the lowest rates (<0.044, Table 3.7).

However, if we observe the trial divided in phases it is possible to verify whether the coefficients were constant through time. Most coefficients were constant, but the PAR area had its coefficients increasing through the phases, revealing a more intense growth. When comparing the PAR area and WH coefficients in the first (0.315/0.01) and last phase (0.276/0.13), for example, it would be possible to visualize a change of proportion in fifteen times, that is, the PAR area grew more proportionally through time.

Table 3.7. Main Allometric components and their standard error for body measurements and PAR area

	Phase 1	Phase 2	Phase 3	Phase 4	Phase 5	Phase 6	All Period
WH	0.315±0.018	0.363±0.016	0.351±0.019	0.35±0.014	0.34±0.016	0.276±0.028	0.3229±0.0037
RH	0.367±0.02	0.377±0.016	0.354±0.019	0.35±0.017	0.339±0.017	0.296±0.029	0.3262±0.0338
CD	0.127±0.016	0.174±0.012	0.187±0.011	0.151±0.013	0.165±0.012	0.137±0.013	0.1754±0.0026
BL	0.291±0.019	0.327±0.016	0.301±0.016	0.283±0.016	0.293±0.015	0.249±0.025	0.3034±0.0036
SL	0.104±0.009	0.102±0.007	0.115±0.008	0.108±0.009	0.112±0.008	0.109±0.012	0.1489±0.0021
LL	0.09±0.01	0.106±0.007	0.097±0.007	0.09±0.008	0.087±0.007	0.102±0.01	0.1071±0.0017
RL	0.084±0.012	0.098±0.01	0.092±0.009	0.089±0.009	0.095±0.007	0.103±0.011	0.1117±0.0020
LP	0.123±0.017	0.195±0.027	0.241±0.013	0.238±0.012	0.211±0.013	0.204±0.019	0.3204±0.0050
SW	0.036±0.008	0.059±0.006	0.059±0.005	0.041±0.006	0.055±0.005	0.043±0.008	0.0754±0.0015
RW	0.078±0.007	0.085±0.007	0.102±0.005	0.115±0.005	0.116±0.005	0.115±0.007	0.1104±0.0013
CW	0.085±0.01	0.095±0.008	0.106±0.007	0.116±0.007	0.126±0.006	0.114±0.011	0.1216±0.0017
HG	0.425±0.02	0.431±0.014	0.457±0.014	0.449±0.013	0.477±0.014	0.514±0.017	0.4681±0.0032
BG	0.652±0.033	0.555±0.029	0.543±0.033	0.571±0.024	0.549±0.021	0.593±0.029	0.4863±0.0070
RTL	0.03±0.005	0.045±0.005	0.049±0.005	0.041±0.006	0.032±0.005	0.014±0.007	0.0444±0.0012
LTL	0.033±0.005	0.043±0.005	0.049±0.005	0.039±0.006	0.027±0.006	0.013±0.008	0.0439±0.0014
TD	0.029	0.022	0.007±0.006	0.016±0.005	0.019±0.005	0.029±0.009	0.0217±0.0013
RTW	0.012±0.002	0.014±0.002	0.018±0.002	0.014±0.002	0.009±0.002	0.002±0.002	0.0106±0.0004
LTW	0.011±0.002	0.015±0.002	0.017±0.002	0.015±0.002	0.009±0.002	0.004±0.002	0.0104±0.0004
Area R	0.01±0.004	0.025±0.005	0.036±0.008	0.063±0.012	0.099±0.014	0.13±0.025	0.1094±0.0032
Area L	0.012±0.004	0.025±0.007	0.036±0.009	0.06±0.012	0.099±0.014	0.137±0.028	0.1139±0.0034
Inertia	80.52	82.49	83.90	85.23	80.75	74.80	91.44

WH=Withers Height, RH=Rump Height, CD=Corporal Depth, SH=Sternum Height, BL=Body Length, SL=Shoulder Length, LL=Leg Length, RL=Rump Length, LP=Leg Perimeter, SW=Shoulder Width, RW=Rump Width, CW=Chest Width, HG=Heart Girth, BG=Body Girth, RTL=Right Teat length, LTL=Left Teat Length, TD=Teat Distance, RTW=Right Teat Width, LTW=Left Teat Width, Area R= Mammary Gland ultrasound area right side, Area L= Mammary Gland ultrasound area left side.

3.5. Discussion

Although many studies have concluded that ADG has the most impactful effect on body growth, mammary gland and future milk production (Sejrsen *et al.* 1982; McCann *et al.* 1989; Stelwagen and Grieve 1990; Bowden *et al.* 1995; Abeni *et al.* 2000), the nutrient intake

level also has an important significance on animal development (Whitlock *et al.* 2002; Brow *et al.* 2005; Davis Rincker *et al.* 2008; Albino *et al.* 2015). Unfortunately, due to a lack of ADG and nutrition impact on the goat's body and mammary growth, it is necessary to use works on heifers and sheep to make comparisons.

In this study a positive impact of increasing ADG for all body measurements was found ($p < 0.002$), especially for WH and RH ($p < 0.000$) that well represent animal growth. Stelwagen and Grieve (1990), on the contrary, in an experiment with forty-one Holstein heifers, with different ADG, found that plane nutrition had no effect on WH and RH at the end of the trial, indicating that even the low plane of nutrition and low ADG were sufficient for skeletal and body development. However, at puberty, WH decreased linearly with the increase of the nutrition status, indicating that a compensatory growth might have occurred. Additionally, Whitlock *et al.* (2002) also did not find differences in WH, BCS and pelvic area for Holstein heifers reared with different dietary protein. However, it must be considered that the used diet also did not affect ADG. So, contemplating this aspect, their findings endorse this study, where CPI did not affect WH ($P < 0.61$), BCS ($p < 0.16$), RL ($p < 0.35$) and RW ($p < 0.57$). MEI but, on the other hand, decreased RL and CW ($p < 0.05$).

Abeni *et al.* (2000), conducting an experiment with Italian Holstein heifers during pre and postpubertal period, found that accelerated gains promoted by protein and energy increasing ratio concentrations, augmented WH, HG and BCS. BCS increased, especially during the postpubertal period, since in early development, before puberty onset, energy and protein intake are converted into body mass, thus, permitting maximum growth potential. After sexual maturity, nutrient deposition becomes less efficient and its excess is converted into lipids or excreted. This explains the higher BCS in these heifers, as in our experiment, since we followed body growth until pregnancy. Nevertheless in both, no statistical differences were found. We observed that from the beginning to the end of the trial, all animals had their BCS increased and statistically, a tendency for ADG increase ($p < 0.06$) and MEI decrease ($p < 0.07$) of this parameter was observed. Though the exact time when the goats reached puberty was not determined, it was possible to observe the changing of the type of growing through time, observing an increased subcutaneous fat deposition. Additionally, since HG is related to ADG and consequently to the animal weight, also used as a method of tape weight, close relation was found ($p < 0.000$), mainly due to lipid deposition on this specific area.

These same findings were reported by Villeneuve *et al.* (2010), in a trial with 72 Dorset ewe lambs, divided in three prepubertal diets given for 75 days. WH ($p < 0.001$), HG

($p < 0.0001$) and BCS ($p < 0.001$) were greater for *ad libitum* diet lambs, as were also their ADG after 75 days of treatment, corroborating with our results. Inasmuch, after that period, restricted lambs achieved near full compensatory growth, equating the previous differences, consuming less DMI during the rearing phase, being economically more efficient. Supporting those findings, working with Dorset and Suffolk ewe lambs McCann *et al.* (1989), despite differences in ADG and BCS, did not find variation on WH among treatments before puberty, however, after reaching maturity, higher ADG increased WH.

Notwithstanding its positive effect on body growth, for years a high ADG was considered the villain of mammary development, especially during the prepubertal period (Sejrsen *et al.* 1982; McFadden *et al.* 1990; Capuco *et al.* 1995; Lammers *et al.* 1999). Teat length, teat width and distance between teats were used as indirect measurements of interior PAR epithelial growth because they increase when exogenous estrogen is administered (Lammers *et al.* 1999). Since estrogen stimulates PAR elongation and branching, it might also have an effect on teat growth. In this experiment, the growth rate of RTL, TLT was of 0.01 cm/day and was only affected by ADG ($p < 0.002$). TD showed also the same growth rate and a tendency to be affected by ADG ($p < 0.10$). MEI and CPI did not interfere in any udder measurements, but DMI showed a tendency to affect teat width ($p < 0.06$). Endorsing our results, Lammers *et al.* (1999) found that teat length was not affected by the rate of Holstein heifers prepubertal growth with different ADG, but was affected by estrogen implants during the treatment period. TD, on the other hand, increased for heifers with higher gains, probably because ADG increased mammary udder weight and extraparenchymal fat and, consequently, udder size increased teat distance. Whitlock *et al.* (2002) and Esselburn *et al.* (2015) however, did not find differences or correlations between mammary parenchyma mass and teat length, disregarding this type of external indicator of mammary development. Since goats have, proportionally, the biggest udders and teats among dairy animals and because of our results, it is also believed that teat morphology is more related to genetics than to nutrient intake.

This set of data is one of the first to characterize the effects of ADG, DMI and nutrient intake on mammary development from prepubertal phase to the early stages of pregnancy in dairy goats, and it was found that PAR area and perimeter were especially influenced by ADG. Only one previous study, conducted by Bowden *et al.* (1995), using the comparative slaughter technique, demonstrated that a high level of nutrient intake had a negative impact on prepubertal alpine goat mammary development. In their study, high nutrient intake before puberty increased ADG, mammary gland and also its extraparenchymal adipose tissue, but lowered the percentage of secretory tissue. Nevertheless, only ten animals were used and all

were slaughtered, not allowing further analyses. It must also be pondered that only the age of the animals was considered for cohort comparison, disregarding their weight.

Villeneuve *et al.* (2010a; b), on the other hand, provided different diets during the post-weaning period, to cover the allometric phase of mammary development. The ewes' growth performance, mammary gland development was recorded through all rearing phase, and lactation yield was recorded throughout two consecutive yearly seasons. The *ad libitum* group had higher ADG in comparison with the other restricted groups. The mammary stroma and MFP were also greater in this group, but there was less dry fat-free tissue, which is correlated with connective mammary tissues. After the end of the feeding program, a compensatory growth of the restricted cohorts was observed, suggesting that prepubertal restricting feeding did not compromise body and mammary growth. The first lactation performance showed no statistical difference among treatments; meanwhile restricted groups produced more liters per day⁻¹. Only during the second lactation, fat, protein and milk yield changed, being higher for the restricted groups. McCann *et al.* (1989), also working with different diets in Dorset and Suffolk ewe lambs, found only differences in fat pad area, which was higher for the high gain ewes, but no differences on total area and gross duct area. Additionally, the ewes with lower ADG tended to produce more milk, as was observed in the previous trial.

In this study, PAR area was positively influenced by ADG ($p < 0.003$) and CPI ($p < 0.003$ for the right side and $p < 0.07$ for the left). MEI in contrast, decreased PAR area, especially on the right side ($p < 0.04$). Whitlock *et al.* (2002) did not find PAR mass alteration or excessive extraparenchymal fat in heifers with different dietary protein. However, this lack of protein effect may have occurred since the diets were high-energy dense, also promoting high ADG. Additionally, the low protein diet, combined with high energy could have decreased mammary growth, limiting its development. Davis Rincker *et al.* (2008), using low and high energy diets through different average times for dairy heifers, concluded that the longer the high energy diet lasted, the more total mammary gland mass and fat increased; whereas DNA, RNA and percentage of proliferating epithelial cells in terminal ductal units decreased. These data are consistent with our findings in which we observed that MEI decreased the PAR area and perimeter, and increased brightness, or pixel value in the ultrasound imaging, showing more fat accumulation focus on the mammary gland. It is possible that a compensatory growth occurred in the last phase of pregnancy, however, considering that the ductwork is formed after puberty, high MEI could have compromised

mammary development and future lactations, therefore, is not recommended for dairy goat rearing nutrition.

Allometry is the variation in morphometric variables associated with size variation, distinguished at three levels: static allometry that reflects individual variation within a population, ontogenetic allometry due to the growth process, and evolutionary allometry in result of phylogenetic variation among animals and species (Klingenberg 1996). In this study only the first type of allometry was considered since we worked with the same specie, the same breed, with animals from the same dairy farm, the same age, during the rearing phase, that is, during the growth phase.

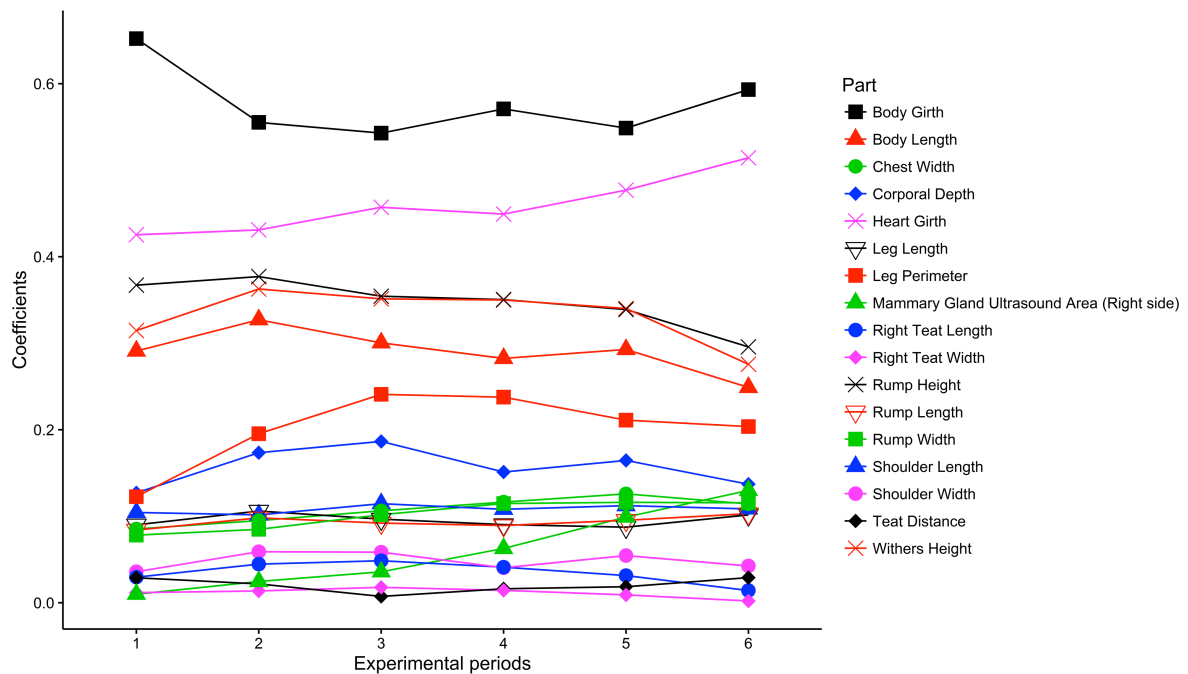


Figure 3.2. Allometric coefficients of body parts and PAR area throughout the experimental periods divided in six parts.

ADG, CPI and MEI are closely related to animal weight increase, protein and energy conversion in connective tissue, muscle and fat deposition. So, most body measurements, despite little variation and considering all six phases, were somehow stable through time. WH and RH are related with long bone growth and consequently with weight gain. Together with BL, this three variables represent main body growth, used as tools to predict and compare animal growth. HG and BG, on the other hand, are highly associated with body weight and since all animals in this experiment gained weight, ADG of 0.14 ± 0.03 Kg, these two coefficients were also stable and high. Other body measurements correlated with long bone growth, LL had a medium coefficient, lower than the body isometry (<0.2234). However, due

to muscle and fat deposition, LP coefficient increased from the first phase (0.123 ± 0.017) to the last (0.204 ± 0.019), having an accumulated coefficient (0.3204 ± 0.005) higher than the body coefficient (0.2234).

The other body parts, CD, SL, RL, CG, LG and LP had intermediate coefficients probably because they are composed by flat bones and do not increase like the long bones or body weight related measurements. The udder variables – RTL, LTL, TD, RTW, LTL –, changed little among all trials, and kept low coefficients, indicating that few changes happened over time, therefore, not ideal for mammary gland growth prediction as was believed previously.

PAR area, on the other hand, despite its low coefficients increased considerably during the trial, more than any other variable. As a matter of fact, none of the other variables had a corresponding pattern growth. Although LL, RL and RW had similar coefficients as the PAR area during the trial period, when this period was subdivided in six parts, it was possible to visualize that these body measurements do not show how PAR develops, characterizing the mammary gland with a unique growth pattern (Figure 3.2).

3.6. Conclusion

High growth rates given by high ADG and nutrient intake did not compromise body and mammary development. Therefore, special attention must be given to a balanced diet, especially to energy intake excess. This is essential for achieving high ADG, diminishing the unproductive period and providing good environment for body and mammary growth. Low protein and high-energy diets might impair this development. Additionally, allometric study revealed that body measurements in this study can be used to predict mammary parenchyma growth.

3.7. Acknowledgments

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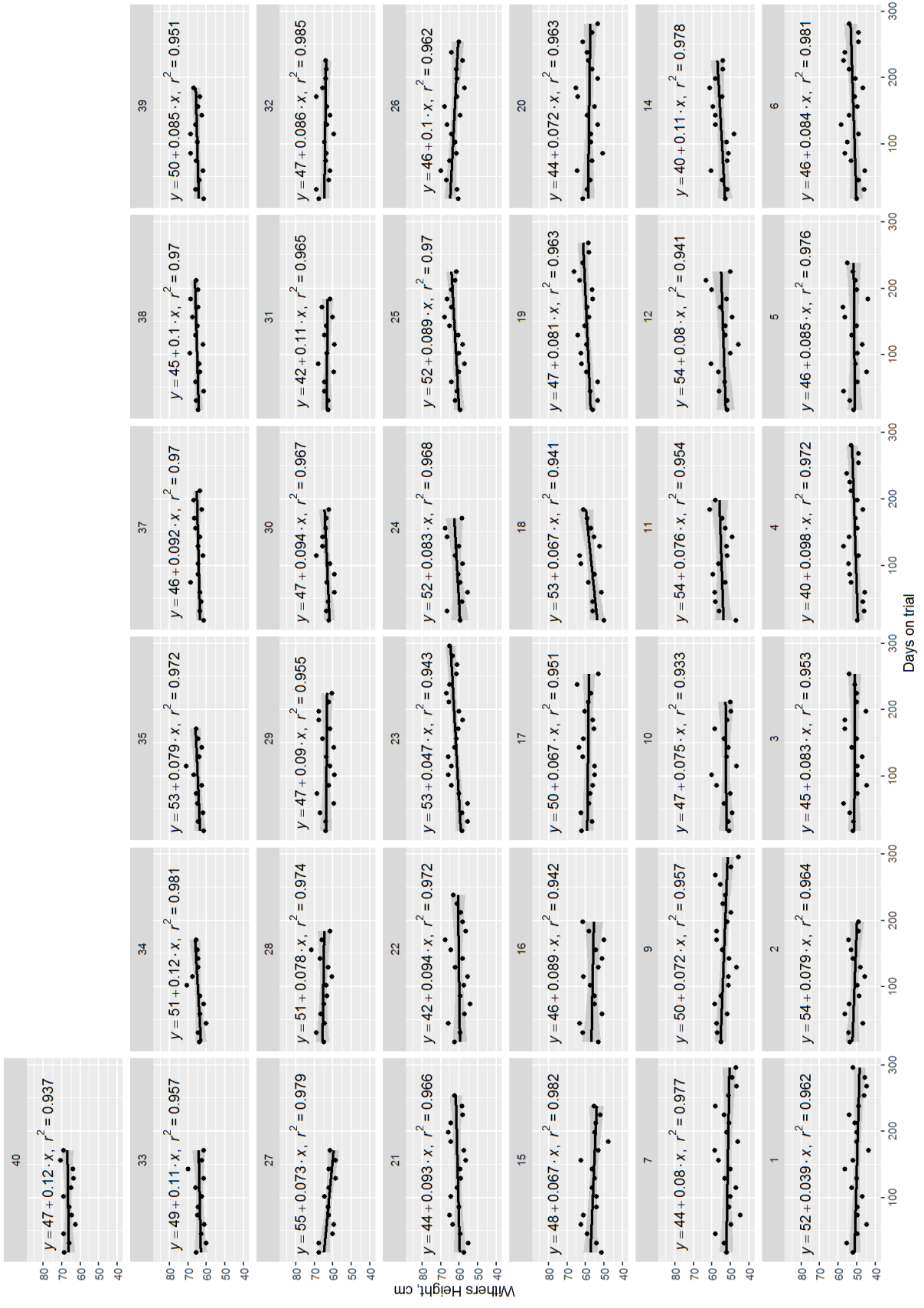
CHAPTER 4. Implications

The lack of works on the nutrition impact on mammary gland and body growth as also their allometry studies in goats makes it difficult for comparison. However, at the same time, this study is precursor since it is one of the first to evaluate these variables.

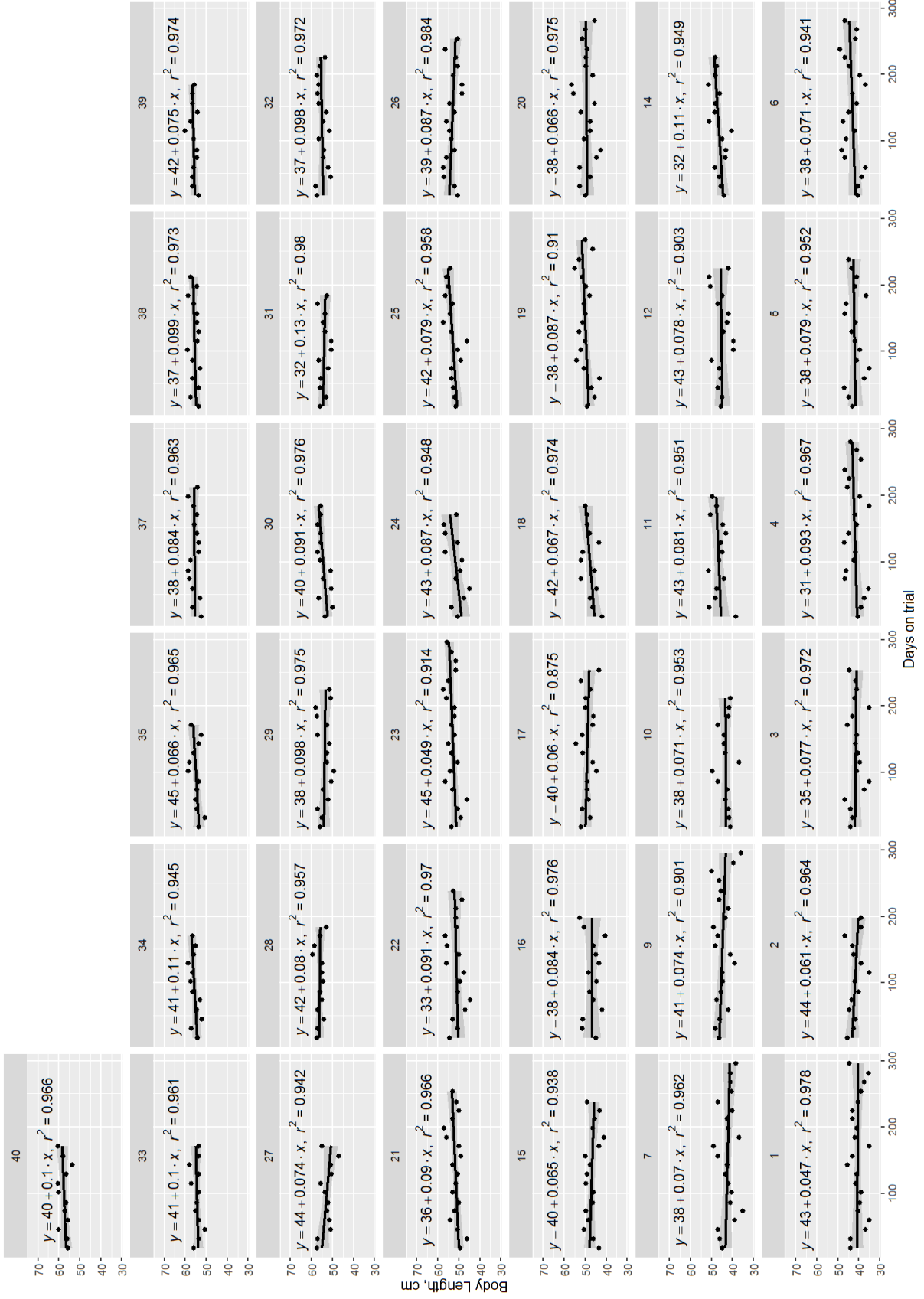
Ultrasound is a useful, easy, cheap and accurate tool for measuring and following PAR area, perimeter and pixels in dairy goats. Further studies should incorporate this method since it allows an internal view of body tissues without compromising them.

ADG did not show to be harmful for mammary and body development, on the contrary, it showed to be beneficial. However, because of the lack of studies involving ADG, CPI, MEI and DMI on dairy goat mammary, body development, and the subsequent implications on milk production, the conclusions observed in this study must be ratified by further experiments.

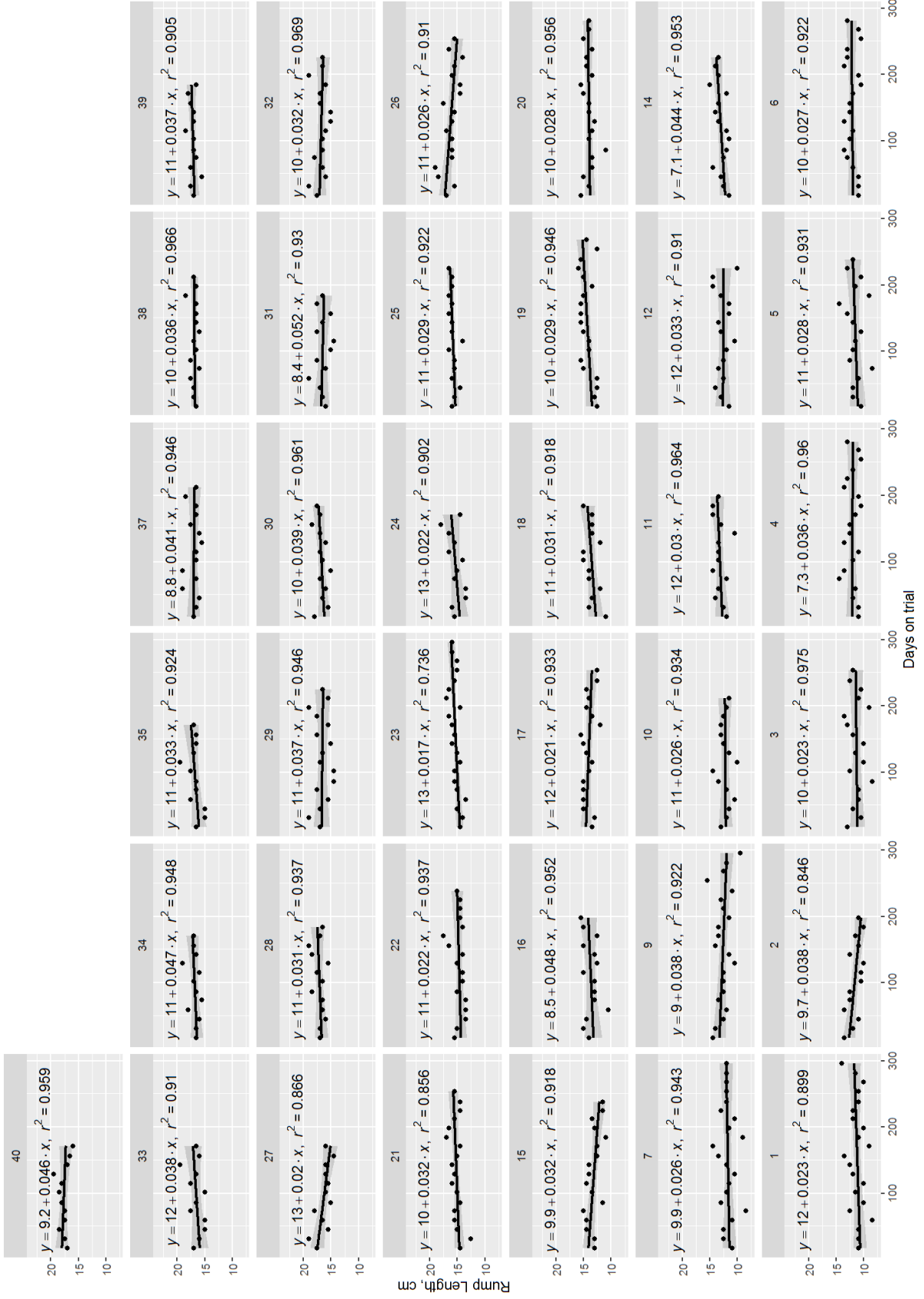
APPENDICES













































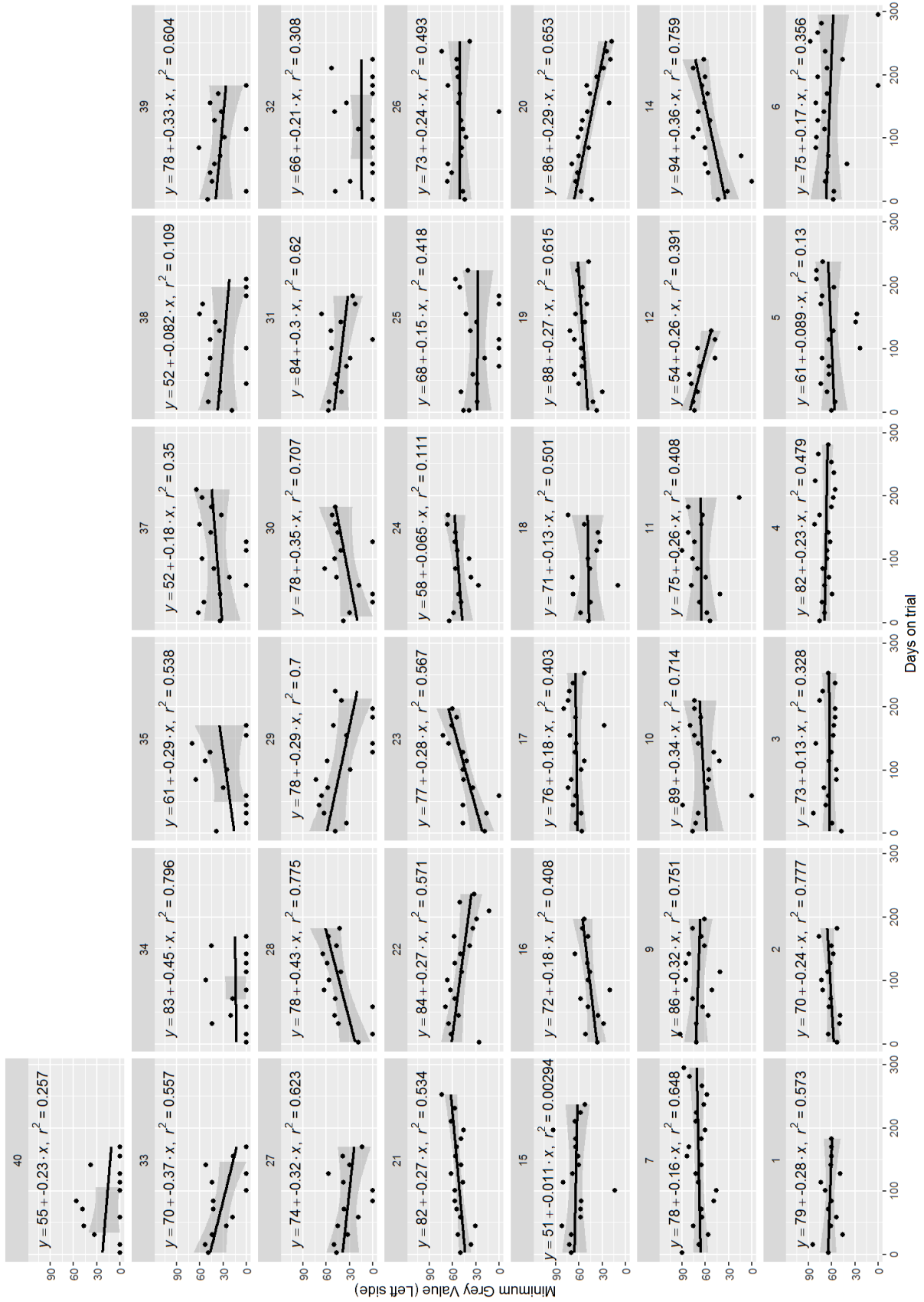
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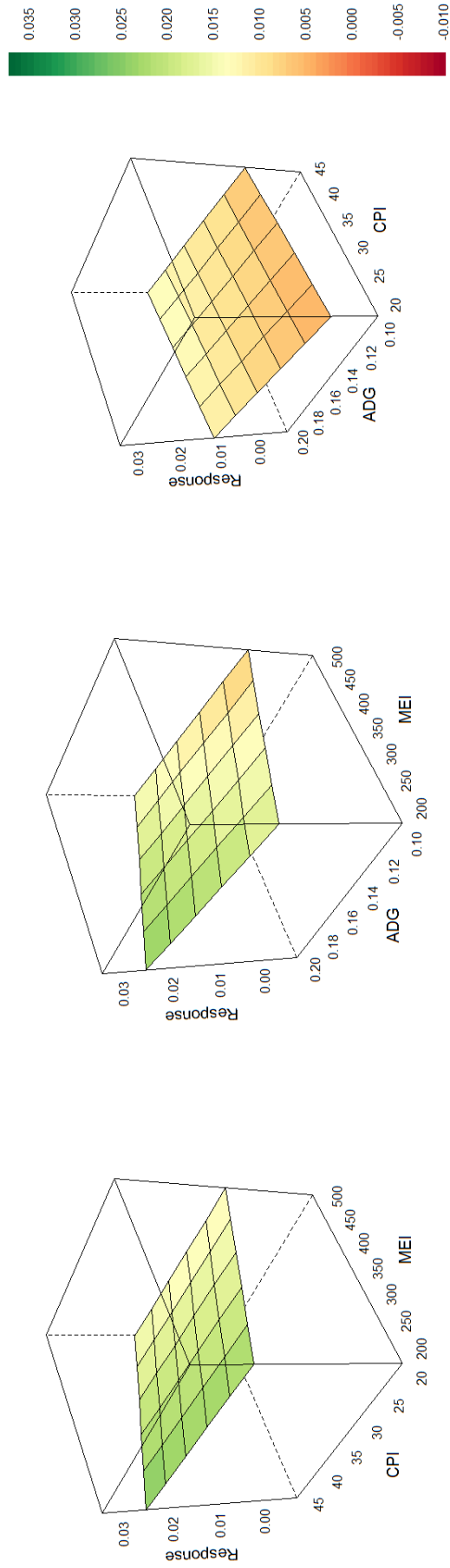




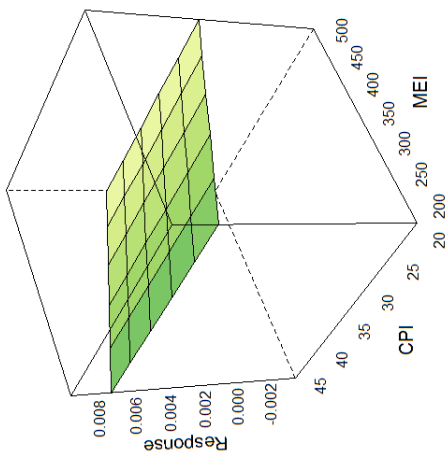
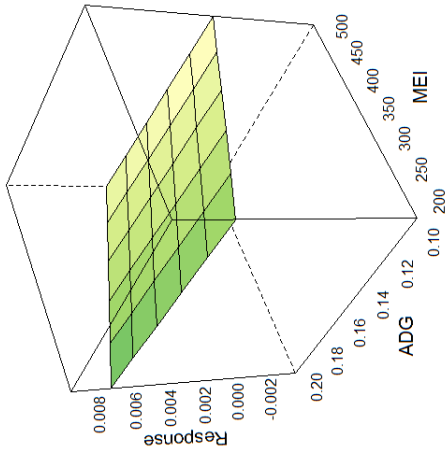
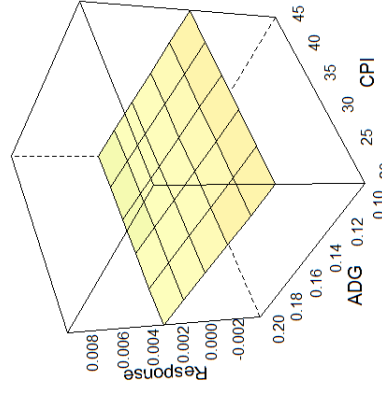
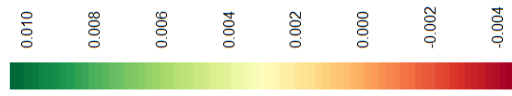




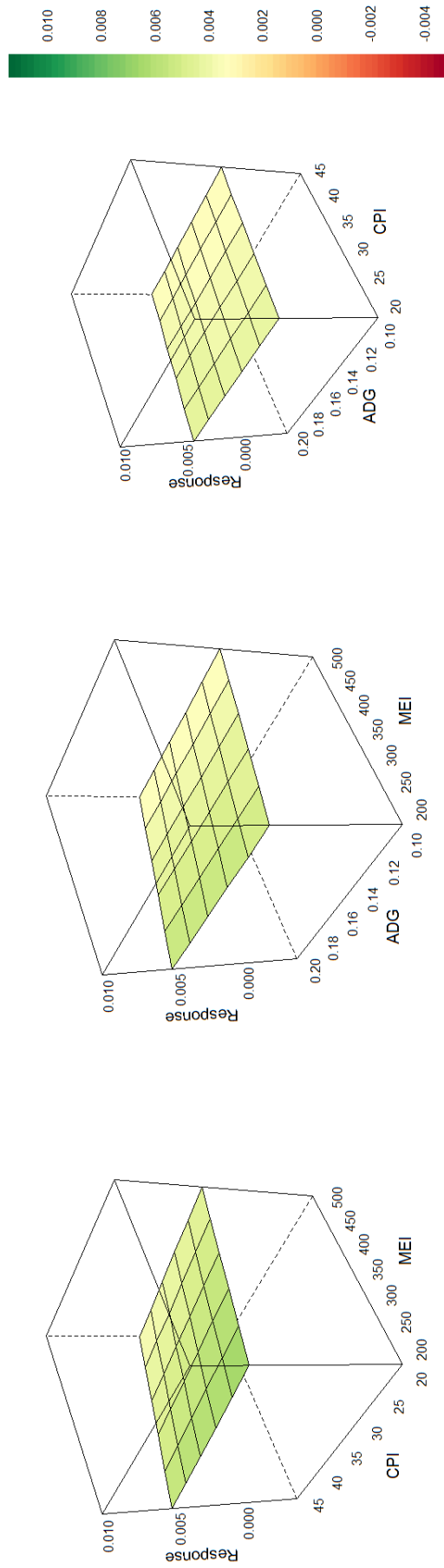
Left Teat Length, cm



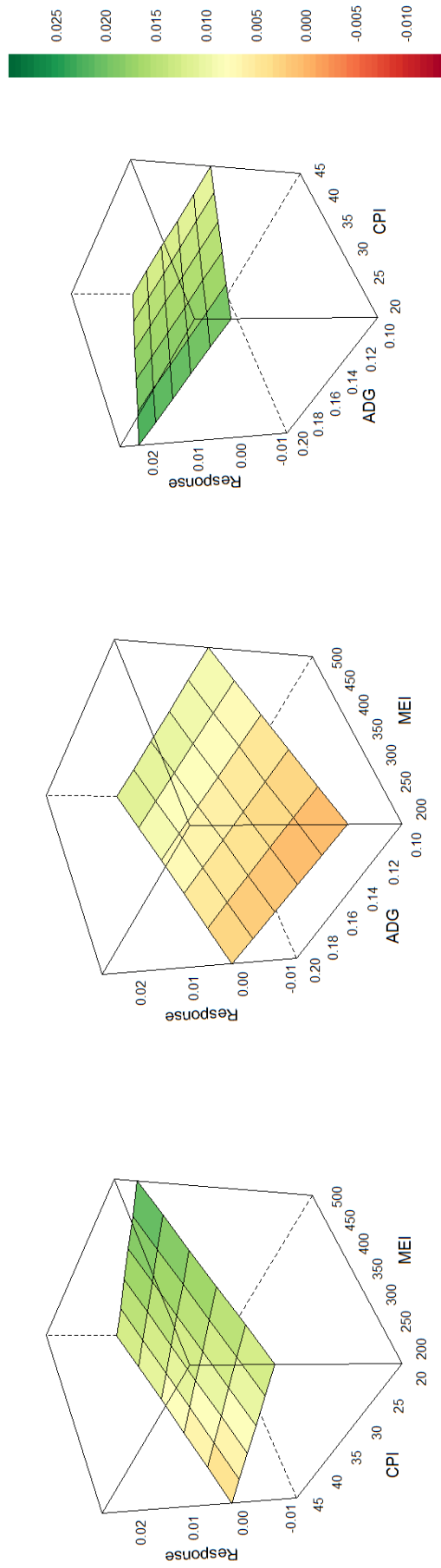
Right Teat Width, cm



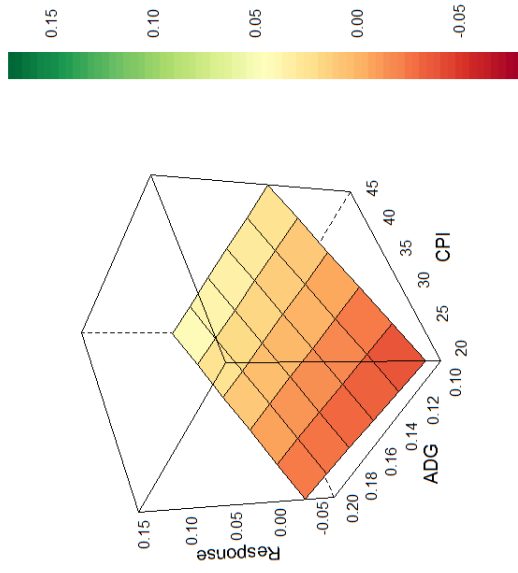
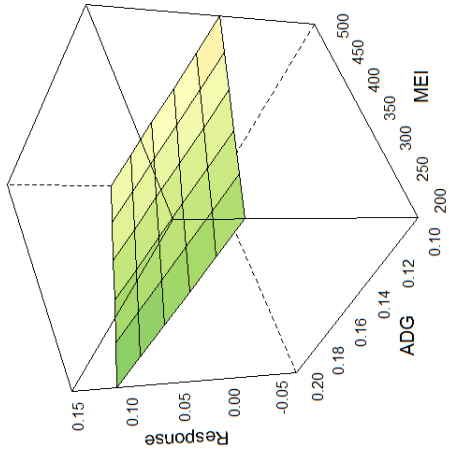
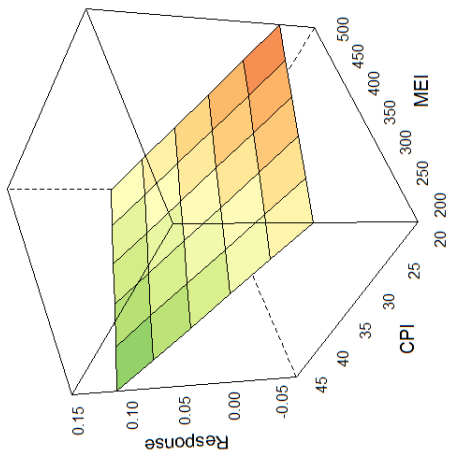
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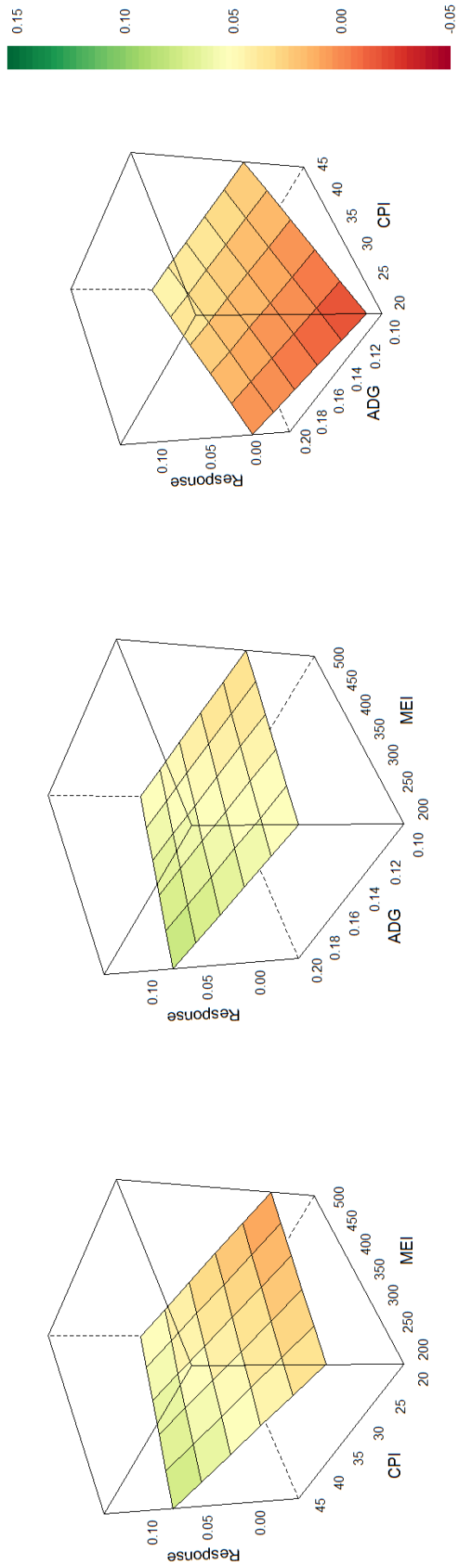
Teat Distance, cm



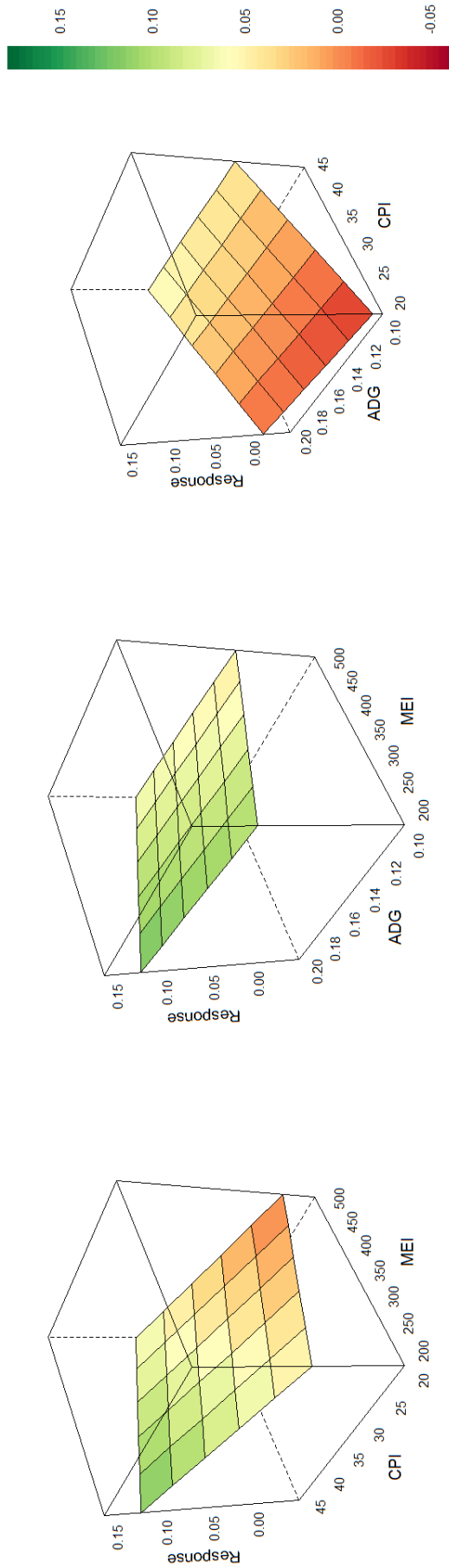
Mammary Gland Ultrasound Area (Right side), cm2



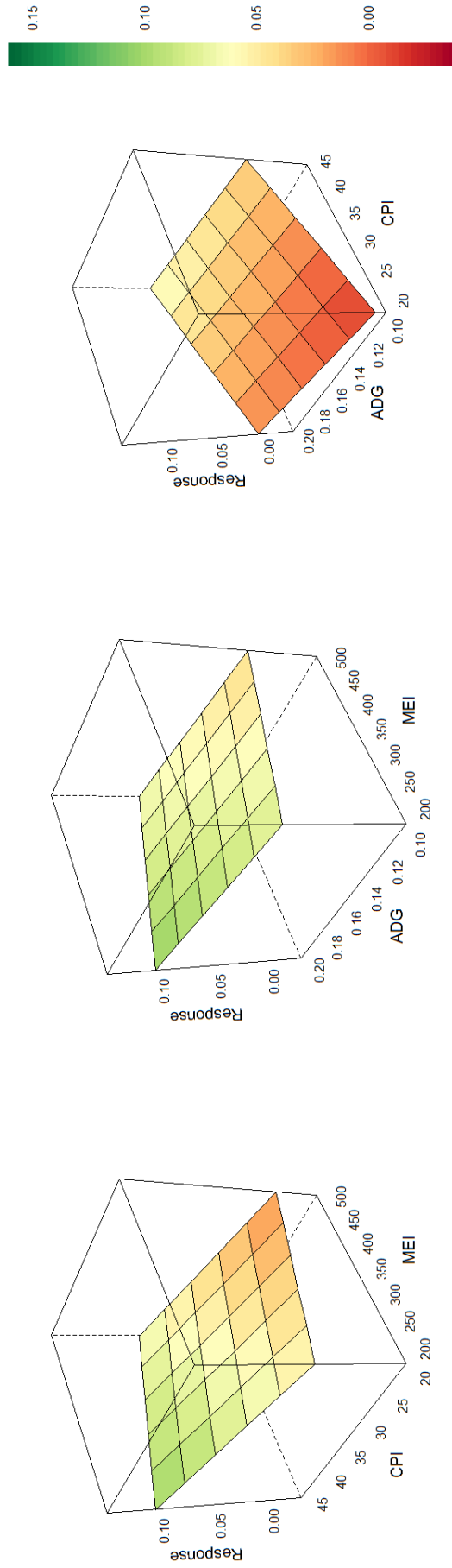
Mammary Gland Ultrasound Area (Left side), cm2



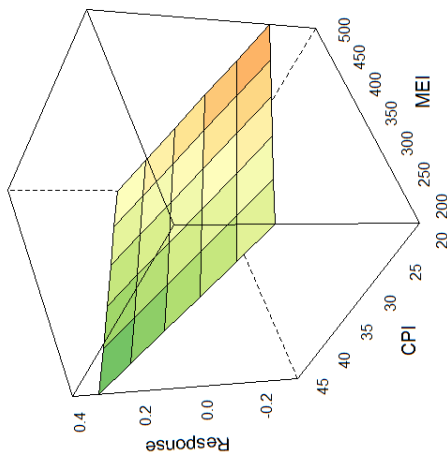
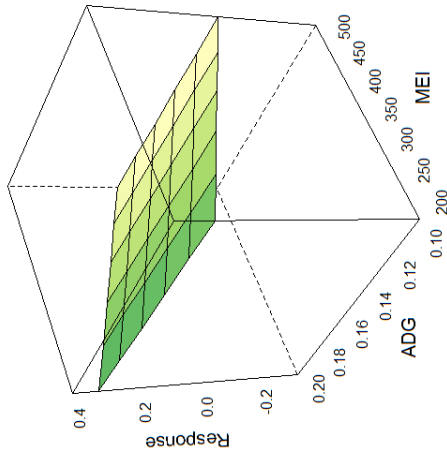
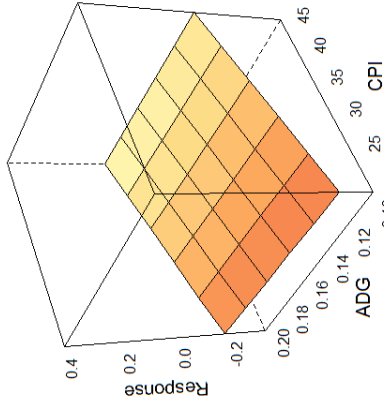
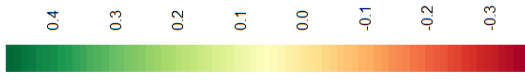
Mammary Gland Ultrasound Perimeter (Right side), cm



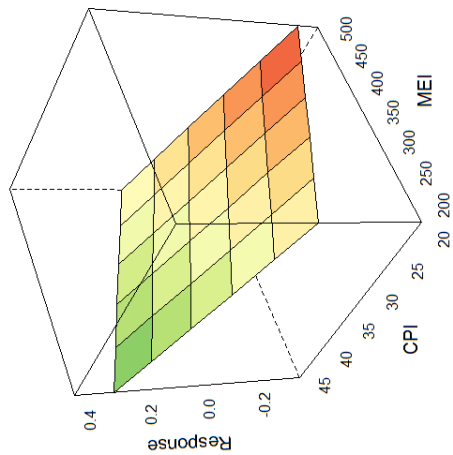
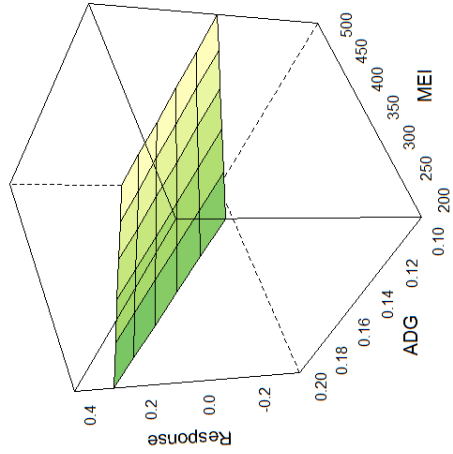
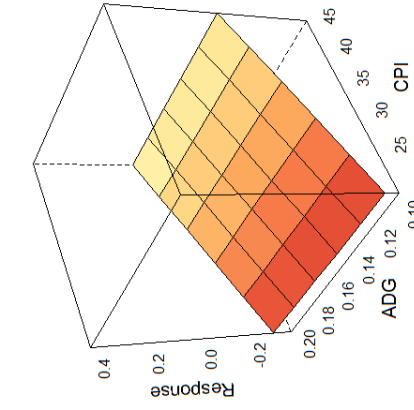
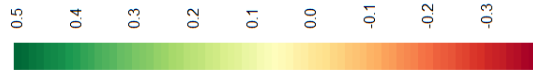
Mammary Gland Ultrasound Perimeter (Left side), cm



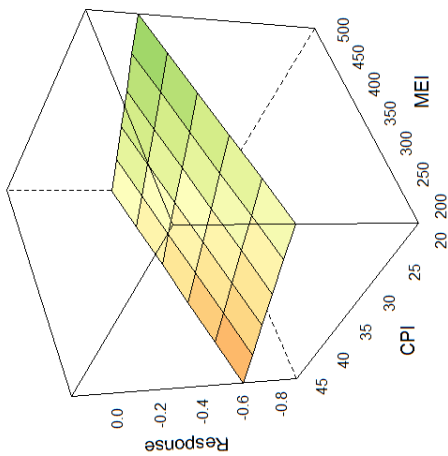
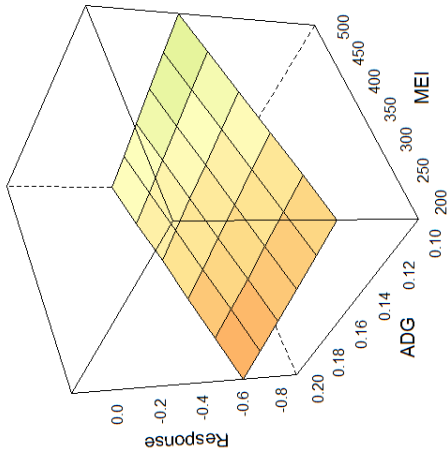
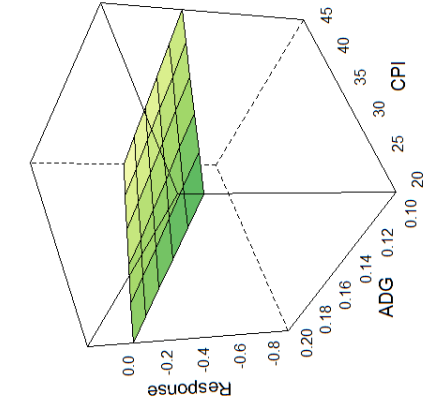
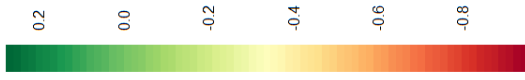
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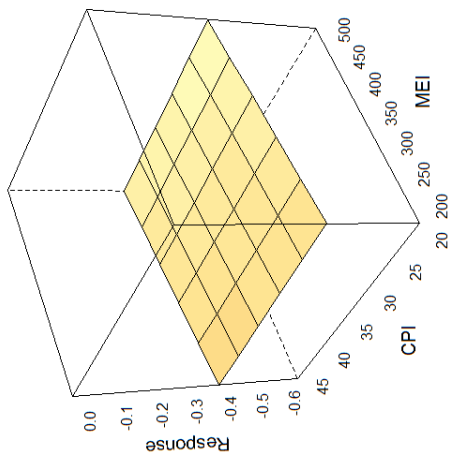
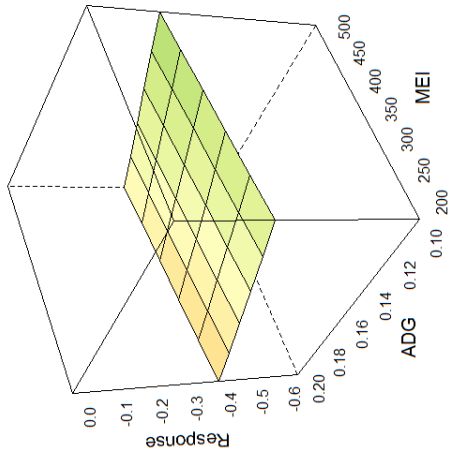
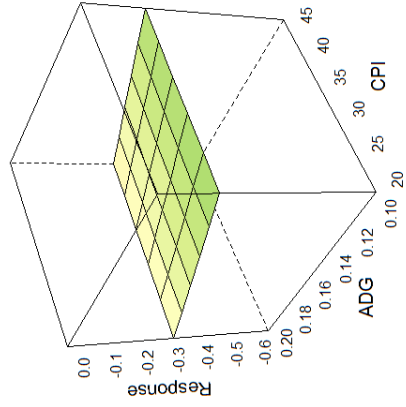
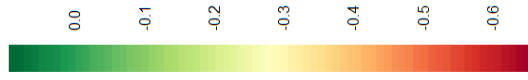
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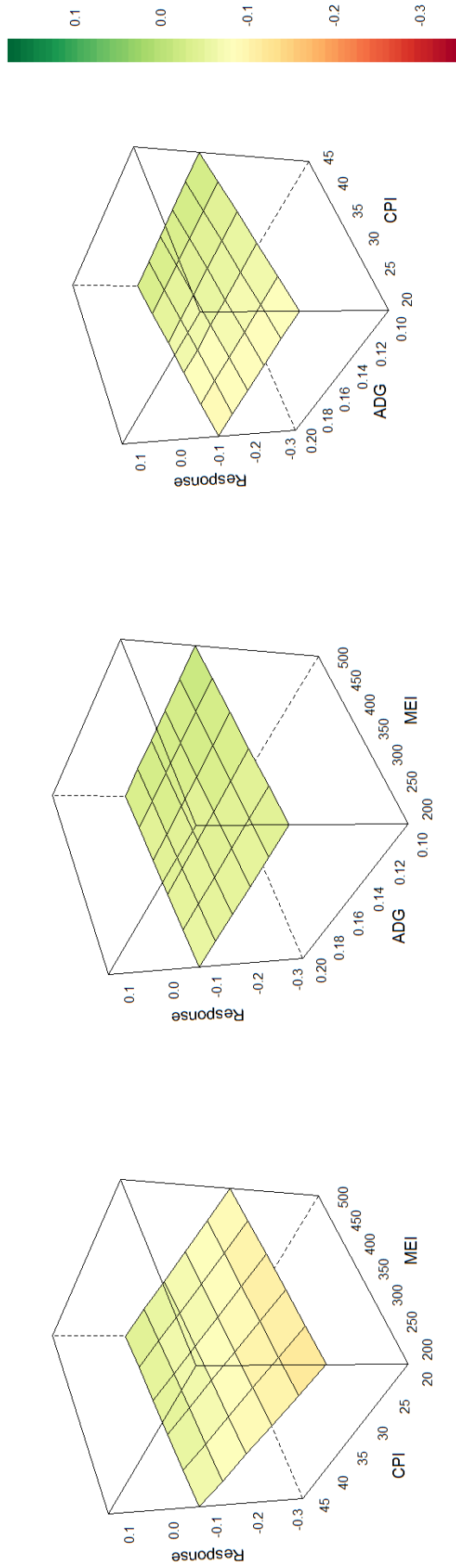
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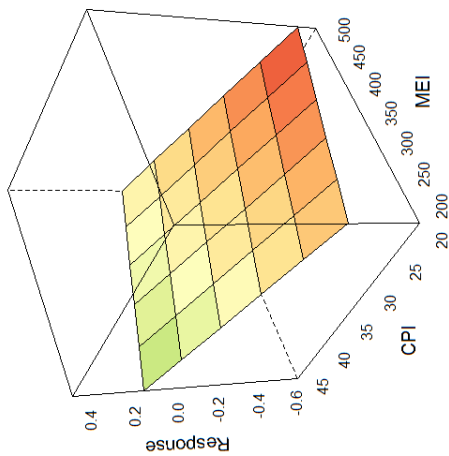
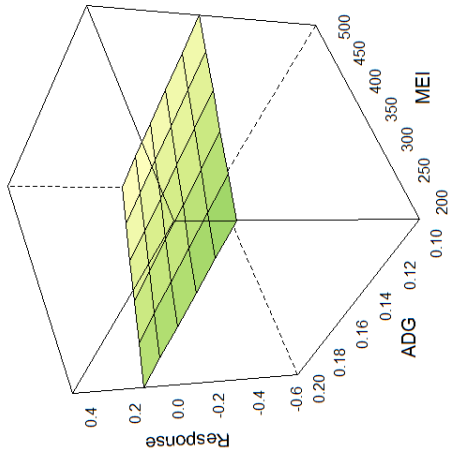
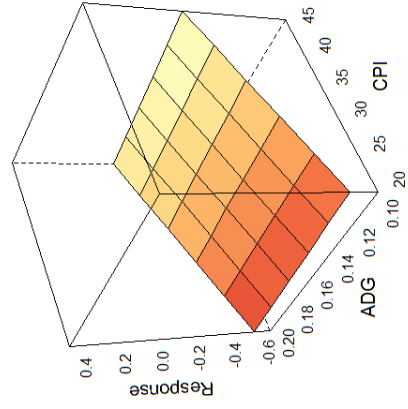
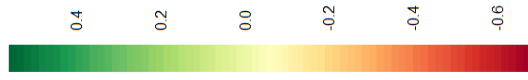
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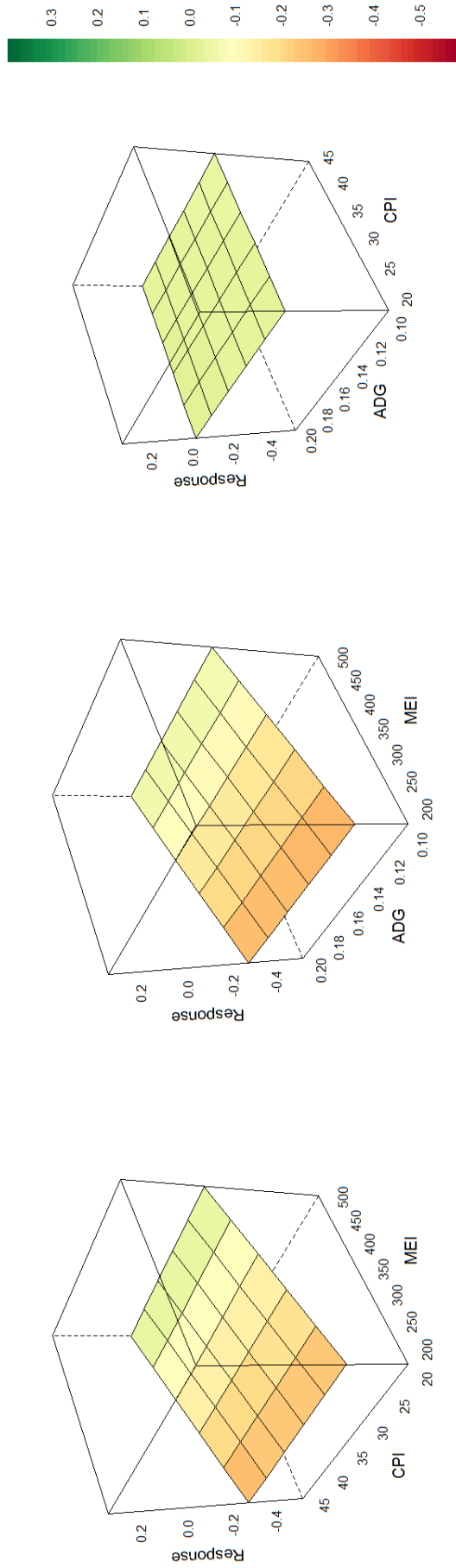
Mean Grey Value (Right side)



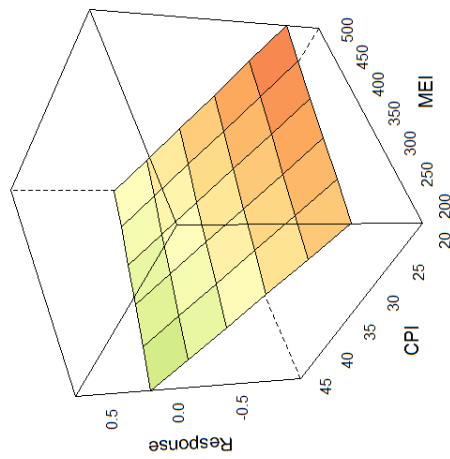
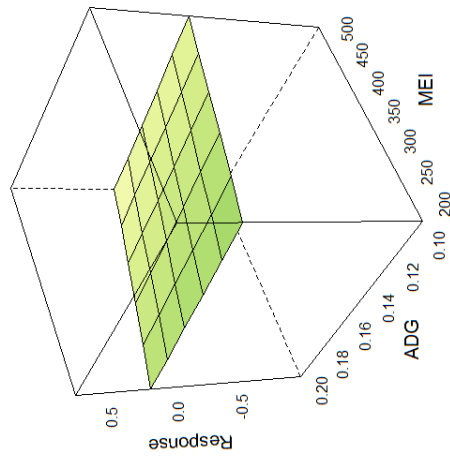
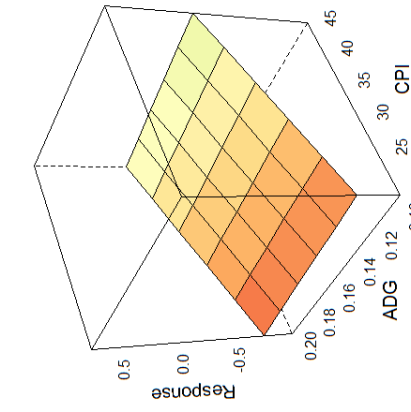
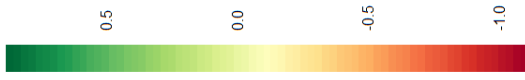
Mean Grey Value (Left side)



Most Frequently Occurring Gray Value (Right side)



Most Frequently Occurring Gray Value (Left side)



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ESCOLA DE VETERINARIA
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2016