Institut für Tierwissenschaften

Effects of different milk replacer feeding levels during a 14-week preweaning phase in heifer calves:

Characterisation of the oxidative status and the mammary gland development by ultrasound imaging

Dissertation

zur Erlangung des Grades

Doktorin der Agrarwissenschaften

(Dr. agr.)

der Landwirtschaftlichen Fakultät

der Rheinischen Friedrich-Wilhelms-Universität Bonn

von

M.Sc.

Katharina Diana Seibt

aus

Bergisch Gladbach

Bonn, 2022

Referentin:	Prof. Dr. Dr. Helga Sauerwein
Korreferent:	Prof. Dr. Karl-Heinz Südekum
Tag der mündlichen Prüfung:	14. Dezember 2021
Erscheinungsjahr	2022

Angefertigt mit Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

Meiner Familie und Freunden

English Abstract

The development of dairy heifer calves and especially their mammary glands is crucial for the productivity of the future dairy cows. Conventional rearing of dairy heifer calves is still based on restrictive milk or milk replacer (MR) feeding regimes, i.e., 4 – 6 L/d or 10% of body weight and weaning at around 8 weeks of age. A paradigm shift in the way of rearing heifer calves from restricted feeding and early weaning towards greater feed allowances and later weaning ages is ongoing. Increasing the daily MR allowance for calves might be better for growth, development, and future milk yields than restricted and low MR feeding. An improvement in animal welfare can be expected by increasing the daily MR allowance and thereby increasing energy and nutrient intake as well as by extending the time until weaning, because hunger and abnormal behaviour associated with restrictive feeding can be avoided. The influence of the MR feeding level on the systemic oxidative status, which describes the balance between the production of oxidants and the neutralizing defense capacity of antioxidants, is less explored in calves during rearing. The aim of this thesis was to investigate the influence and effects of different MR feeding levels during a 14-week preweaning phase on growth, mammary gland development, and systemic oxidative status in dairy heifer calves. In this study, 37 German Holstein heifer calves were examined during the first 5 months of life (week 20). The calves were allocated either to a high feeding level of MR (14% solids, 140 g MR/L) of 10 L/d (1.4 kg MR/d; HIGH, n = 18) or to a restrictive low feeding level of 5.7 L/d (0.8 kg MR/d; RES, n = 19) until the linear weaning in week 13 and week 14 of life. Blood samples were initially collected 36 – 48 h after birth, then fortnightly from week 8 – 16, and in week 20 of life. Variables characterizing the oxidative status were measured in plasma. Ultrasound scans of the mammary glands were performed from each udder quarter of each calf first in week 3 of life and then at the same time points as blood sampling. The RES calves had lower growth rates and showed more signs of hunger than HIGH-fed calves, but did not differ from HIGH in their intake of solid starter feed and health status. While the level of derivatives of reactive oxygen metabolites (dROM) and the ferric reducing ability of plasma (FRAP) increased with age, the oxidative damage to lipids (TBARS) and to proteins (AOPP), as well as the glutathione peroxidase (GSH-Px) activity decreased during the first 20 weeks of life, whereby differences between feeding groups were limited to AOPP and FRAP. The developmental stages and tissue structures of the bovine mammary gland visible in ultrasound images, i.e., the parenchyma (PAR) and surrounding tissue (SURR), were defined, described, and schematically delineated. The resulting and classified 11 visible stages of the developing mammary gland thus formed an atlas of the developing bovine mammary gland. The brightness of these tissue structures,

measured as pixel values, was not affected by the MR allowance, but showed an increase in brightness of PAR from week 8 of life onwards and a decrease in brightness of SURR, indicating the spread of PAR into SURR with increasing developmental stage. The present thesis characterizes the changes in oxidative status of dairy heifer calves with increasing age and confirms the benefits of a high feeding level of MR with regard to improved welfare, growth, and development. Even though an effect of MR feeding level on the brightness of structures and visible developmental stages of the mammary gland could not be detected in this study, the structural development and change of PAR could be seen in its increasing brightness and the categories of the herein developed atlas. Therefore, this study and the atlas can serve as a basis for further studies on the investigation of mammary gland development by ultrasound scanning.

German Abstract

Die Entwicklung von Färsenkälbern und insbesondere ihrer Milchdrüsen ist entscheidend im Hinblick auf die Produktivität als zukünftige Milchkühe. Die konventionelle Aufzucht von Kälbern basiert immer noch auf einer restriktiven Fütterung mit Milch oder Milchaustauscher (MR), d.h. 4 - 6 L/Tag oder 10% des Körpergewichts pro Tag und Absetzen im Alter von ca. 8 Wochen. Derzeit findet ein Paradigmenwechsel in der Aufzucht von Färsenkälbern von restriktiver Fütterung und frühem Absetzen hin zu größeren Futtermengen und einem späteren Absetzalter statt. Eine Erhöhung der täglichen MR-Menge für Kälber könnte das Wachstum, die Entwicklung und die zukünftige Milchleistung im Vergleich zu einer niedrigen restriktiven MR-Fütterung verbessern. Auch eine Verbesserung des Tierwohls kann durch die Erhöhung der täglichen MR-Zulage und der dadurch erhöhten Energie- und Nährstoffzufuhr als auch durch die Verlängerung des Zeitraums bis zum Absetzen erwartet werden, da Hunger und abnormes Verhalten, die mit einer restriktiven Fütterung einhergehen, vermieden werden können. Der Einfluss des MR-Fütterungsniveaus auf den systemischen oxidativen Status, der das Gleichgewicht zwischen der Produktion von Oxidantien und der neutralisierenden Abwehrkapazität von Antioxidantien beschreibt, ist bei Kälbern während der Aufzucht bislang wenig erforscht. Ziel dieser Arbeit war es daher, den Einfluss und die Auswirkungen unterschiedlicher MR-Fütterungsniveaus während einer 14-wöchigen Tränkephase auf das Wachstum, die Entwicklung der Milchdrüse und auf den systemischen oxidativen Status bei Kälbern zu untersuchen. In dieser Studie wurden dazu 37 Deutsche Holstein Färsenkälber während ihrer ersten 5 Lebensmonate (bis zur 20. Lebenswoche) untersucht. Die Kälber erhielten entweder eine hohe Menge an MR (14% Trockenmasse, 140 g MR/L) mit 10 L/Tag (1,4 kg MR/Tag; HIGH, n = 18) oder eine restriktive niedrige Menge von 5,7 L/Tag (0,8 kg MR/Tag; RES, n = 19) bis zum linearen Absetzen in der 13. und 14. Lebenswoche. Blutproben wurden zunächst 36 - 48 h nach der Geburt, dann in vierzehntägigen Abständen von Woche 8 - 16 und in der 20. Lebenswoche genommen. Parameter, die den oxidativen Status charakterisieren, wurden im Plasma bestimmt. Ultraschalluntersuchungen der Milchdrüsen wurden von jedem Euterviertel eines jeden Kalbes zuerst in der 3. Lebenswoche und dann zu den gleichen Zeitpunkten wie die Blutentnahme durchgeführt. Die restriktiv gefütterten Kälber (RES) zeigten geringere Wachstumsraten und mehr Anzeichen von Hunger als die auf hohem Niveau gefütterten Kälber (HIGH). Dabei unterschieden sich die beiden Gruppen jedoch nicht in der Aufnahme von festem Starterfutter und auch nicht im Gesundheitszustand. Während im Plasma der Gehalt an reaktiven Sauerstoffmetaboliten (dROM) und das antioxidative Potential, gemessen als das Eisen(III)-Reduktionsvermögen des Plasmas (FRAP), mit dem Alter

zunahmen, nahmen die oxidativen Schädigungen von Lipiden (TBARS) und von Proteinen (AOPP) sowie die Aktivität der Glutathionperoxidase (GSH-Px) in den ersten 20 Lebenswochen ab, wobei die Unterschiede zwischen den Fütterungsgruppen auf AOPP und FRAP beschränkt waren. Die in Ultraschallbildern sichtbaren Entwicklungsstufen und Gewebestrukturen der bovinen Milchdrüse, d.h. das Parenchym (PAR) und das umgebende Gewebe (SURR), wurden definiert, beschrieben und schematisch abgegrenzt. Aus den daraus resultierenden und klassifizierten 11 sichtbaren Stadien der sich entwickelnden Milchdrüse wurde somit ein Atlas der sich entwickelnden bovinen Milchdrüse erstellt. Die Helligkeit dieser Gewebestrukturen, gemessen als Pixelwerte (pixel values), wurde durch die Fütterungsmenge von MR nicht beeinflusst. Dagegen zeigte sich eine Zunahme der Helligkeit von PAR ab der 8. Lebenswoche und eine Abnahme der Helligkeit von SURR, was auf die Ausbreitung des PAR in SURR mit fortschreitendem Entwicklungsstadium hindeutet. Die vorliegende Arbeit charakterisiert die Veränderungen des oxidativen Status von Färsenkälbern mit zunehmendem Alter und bestätigt die Vorteile eines hohen Fütterungsniveaus von MR im Hinblick auf eine Verbesserung des Wohlbefindens, des Wachstums und der Entwicklung. Obwohl ein Effekt des Fütterungsniveaus von MR auf die Helligkeit der Gewebestrukturen und auf die sichtbaren Entwicklungsstadien der Milchdrüse im Rahmen dieser Studie nicht nachgewiesen werden konnte, ist es gelungen die strukturelle Entwicklung und Veränderung von PAR durch dessen zunehmende Helligkeit und durch die Kategorien des hier entwickelten Atlasses zu zeigen. Daher können diese Studie und der Atlas als Grundlage für weitere Studien zur Untersuchung der Milchdrüsenentwicklung mittels Ultraschall dienen.

List of abbreviations

ADF	acid detergent fiber
ADG	average daily gain
AI	artificial intelligence
AOPP	advanced oxidation products of proteins
a.p.	ante partum
bbp	bits per pixel
BFT	back fat thickness
BW	body weight
CP	crude protein
CV	coefficient of variation
DEPPD	*N*,*N*-diethyl-*para*-phenylendiamine
DM	dry matter
dROM	derivatives of reactive oxidative metabolites
ECM	energy corrected milk
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
FRAP	ferric reducing ability of plasma
GH	growth hormone
GSH-Px	glutathione peroxidase
HIGH	high feeding level
Hp	haptoglobin
IgG	immunoglobulin G
MDA	malonyldialdehyde
ME	metabolizable energy
MFP	mammary fat pad
MG	mammary gland
MR	milk replacer
MVC	mean visible category
NADPH	nicotinamide adenine dinucleotide phosphate
ND	not determined

NDF	neutral detergent fiber
NFE	nitrogen-free extract
NOS	nitric oxide synthase
PAR	mammary parenchyma
p.n.	post natum
p.p.	post partum
RES	restrictive feeding level
RFT	rib fat thickness
RNS	reactive nitrogen species
ROI	regions of interest
ROM	reactive oxygen metabolites
ROS	reactive oxygen species
RT	room temperature
SCC	somatic cell count
SD	standard deviation
SEM	standard error of the mean
SG	specific gravity
SOD	superoxide dismutase
SURR	surrounding tissue
TBARS	thiobarbituric acid reactive substances
TMR	total mixed ration
USAtlasMG	ultrasonographic atlas of the developing bovine mammary gland

Table of contents

1 Introduction 1
1.1 Rearing and feeding management of dairy calves 1
1.2 Growth and development of calves 3
1.3 Mammary gland development 5
1.4 Ultrasound technologies for recording the mammary gland development 9
1.5 Oxidative status 11
2 Objectives 15
3 Manuscript I (published in Antioxidants) 16
4 Manuscript II 49
5 Manuscript III 74
6 General Discussion 118
6.1 Starter intake influenced by milk replacer (MR) intake 118
6.2 Calves' feeding behaviour and welfare 120
6.3 Health status of dairy heifer calves 122
6.4 Development and body growth of dairy heifer calves 124
6.5 Mammary gland development of dairy heifer calves 125
6.6 Milk yield in first lactation and performance 128
6.7 Oxidative status and antioxidative potential in calves 130
7 Conclusions and future perspectives 134
8 Summary 135
9 Zusammenfassung 140
10 References 146
11 Acknowledgments/Danksagungen 159
12 Publications derived from this doctorate thesis 160

1 Introduction

Since cows have been domesticated for dairy production, calving is essential for a new lactation period. Previously, calves were reared and nursed by their dams, while dams were additionally milked to obtain milk for human consumption. Nowadays, in conventional farming, the mother cow is milked and her calf is reared separately. It is still common practice to separate the calf from its dam after birth and colostrum feeding. Hereby, dairy farming is an exception in comparison to husbandry systems of other mammalian farm animals in which animals are kept with their dams during the milk feeding period. Female calves are reared on farms as future cows, but they often do not get the same attention as their dams, the dairy cows, since calves are not yet productive at that age. Therefore, more care should be given to the feeding and rearing management of heifer calves to ensure good development, health status, animal welfare, and also good performance in adult life. The following sections describe conventional rearing practices as well as the physiological and developmental consequences thereof.

1.1 Rearing and feeding management of dairy calves

The level of nutrition in the early life is an effective factor in the development of calves which can be easily influenced in farm animals by daily farm management. Metabolic programming and imprinting are also mentioned in this context, implying that the early life exerts long-term effects on a cow´s entire life (Kesser et al., 2017; Kenéz et al., 2018). Not only the prenatal period and the nutritional level of the mother cow during pregnancy influence the calf´s development (Bach, 2012; Noya et al., 2019), but also the nutritional level provided after birth in the first weeks of life (Kesser et al., 2017; Korst et al., 2017). Hence, the feeding regime of dairy calves especially in the preweaning period is a crucial tool for such programming of future health and profitability of dairy cows.

Naturally, dairy calves are offered whole milk *ad libitum* when they stay with their mother. They consume up to 12 L/d in several small portions throughout the day, i.e., 8 - 12 meals/d (Reinhardt and Reinhardt, 1981; Passillé et al., 2011; Passillé and Rushen, 2012). The natural weaning process occurs gradually over several weeks and at the age of approximately 10 months (Reinhardt and Reinhardt, 1981; Eckert et al., 2015).

The conventional dairy farming system contradicts in many points this natural behaviour. This may influence the development and health of the dairy calf until reaching its productive status as a dairy cow. In conventional rearing, it is still common to feed milk or milk replacer in

quantities which are substantially lower than the amount they would drink in an *ad libitum* situation (Khan et al., 2011; Kesser et al., 2017; Korst et al., 2017). Conventional rearing of dairy calves is still based on restrictive feeding regimes, i.e., 4 – 6 L/d or 10% of body weight per day (Jasper and Weary, 2002), and calves are commonly weaned around week (wk) 8 of age to stimulate solid feed intake which should enhance early rumen development (Kiezebrink et al., 2015; Khan et al., 2016), and also to reduce rearing costs by achieving greater weight gains from starter feed which in turn decreases the age at first calving (Lohakare et al., 2012; Kertz et al., 2017).

For a long time, mainly in the last century, the rearing of Holstein dairy calves was only considered in terms of economic efficiency and profitability in agricultural dairy systems. The early life of calves is considered as a non-productive time period in the life cycle of dairy cows when the animal generates costs without any productive yield (Geiger et al., 2016). Milk replacer (MR) or whole milk fed to calves, among other things, are a major cost factor in rearing (Raeth-Knight et al., 2009; Korst et al., 2017). Hence, this was supposed to be an economical starting point to save money. The long-term physiological disadvantages for restricted milk or MR-fed calves in their future life as dairy cows were not considered sufficiently.

It should be noted that calves are born as functional monogastrics and their nutrient and energy intake is initially based on the intake of whole milk or MR until their transition to mature ruminants (Drackley, 2008; Khan et al., 2016; Steele et al., 2016). The transition from monogastric to ruminant digestion is a slowly evolving process and its mechanisms controlling ruminal differentiation are not entirely understood, but also affected by diet (Klein et al., 1987; Baldwin et al., 2004; Roth et al., 2009). However, most restrictively fed or early-weaned calves are not able to meet their energy requirements by solid feed due to their not yet completely developed rumen and insufficient digestion of solid feed. Hence, liquid feed like milk and MR is still the basis for calves to gain energy for growth and development, while the intake of starter feed and forage is also essential for rumen development (Kertz et al., 2017; Huber, 2018; Diao et al., 2019).

For several years, there has been a growing interest in feeding calves increased amounts of milk or MR in the first weeks of life, as this may improve growth rates, and with regard to long-term consequences, also their performance as cows (Drackley, 2008; Passillé et al., 2011; Korst et al., 2017; Gerbert et al., 2018). There is growing evidence that an intensive preweaning feeding regime, i.e., increasing the daily allowance for MR to amounts that are close to calves' *ad libitum* intake (10 - 14 L/day), is superior to a restricted and low allowance in terms of growth,

development (Frieten et al., 2017; Korst et al., 2017), and also welfare by reducing hunger and avoiding abnormal behaviour (Webster, 1997; Paula Vieira et al., 2008). Furthermore, an increased daily MR allowance supports greater body weight gain, improves feed efficiency, reduces the incidence of disease, provides a greater opportunity to express natural behaviours, which in combination suggests improved welfare (Webster, 1997; Khan et al., 2016), and augments the average daily gain (ADG) in calves, which seems to promote milk yields in the first lactation and cows productive performance (Zanton and Heinrichs, 2005; Soberon et al., 2012; Kenéz et al., 2018).

1.2 Growth and development of calves

In general growth and development depend on the availability of nutrient resources including energy as well as on time (Huber, 2018). Furthermore, growth and development are determined by an animal's genetic potential and its voluntary feed intake, i.e., the intake of nutrients and energy (Huber, 2018). Several external factors in the environment of animals such as temperature, housing, keeping, and feeding conditions can influence growth and development as well (Huber, 2018; Diao et al., 2019).

In young farm animals which are growing and developing, the partitioning and allocation of nutrient and energy resources deriving from the diet are clearly defined (Rauw, 2008; Huber, 2018): first and foremost for maintenance of important and essential body and physiological functions and secondly for the assembling of body-own tissue and structural development that lead to an increase in body growth or ontogenic growth, i.e., growth of organs, muscles, and the skeleton (Rauw, 2008; Kirchgeßner et al., 2014; Huber, 2018). In addition, energy losses such as heat loss via the skin must be considered and compensated by energy intake (Kirchgeßner et al., 2014; Huber, 2018). Maintenance is defined as the condition in which an animal is in a nutritive equilibrium, when the intake is in balance with losses and with neither weight gain nor weight loss (Huber, 2018). When the feed intake exceeds that required for maintenance, then there is energy available for growth (Lawrence et al., 2012).

The amount of nutrients and energy available to the animal also depends on the type of feed and its digestibility and absorptive capacity, or bioavailability (Huber, 2018). In young ruminants, such as calves, this is also influenced by the developmental stage of the gastrointestinal tract. After birth, the abomasum is the only fully and functionally developed stomach and is therefore important for milk digestion (Diao et al., 2019). The development of the rumen in particular takes time until it is functionally developed and mature rumination is

possible, which is in calves at the age of around 12 - 16 weeks of life (Huber, 1969; Huber, 2018). Therefore, young ruminants are initially and primarily dependent on the supply of milk for energy.

An early weaning age and abrupt weaning procedure can result in decreased solid feed intake and weight loss postweaning because the gastrointestinal tract is premature in its development to effectively digest solid feed (Sweeney et al., 2010; Eckert et al., 2015). Eckert et al. (2015) found that calves fed an elevated plane of nutrition preweaning had higher starter intakes and ADG during the weaning period when weaning was extended from wk 6 to wk 8 of age. With increasing time and solid feed intake, the rumen develops and increases in size and functionality (Diao et al., 2019). This underlines the impact of time on developmental processes. Also other specific body structures like the mammary gland, the skeletomuscular system, and the central and peripheral neuronal systems need sufficient time to develop and mature (Huber, 2018).

In any metabolic situation, the allocation of resources to maintenance must be maintained; anything over and above maintenance needs can be invested in growth and development (Huber, 2018). If nutrient resources are limited like in a restrictive feeding regime, then the processes of maintenance are no longer sufficient to maintain proper functional body tissues or to invest in the growth of the developing young animal (Huber, 2018). This may cause stagnation of growth and can lead to compensatory growth, which is a phenomenon whereby the animal accelerates its growth after a period of relatively slow growth, usually due to reduced feed intake, in order to reach the weight of animals whose growth was never reduced (Donovan et al., 1998; Hornick et al., 2000; Geiger et al., 2016).

The growth potential of a young animal can only fully develop if it is provided with sufficient energy and nutrients beyond the basal requirements for maintenance. Therefore, both growth and development of the dairy calves can be influenced by feeding and weaning management, i.e., the allowance for nutrients and energy intake, the time until weaning of calves, and until the first insemination of young heifers. Hence, the nutrition of calves should be appropriate and adapted with regard to their gastrointestinal stage of development. The overall aim should be the creation of the best conditions for healthy and optimal growth through farm management.

1.3 Mammary gland development

The development of the calf´s mammary gland is one of the most important topics of interest with regard to productivity in later life as a dairy cow. Hence, it is important to gain a deeper insight into the development of the mammary gland in the early life as well, to improve management in the early life for supporting the development of the mammary gland and future productivity, and to ensure a good health status of the udder. Previous studies mainly focused on mammary gland development from puberty to the first lactation, because mammary gland development was often considered to be decelerated or quiescent in the early lifetime of calves (Geiger et al., 2016; Geiger, 2019).

The mammary gland itself is a high differentiated apocrine gland of the epidermis, the skin (Lawrence et al., 2012; Kressin et al., 2019). It develops from the germ layer ectoderm in the embryonic stage building a first bud (Hovey et al., 1999; Franz et al., 2009; Rowson et al., 2012; Berryhill et al., 2017) as shown in Figure 1. Due to an inductive signalling from the mammary mesenchyme, the epithelial *anlage* begins its formation into the mammary ducts (Berryhill et al., 2017). During the fetal stage, the developing mammary gland contains different fractions of tissue: the mammary parenchyma (PAR) and the early formation of an adjacent mammary fat pad (MFP, Hovey et al., 1999; Rowson et al., 2012). These tissue fractions largely determine the postnatal growth of the mammary gland. In a female bovine fetus, the primary sprout appears when it reaches a size of approximately 12 cm in length (Berryhill et al., 2017). This primary sprout continues to develop into secondary and tertiary branches, which appear when the fetus reaches a size of approximately 20 cm in length (Berryhill et al., 2017).

In neonatal ruminants, a single primary duct extends from the teat to the gland cistern and ends with the epithelial ducts (Rowson et al., 2012). The PAR at this stage is described by Meyer et al. (2006a, 2006b) as a threadlike mass that extends dorsally above each teat. The ducts are simply branched *anlagen* which are embedded within distinct embryonic mesenchyme positioned adjacent to a mature depot of differentiated white adipose tissue, the MFP, into which it subsequently extends (Berryhill et al., 2017). At birth, the structure of the bovine udder with four quarters, i.e., each quarter is an individually secretory gland culminating in one cistern and one teat with a single canal, is already established (Franz et al., 2009). Therefore, an internal crossover of the parenchymal tissue and the mammary duct system within the four quarters is not possible (Franz et al., 2009). However, it can be expected that the velocity of development and the early developmental stages may vary between quarters.

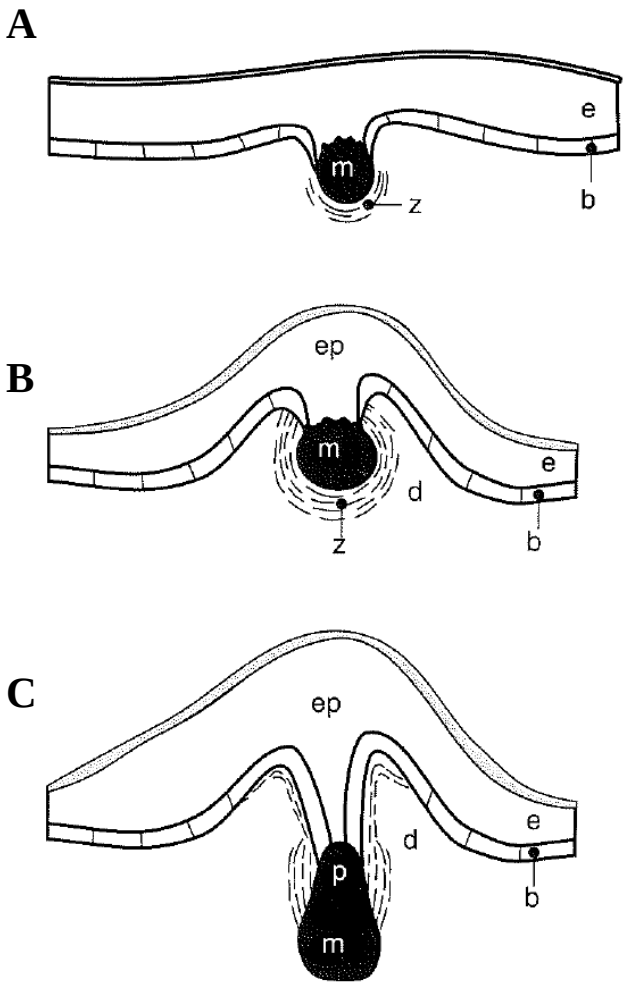


Figure 1. The formation of the epidermal cone supporting the mammary bud in the bovine fetus: (A) female bovine fetus of 8.5 cm length, (B) female fetus of 9 cm length, and (C) male fetus of 15 cm length. Under the epidermis (e) lays the epidermal basal layer (b) followed by the embryonic dermis (d). When the female fetus reaches 5 - 10 cm in length, mesenchymal cells accumulate around each mammary bud (m) to from fairly dense conglomerates, the areolar zone (z). This can also be detected in the male fetus. At around 7 - 8 weeks after conception, when fetus reaches 8 - 9 in length, cellular differentiation occurs to give a neck-line epidermal formation, the pedicle (p), that connects the epidermis to the mammary bud (m). This results in an upward thrusting of the bud so that it gradually rises above the basement epithelium. This in turn leads the formation of an epithelial cone (ep). Modified from Lawrence et al. (2012).

In dairy animals, mammary gland growth and development are characterized by isometric and allometric phases of mammary growth (Berryhill et al., 2017). It is mostly reported that after birth, the mammary gland PAR growth in calves is initially isometric followed by a prepubertal developmental phase of allometric growth (Tucker, 1987; Hovey et al., 1999; Berryhill et al., 2017). There is still the dogmatic assumption that PAR grows isometrically during the first 2 months of life (Soberon and van Amburgh, 2017), albeit several studies indicated rather allometric growth of PAR in relation to body weight in this phase (Esselburn et al., 2015; Meyer et al., 2006a; Geiger, 2019). The allometric growth is assumed to begin already before 2 months of age and continue until the onset of puberty at around 9 months of age (Capuco and Akers, 2010; Geiger et al., 2016; Berryhill et al., 2017). During puberty, PAR growth becomes isometric again until the beginning of the first pregnancy (Hovey et al., 1999). Hence, puberty is a transition phase in which the mammary gland changes from allometric to isometric growth. The growth of the mammary PAR is modulated and stimulated by ovarian steroid hormones (Akers, 2017) such as oestrogens and progesterone which act in conjunction with the proteohormones prolactin and growth hormone (GH). After several oestrous cycles during puberty, the growth changes to an isometric form until the first pregnancy (Tucker, 1987; Lawrence et al., 2012).

The mammary PAR is the tissue region where milk synthesis takes place in cows. The milk is synthesized by the secretory cells of PAR, the alveoli. Alveoli are bundled into lobules and are surrounded by myoepithelial cells (Franz et al., 2009). During mammary gland development, the ducts grow and spread into the surrounding which contains adipose and connective tissue and is referred to as the MFP (Hovey et al., 1999; Albino et al., 2015). Hovey et al. (1999) designated the MFP as a central factor in regulating mammary PAR development and mammogenesis in general. It is assumed that the higher the amount of mammary adipose tissue is, the more the duct and PAR have to penetrate and grow into the MFP, which might lead to a reduced overall mammary gland development depending on its dimensions (Albino et al., 2017). The MFP is not only a reservoir for triglycerides, but also for other lipids or their derivatives, which in turn might impact the signalling and the responsiveness of adjacent epithelial cells to external signals such as hormones and growth factors (Hovey and Aimo, 2010; Berryhill et al., 2017).

Nevertheless, pregnancy is assumed to be the major stimulus for ductal growth, development, and for the particular differentiation of alveoli to provide the characteristic structure of the matured lobulo-alveolar structure of the mammary gland (Lawrence et al., 2012).

Recently, the pre-pubertal development of the mammary gland is standing more in the focus of interest (Geiger, 2019). Previous studies already showed that mammary gland development begins in the early life of calves and can be influenced by preweaning nutrition (Brown et al., 2005; Meyer et al., 2006a, 2006b; Geiger, 2019). An intensive preweaning feeding regime until wk 8 of life, i.e., increased energy and protein intake, can positively influence mammary gland development, especially of the growth of PAR mass (Brown et al., 2005; Meyer et al., 2006b; Geiger et al., 2016). This greater growth of mammary gland in calves receiving an intensive preweaning MR feeding in comparison to calves at restrictive feeding regime was illustrated in the study by Geiger et al. (2016): Figure 2 shows the dissected mammary glands of 2 of these calves at different feeding levels. A trend for an increase in mammary epithelial cell proliferation in PAR in response to higher preweaning (wk 6) energy intake was also observed (Meyer et al., 2006b).

It is becoming evident that the time period between wk 8 and 10 of life during rearing, which is the time point of weaning in conventional calf rearing management, is a critical time for imprinting the capacity of the mammary gland for milk production (Brown et al., 2005; Meyer et al., 2006a). Hence, the development of the mammary gland depends on both the length of MR feeding before weaning as well as the daily allowance for milk, MR, and energy intake.

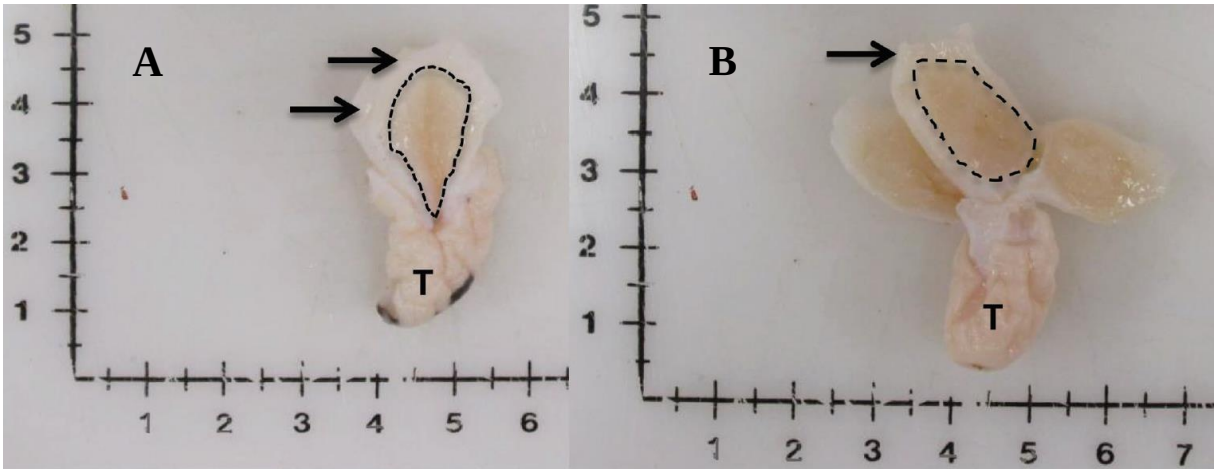


Figure 2. Dissected and trimmed mammary glands from 8-week old heifer calves fed either a restricted (A, left) or enhanced (B, right) diet preweaning. This visual depiction of mammary gland parenchyma (PAR) development shows the differences between calves fed a low plane of nutrition (A, left; 20% protein and 20% fat milk replacer) or a high plane of nutrition (B, right; 28% protein and 25% fat milk replacer). The scales on both pictures indicate cm. The teat (T) appears at the bottom of each sample. Arrows indicate the remnants of the mammary fat pad (MFP) that was trimmed and removed to prepare the tissue for fixation. The light brown tissue within the dashed line indicates the PAR. Modified from Geiger et al. (2016) and Geiger (2019).

1.4 Ultrasound technologies for recording the mammary gland development

There are different possibilities to gain insights and to measure mammary gland development. In previous studies mainly weight (Swanson and Poffenbarger, 1979; Geiger et al., 2016), the volume of mammary glands, or the composition of the gland were investigated (Brown et al., 2005; Akers, 2017). Due to the spread of the mammary PAR into the MFP and thus its gradual displacement, Swanson and Poffenbarger (1979) concluded that the total udder weight does not adequately reflect the development of the mammary gland. Several previous studies also focused on mammary gland growth by weighing the different tissue components of the growing gland, i.e., the mammary PAR, the MFP (Meyer et al., 2006a, 2006b; Esselburn et al., 2015; Albino et al., 2015; Albino et al., 2017; Furini et al., 2018; Silva et al., 2018). For these examinations and measurements, the calves had to be slaughtered before.

Ultrasound technologies allow for non-invasive observation of mammary gland development *in vivo*. The main advantage of ultrasound measurements is that it is a non-invasive technique and allows for examining the developmental process throughout life. Furthermore, it offers the possibility to gain a detailed insight into the different structures and characteristics of tissues in order to differentiate and define them, without the need for dissection. The differentiation between the different tissues of the mammary gland and the evaluation of a shift in the composition of mammary gland is also possible: various recent studies already used ultrasound techniques for this purpose (Albino et al., 2015; Esselburn et al., 2015; Furini et al., 2018; Silva et al., 2018). The tissues' different ability to reflect or to absorb ultrasonic waves forms the basis for their differentiation via ultrasound: the more the ultrasonic waves are reflected, the brighter the structures appear and thus show higher pixel values within the ultrasonographic image and vice versa (Delorme and Debus, 2005; Penninck and d'Anjou, 2015). This is exemplified in Figure 3 (Rowson et al., 2012; Esselburn et al., 2015): the dissected light brown pigmented parenchymal region can be related to the dark (hypoechogenic) region in the ultrasound image, whereas the dissected white surrounding, in which PAR is embedded, correspond to the white (hyperechoic) region. In the study of Esselburn et al. (2015), the structure of the mammary gland was described after a dissection directly after the ultrasonic measurement. Hence, they could relate the different parts of the mammary gland visible in the ultrasonic image to the different tissues they identified.

One of the first B-mode ultrasound measurements of the bovine mammary gland was performed in 1986 by Cartee et al. (1986). Over the years, various studies which used ultrasound examinations focused mainly on the structure of the teat and gland cistern in context with

milking-induced alterations (Bruckmaier and Blum, 1992). In recent years, ultrasound sonography became more attractive to evaluate tissue development or structures of specific parts of the body. Since Esselburn et al. (2015) have postulated that in heifers from birth to 2 months of age (8 wk), weekly PAR measurements using ultrasound are an effective quantitative tool for measuring changes in PAR development *in vivo* and evaluation of ultrasound images by measuring the pixel value with the software program ImageJ; several studies followed this example of a non-invasive evaluation (Albino et al., 2015; Silva et al., 2018; Furini et al., 2018). The methods of Nishimura et al. (2011) and (Esselburn et al. (2015) were adapted for ultrasound image evaluation and continuously improved and developed. The whole mammary PAR area (Esselburn et al., 2015), the circularity of PAR (Furini et al., 2018), and the brightness of different tissues in ultrasound images (pixel value) were measured (Albino et al., 2015; Albino et al., 2017; Furini et al., 2018; Silva et al., 2018). Additionally, Esselburn et al. (2015) and Furini et al. (2018) conducted out palpation according to a palpation score as another non-invasive method and also teat length measurements were performed. Thus, there are several non-invasive ways to examine and monitor the growth of the mammary gland, while ultrasound imaging can provide the most detailed insights.

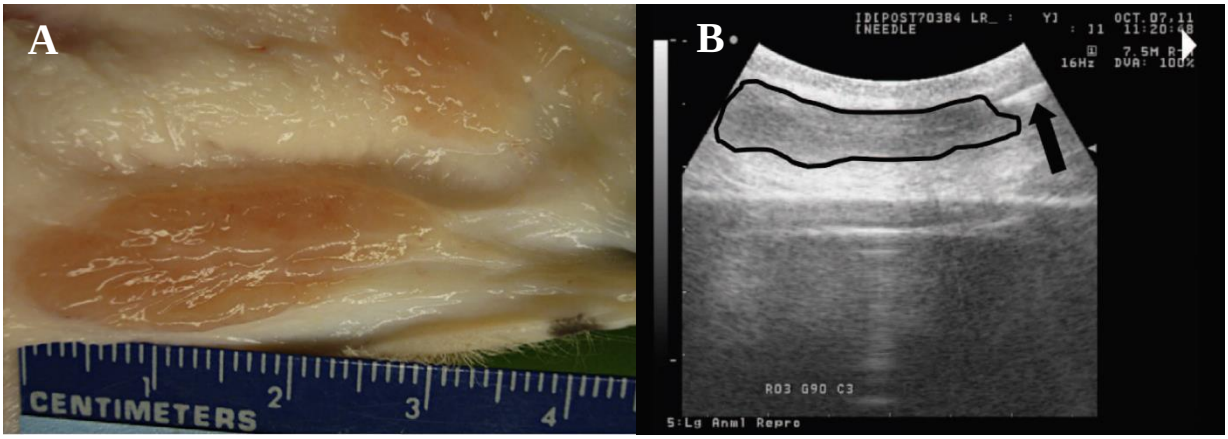


Figure 3. Sagittal bisected mammary gland quarter (A, left) and in comparison an ultrasound image of one mammary gland quarter (B, right) both from 8-week old heifer calves. The picture of the dissected mammary gland (A, left) shows the light brown pigmented parenchymal region within the surrounding mammary fat pad (MFP). In the upper right corner of the ultrasound image (B, right) a needle (hyperechoic, white) appears, which was inserted into the teat (black arrow). The mammary parenchyma (PAR) in this gland (hypoechoic, dark) is outlined with a solid black line. The white tick marks on the left vertical axis of the ultrasound image are spaced 0.5 cm apart. Modified from Rowson et al. (2012) and Esselburn et al. (2015).

1.5 Oxidative status

The oxidative status describes the amounts of oxidants and antioxidants that occur in blood circulation or in cells (Costantini, 2019). The oxidative status depends on the different levels of the production of reactive oxygen species (ROS) or free radicals and the present amount of antioxidants (Sies, 1985).

The ROS are oxygen-containing molecules that can be generated by enzymatic or non-enzymatic redox reactions and during cellular aerobic metabolism (Kaushal et al., 2019). The ROS include radicals such as superoxide anion ($O_2^{\bullet-}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide ($^{\bullet}NO$), as well as non-radicals like hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), peroxynitrite anion ($ONOO^-$), singulett oxygen ($^1O_2$), and ozone (O_3), which can cause oxidative damage to cellular components, the DNA, proteins, and lipids (Sies, 1985; Aruoma, 1998). Most ROS are generated as by-products of mitochondrial oxidative phosphorylation or formed as intermediates of oxidoreductases and metal-catalysed oxidation (Sies, 1985). Several mechanisms of ROS generation in the cell are shown in Figure 4. The ROS occur during metabolic processes and are highly reactive signalling molecules that also maintain redox homeostasis in mammalian cells (Kaushal et al., 2019). Antioxidants are their antagonists and are represented either by enzymes or small compounds derived from the diet, such as vitamins, polyphenols, or flavonoids (Sies, 1985). Antioxidant enzymes and non-enzyme agents such as superoxide dismutase (SOD), catalases, peroxidases, glutathione peroxidase (GSH-Px), and thioredoxin are able to detoxify ROS (Aruoma, 1998; Kaushal et al., 2019). For example, the SOD removes $HO^{2\bullet}$ superoxide by accelerating its conversion to H_2O_2, then the catalases in the peroxisomes convert H_2O_2 into water (H_2O) and oxygen (O_2). The GSH-Px also catalyses the conversion of H_2O_2 into H_2O and thus removes it in cells (Aruoma, 1998; Buddecke, 1994).

Therefore, the oxidative status is a balance between oxidative and antioxidative activities within the cells as well as in blood circulation (Sies, 1985). Furthermore, the systemic oxidative status is a balance between oxidants and antioxidants present in blood circulation (Ranade et al., 2014). Oxidative stress is caused by an imbalance between oxidants and antioxidants or between the amount of the generation of ROS and of the sufficiency antioxidant defence mechanism to cope with increasing ROS (Sies, 1985; Aruoma, 1998; Ranade et al., 2014). During oxidative stress, the balance is in favour of the excessive generation of oxidative molecules, mostly ROS. This leads to cell damages to the membrane, lipids, proteins, mitochondria, or DNA (Sies, 1985; Kaushal et al., 2019). Therefore, oxidative stress is associated with inflammatory processes and diseases (Aruoma, 1998; Kaushal et al., 2019) also

in ruminants including sepsis, mastitis, acidosis, ketosis, enteritis, pneumonia, and respiratory diseases (Miller et al., 1993; Celi, 2011; Ranade et al., 2014). ROS are also generated in phagocytic white blood cells, neutrophils, in order to kill invading pathogens (Leeuwenburgh and Heinecke, 2001). Overproduction of ROS within the cells can cause tissue injury, and tissue injury itself also induces ROS generation by activation of phagocytic cells or by releasing transition metal ions from damaged cells, which may then force the injury (Aruoma, 1998).

There are several approaches to determine the oxidative status of mammals: the measurements of ROS, the amount of antioxidants or antioxidative activity, and the analysis of damages or products as consequences of ROS reactions (Briviba et al., 2008). Although the oxidative status has been largely investigated in several studies, there are limits to the detection of oxidative status: it is still difficult to detect ROS or reactive intermediates directly *in vivo*, because of their short half-lives (10^{-6} to 10^{-12} seconds) and because they are typically present at low levels in biological material which is used for detection, such as urine or blood (Leeuwenburgh and Heinecke, 2001). For characterizing the systemic oxidative status in humans and animals several variables can be measured: out of the various assays, the damage caused by oxidative stress to lipids can be measured, for example, as thiobarbituric acid reactive substances (TBARS; Gutteridge and Halliwell (1988). Damage to proteins can be assessed as advanced oxidation products of proteins (AOPP; Witko-Sarsat et al. (1996). Oxidants can be assessed as derivatives of reactive oxygen metabolites (dROM; Alberti et al. (2000). In addition, for assessing the antioxidative activity the ferric reducing ability of plasma (FRAP) is a common assay (Benzie and Strain, 1996) and the activity of antioxidative enzymes such as GSH-Px can be analysed as well (Paglia and Valentine, 1967).

In cattle, the oxidative stress is known to be mainly associated with compromised immune responses and increased incidence of health disorders around the time of calving and at the onset of lactation due to the increased metabolic activity and higher production of ROS as by-products of the enhanced cellular metabolism (Sordillo and Aitken, 2009; Sordillo, 2016). Many antioxidants such as vitamins and minerals which could balance the ROS production of dairy cattle are derived from their diet (Sordillo, 2016). Hence, it was assumed that the oxidative status in dairy cows is also influenced via nutritional factors derived from plants such as tannins, which are known to have antioxidative capacity, or other antioxidants supplemented to cows' diet (Ciampi et al., 2020).

The effects of different levels of nutrition on oxidative status in calves are less explored and data about the oxidative status in calves until weaning and thereafter are limited (Ranade et al.,

2014). There are some studies that focused on newborn calves (Albera and Kankofer, 2011; Vannucchi et al., 2019) or tested the effect of supplementation of vitamins or minerals on the calves' antioxidant system around weaning (Bordignon et al., 2019).

Due to the abrupt environmental change at birth, from the hypoxic intrauterine to the hyperoxic environment, the cells of neonates react by generating large amounts of ROS which can cause oxidative stress in neonates (Mutinati et al., 2014; Ranade et al., 2014). In addition, it was assumed that the cellular antioxidative defence mechanisms in human neonates are not completely developed and could not adequately compensate for the increase in ROS which may lead to several neonatal diseases (Saugstad, 1990, 1996). In contrast, Gaál et al. (2006) concluded that newborns are prepared to deal with oxidative stress.

The colostrum intake might also influence the oxidative status in neonates since several ROS generated by activated phagocytes, enzymes such as the xanthine oxidase and the lactoperoxidase are present in colostrum (Mutinati et al., 2014). Other factors influencing the antioxidative capacity such as caseins and whey proteins, SOD, catalases, GSH-Px, and non-enzymatic antioxidants such as lactoferrin, vitamins, and flavonoids also play a role (Przybylska et al., 2007). Selenium, copper, zinc, manganese, and iron are important components of the antioxidative enzymes mentioned before (Sordillo, 2016). Hence, a balanced supplementation of dietary micronutrients such as vitamins and trace elements is crucial for the antioxidative capacity and oxidative status in animals (Sordillo, 2016).

Few studies are investigating the oxidative status in calves and limited information is available on physiological changes in oxidative biomarkers in calves (Ranade et al., 2014). Therefore, one reason for the study which is presented in this thesis was to contribute more basic data to this research field in calves.

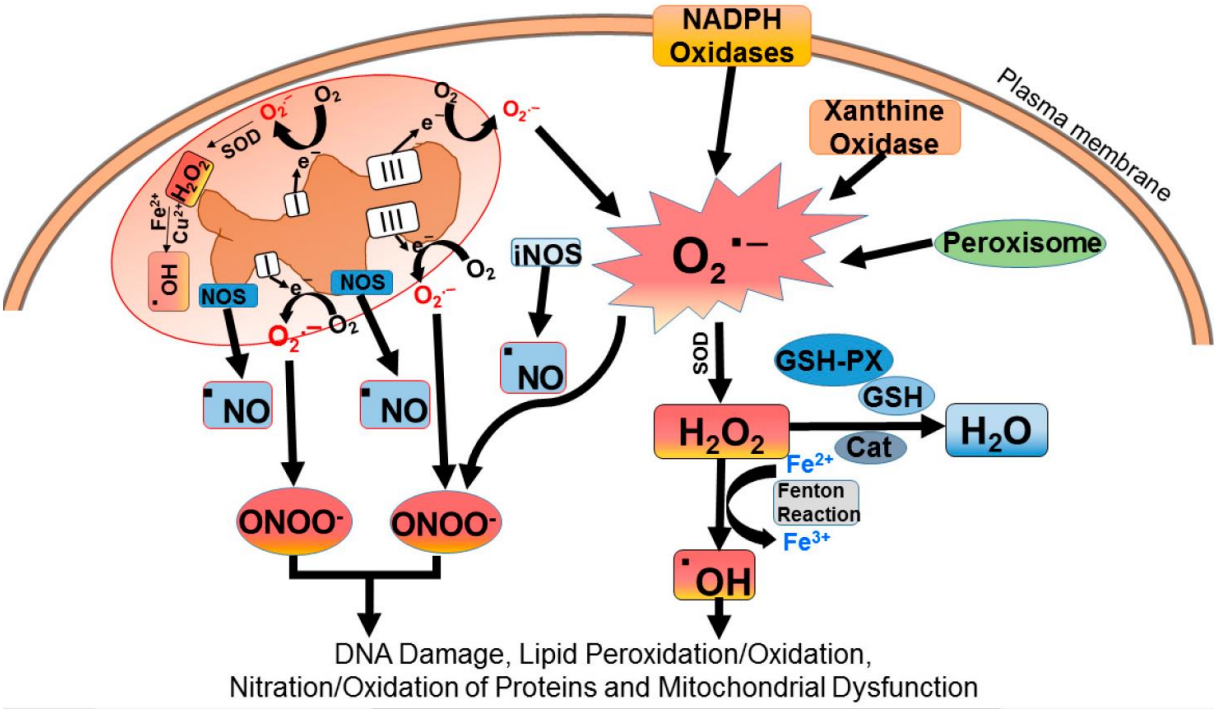


Figure 4. Generation of reactive oxygen species (ROS) in mammalian cells. ROS are generated by enzymatic and non-enzymatic redox reactions during cellular metabolism under normal and pathological conditions. Within mitochondria, the plasma membrane enzyme NADPH oxidase, peroxisomes, and the cytosolic xanthine oxidase generate first the superoxide anion ($O_2^{\bullet-}$), which becomes the free radical precursor for the generation of other ROS molecules. Cytosolic Cu, Zn superoxide dismutase (SOD) and mitochondrial Mn-SOD dismutate $O_2^{\bullet-}$ to H_2O_2, which yields in highly reactive hydroxyl radicals ($^{\bullet}OH$) by interaction with reduced transition metal ions (such as Fe or Cu) in a Fenton reaction (Haber-Weiss reaction). In addition, cells also generate reactive nitrogen species (RNS). The major RNS include nitric oxide ($^{\bullet}NO$), peroxynitrite anione ($ONOO^-$), and nitrogen dioxide ($^{\bullet}NO_2$). Nitric oxide ($^{\bullet}NO$) is produced by three isoforms of nitric oxide synthase (NOS). The ROS produced cause oxidative damage, including DNA damage, lipid and protein oxidation, protein nitration, and mitochondrial dysfunction. The glutathione peroxidase (GSH-Px) catalyses the conversion from H_2O_2 to H_2O and thus prevents the accumulation of H_2O_2 in the cell. Modified from Kaushal et al. (2019).

2 Objectives

The objectives of this doctoral thesis and project were to test for potentially positive effects of a high allowance for MR (HIGH, 10 L/d) as compared to restrictive MR allowance (RES, 5.7 L/d) until weaning at wk 14 of age and postweaning until wk 20 of life on growth, development, and especially of the developing mammary gland parenchyma (PAR) and its attached tissue structures, as well as on health, and the oxidative status. Additionally, we assessed the effects of a high and restrictive MR allowance on feed intake and feeding behaviour, body weight gain, and body growth.

The overall objectives of this thesis project were:

1) The characterisation of the systemic oxidative status in the early life, by assessing both the damage caused by oxidative stress to lipids (TBARS) and to proteins (AOPP). In addition, the measurement of the antioxidative capacity as FRAP, of the antioxidant selenoenzyme GSH-Px as well as of oxidants as dROM in plasma.

2) The investigation of the mammary gland and its structural development in calves' preweaning and postweaning phase in the early life by using non-invasive ultrasonographic technology, and the evaluation of changes in tissue structures via brightness measurements as pixel values in ultrasonic images.

3) The analysis of the effects of the MR feed allowance within an prolonged preweaning period of 14 weeks on feeding behaviour assessed by an automated feeding system for both MR and concentrate on body growth by measuring several variables of calves' body dimensions and health by weekly veterinary health checks.

It was hypothesized that a higher allowance for MR for 14 weeks of age in comparison to restrictive MR feeding on levels fed conventionally, will improve body growth, mammary gland development, and calf welfare, forming the basis for a long-term positive development for a good performance in the first lactation.

3 Manuscript I (published in Antioxidants)

antioxidants

MDPI

Antioxidants 2021, 10, 260.

https://doi.org/10.3390/antiox10020260

Article

Characteristics of the oxidative status in dairy calves fed at different milk replacer levels and weaned at 14 weeks of age

Katharina Diana Seibt [1], Morteza Hosseini Ghaffari [1], Theresa Scheu [2], Christian Koch [2], and Helga Sauerwein [1,*]

[1] Institute of Animal Science, Physiology Unit, University of Bonn, Katzenburgweg 7, 53115 Bonn, Germany; katharina.seibt@uni-bonn.de (K.D.S.); morteza1@uni-bonn.de (M.H.G.);
[2] Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, 67728 Münchweiler an der Alsenz, Germany; C.Koch@neumuehle.bv-pfalz.de (C.K.); T.Scheu@neumuehle.bv-pfalz.de (T.S.);
* Correspondence: sauerwein@uni-bonn.de (H.S.)

Abstract: A paradigm shift in the way of rearing heifer calves from restricted feeding and early weaning towards greater feed allowances and later weaning ages is ongoing. We aimed at characterizing the oxidative status in Holstein heifer calves fed with milk replacer (MR) at either a restrictive (RES) or a high (HIGH) level for 14 weeks. We compared two groups: HIGH (10 L MR/d, n = 18) and RES (5.7 L/d, n = 19) from day five until week 14 of life. In blood samples collected at birth, and then fortnightly from week 8–16, and in week 20, the antioxidative capacity measured as ferric reducing ability of plasma (FRAP), oxidative damage of lipids measured as thiobarbituric acid reactive substances (TBARS) and oxidative damage of proteins measured as advanced oxidation products of proteins (AOPP), free radicals measured as reactive oxidative metabolites (dROM), and the glutathione peroxidase (GSH-Px) activity, as well as leptin, adiponectin and haptoglobin were assessed. The time course of these variables during the first 20 weeks of life showed characteristic patterns; group differences were limited to adiponectin, AOPP, and FRAP. RES calves had lower growth rates, showed signs of hunger, but did not differ from HIGH in their intake of solid starter feed and in health status. This work characterizes the changes in oxidative status of dairy calves with increasing age and confirms the benefits of a high feeding plane with regard to welfare and development.

Keywords: dairy heifer; calf; oxidative status; development; adiponectin; leptin; haptoglobin

Academic Editor: G. Andres Contreras

Received: 18 December 2020

Accepted: 2 February 2021

Published: 8 February 2021

1. Introduction

The development of dairy heifers in early life is considered as one of the most influential factors for the future health and profitability of dairy cows [1]. Growth and development are mainly affected by energy intake and the genetic potential of a calf [2]. Calves are born as functional monogastrics and their nutrient and energy intake is initially based on the intake of whole milk or milk replacer (MR) until transformation to mature ruminants [3–5].

If a calf is reared with its dam, the calf receives whole milk ad libitum, and up to 12 L/d are consumed divided into 8 to 12 meals/d [6,7]. The natural weaning process occurs gradually over several weeks at approximately 10 months of age [8,9]. In contrast, it is still common practice to feed dairy calves restrictively, i.e., 4–6 L/d, or 10% of body weight (BW) [10] and they are commonly weaned at 8 weeks of age (2 months) [2] to stimulate solid feed intake, which should advance rumen development [3] and thus reduce rearing costs by achieving greater weight gains from starter feed, which, in turn decreases age at first calving [2]. Hence the weaning process in conventional dairy farming is conflicting in several aspects with the natural weaning process.

However, there is growing evidence that increasing the daily allowance of MR for calves is superior to the restricted intakes in terms of growth, development [5,11] and also of welfare, by reducing hunger and avoiding abnormal behaviors [12]. Furthermore, an increasing daily MR allowance supports greater BW gain, improved feed efficiency, reduced incidence of disease and greater opportunity to express natural behaviors, which in combination suggest improved welfare [13]. An intensive preweaning feeding regime, i.e., an increase of the daily allowance of MR to amounts that are close to calves' ad libitum intake (10–14 L/day) [5,11] augments average daily gain (ADG) in calves which seems to promote milk yields in the first lactation [1].

Not only the plane of milk feeding is important, but also the weaning age. The calves' energy intake via milk is crucial for development until they are mature ruminants. In conventional rearing, calves are weaned at 8 weeks (wk) of life, but the number of studies with

weaning ages up to 10 wk of life increased in the past decades [2,14], but there are just a few studies on later weaning ages around 11 wk of life [15] and 14 wk [7] or 17 wk of life [16]. These studies consistently showed that delaying the weaning age is beneficial for development and reduces weaning stress by reducing the drop in energy intake and the behavioral signs of hunger that commonly occur during weaning [6,15]. The effects of different planes of nutrition in extended preweaning periods are thus less explored, and data about the oxidative status in calves until weaning and thereafter are limited [17]. Some studies focused on newborn calves [18,19] or tested the effect of supplementing vitamins or minerals on the calves' antioxidant system around weaning [20]. In order to extend the existing knowledge, our objectives were to test for potentially positive effects of a high allowance for MR (HIGH, 10 L/d) as compared to restrictive MR allowance (RES, 5.7 L/d) for 14 wk MR feeding on growth and health, and to characterize the oxidative status until weaning and postweaning until wk 20 of life. We therefore assessed the effects of a high and restrictive milk replacer allowance on feed intake, feeding behavior, weight gain, health status and the circulating concentrations of the acute phase protein haptoglobin (Hp) of two adipokines, i.e., adiponectin and leptin, which are known to be associated with insulin responsiveness and body fat content, respectively. For characterizing systemic oxidative status, we addressed both the damage caused by oxidative stress on lipids (measuring thiobarbituric acid reactive substances, TBARS) and on proteins (assessing advanced oxidation products of proteins, AOPP). In addition, antioxidant activity (measured as ferric reducing ability of plasma, FRAP), the antioxidant selenoenzyme glutathione peroxidase (GSH-Px) as well as pro-oxidants (derivatives of reactive oxygen metabolites, dROM), were investigated.

We hypothesized that HIGH fed calves in comparison to RES would show higher rates of development without extreme drops after weaning, fewer signs of hunger and good health condition. We further assumed that feeding high planes of MR would reduce weaning stress and also alter the blood profiles of markers of oxidative status, as well as of Hp, leptin, and adiponectin.

2. Materials and Methods

The present study was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a. d. Alsenz, Germany, following the guidelines of the German Law for AnimalWelfare by permission of the local authority in charge (G 17-20-071; Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

2.1. Animals, Diets, Feeding and Management

Thirty-seven German Holstein heifer calves were studied from birth until wk 20 of life, from February 2018 until March 2019. To ensure a controlled colostrum intake within the first three hours of life, the parturitions were monitored by a calving sensor system Moocall (Moocall LTD, Bluebell, Dublin, Ireland). Only healthy calves born without dystocia were included in this study. At least 3 L colostrum of the respective dam were administered via bottle to each calf within the first 3 h after birth. If a calf was not drinking the whole amount colostrum by itself, it was drenched. In the case of insufficient colostrum quantities available from the dam (n = 4), high-quality colostrum stored at -20 °C was added to reach the amount of 3 L. The quality was assessed with a Brix refractometer (Digital hand-held PAL-S refractometer, ATAGO, Tokyo, Japan) and specific gravity (SG) by a spindle (Kruuse Colostrum Densimeter, Kruuse UK Ltd., Langeskov, Denmark). The total protein content in calves' serum within 48 h of life was also assessed by optical refractometer measurements (Euromex Microscopen bv, Arnhem, The Netherlands). Each calf received 10 mL of an iron suspension per oral administration (Sinta fer-o-bac, 115 mg Fe 3^{+}/mL, Sinta GmbH, Schwarzenborn, Germany), and navels were disinfected with a 10% povidone-iodine solution (Vet-sept solution, aniMedica GmBH, Senden-Bösensell, Germany) or Engemycin® spray (Intervet Productions Srl., Aprilia, LT, Noale, Italy) to protect against bacterial infections.

From birth until day 10 of life, the calves were kept in straw-bedded single hutches and were fed manually twice per day with teat buckets at the regular feeding times of 12 h intervals. Until the 9th meal (day five of life) all calves received 5 L twice per day of their dam's acidified transition milk (2 mL/L of Schaumacid acidifier, H.Wilhelm Schaumann GmbH, Pinneberg, Germany) in the morning and evening, respectively. Transition milk was also supplemented with a mix of trace elements and vitamins (1 mL/L Milkivit Quick- Mix, Trouw Nutrition Deutschland GmbH, Burgheim, Germany). Thereafter the calves were assigned to two different feeding groups considering their birth weight, sire, and parity of the dam to create equal groups. Half siblings were also considered and were equally distributed on the feeding groups. From the 10th meal (day five of life) onwards calves received milk replacer (MR; 14% solids; Milkivit Titan, Trouw Nutrition Deutschland GmbH) either at 10 L/d (HIGH; 1.4 kg MR powder/d; n = 18) or at 5.7 L/d (RES; 0.8 kg MR powder/d; n = 19). During this time, from birth until the 20th meal (day 10 of life), the calves were kept in straw-bedded single hutches and the intake of transition milk and MR was documented by weighing the amount in buckets before feeding and the residues in the bucket before offering the next meal. While the calves were in single hutches, they were provided with fresh water ad libitum in buckets and small

hayracks every day. Afterwards, at day 10 of life, the calves were moved to an open straw-bedded stable where they were kept in a group. Newly incoming calves were assigned to a "baby group", in a separated compartment within the stable for the first five days. This "baby group" was created for enabling the calves to learn to suckle and drink on the automated feeding system without having to compete with older calves.

In the stable, from day 10 (wk 2) to day 98 (wk 14) of life, an automated feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany) for calf starter concentrate and MR ensured milk feeding according to respective feeding regime. The intake of both MR and pelleted calf starter concentrate (Blattin Kälberstart Gold, Höveler Spezialfutterwerke GmbH & Co. KG, Dormagen, Germany), as well as the number of rewarded and unrewarded visits and the drinking speed were recorded individually by the feeding system and its software (Förster-Technik GmbH).Water, hay and calf starter were offered ad libitum. The daily water intake was not recorded, neither in single hutches nor in group pens. The ingredients and the chemical composition of the MR and starter (concentrate) are given in Table 1. At day 15 of life, the calves were moved from the "baby group" to group pens that were also equipped with an automated feeding system (Förster-Technik GmbH) until weaning starting at day 84 (wk 12 of life). In week five5 of life, all calves were dehorned under sedation and with local anaesthesia. The weaning process comprised a gradually reduction of daily MR allowance (by 0.3 L/d in RES and by 0.6 L/d in HIGH) over 2 weeks, i.e., until day 98 (week 14) of life, i.e., the last two days of MR feeding the maximum amount was 2.6 L/d in HIGH and 2.2 L/d in RES and on the last day for both feeding groups 2 L/d. From day 99 onwards the MR supply was stopped (0 L/d) and calves were moved to a new group pen in another stable where they had free access to water and a total mixed ration (TMR) for heifers (Table 1).

Table 1. Ingredients and composition of milk replacer (MR) and other feed.

Item (% of DM)	MR[1]	Starter / Concentrate[2] (g/kg)	TMR[3]
Grass silage	-	-	33.3%
Maize silage	-	-	20.7%
Hay	-	-	8.5%
Wheat straw	-	-	5.7%
Concentrate	-	-	31.8%
Chemical composition, % of DM			
DM, g/kg FM	-	-	49
CP	22	20	13.8
Crude fat	19	3.9	3.7
Crude fiber	0.1	5.2	21
$aNDF_{OM}$[5]	-	-	43.8
ADF_{OM}[5]	-	-	22.9
Ash	6.5	7.1	8.5
NFE[6]	52.4	63.8	-
Total sugar	45.0	ND[4]	-
ME[7], MJ/kg of DM	18.6	11.2	10.7
Ca	1	1	0.58
P	0.7	0.6	0.33
Na	0.4	0.4	0.1
Mg	ND[4]	ND[4]	0.18
K	ND[4]	ND[4]	18.5
Lysine	1.9	ND[4]	ND[4]
Methionine	0.7	ND[4]	ND[4]

[1] MR ingredients: 50% skim milk powder, 24.5% whey powder, 17.5% vegetable oil, 2% glucose, 1.5% wheat soak powder, 0.5% whey protein powder (Milkivit Titan, Trouw Nutrition Deutschland GmbH, Burgheim, Germany). Additional MR ingredients per kg of DM: 200 mg Vitamin E; 150 mg Vitamin C; 75 mg Fe; 6 mg Cu; 85 mg Zn; 30 mg Mn; 1 mg calcium iodate; 0.3 mg Se (Milkivit Titan, Trouw Nutrition Deutschland GmbH, Burgheim, Germany). [2] Starter ingredients (per kg): 50 mg Vitamin A, Vitamin D3, and Vitamin E; 6 mg Cu, 40 mg Zn; 50 mg Fe; 40 mg Mn; 1.2 mg calcium iodate Ca; 0.2 mg Se; soya extraction meal, wheat bran, wheat, maize, wheat gluten, beet pulp, linseed, beet molasses, barley, calcium carbonate, sodium chloride, and monocalcium phosphate (Blattin Kälberstart Gold, Höveler Spezialfutterwerke GmbH & Co. KG, Dormagen, Germany). [3] Total mixed ration (TMR) fed after weaning from wk 14 of age onwards. [4] not determined (ND). [5] Neutral detergent fibre (aNDF) was assayed with a heat-stable amylase. aNDF and acid detergent fiber (ADF) are expressed as related to organic matter ($_{OM}$), exclusive of residual ash. [6] Nitrogen-free extract (NFE), calculated as NFE = 100 − (CP + crude fat + crude fiber + ash); according to Frieten et al. (2017) [11] and the National Research Council [21]. [7] ME (metabolizable energy). The ME content of MR was calculated using the equation: ME, MJ/kg of DM = (24.2 × CP + 36.6 × fat + 17.0 × total sugar)/100 × 0.97 GE × 0.96 DE; according to Frieten et al. (2017) [11] and the National Research Council [21].

2.2. Records of Health Status and Body Weight

Birth weight was measured after colostrum intake whereby the amount of ingested colostrum was subtracted. The health status was checked weekly by veterinarians over the entire 20 weeks of the trial. On the day of the weekly health check, body weight (BW) was recorded until week 20 of life using a mobile electronic scale (KWM GmbH Waagen- und Metallbau, Thiersheim, Germany). The variables assessed during the health check and the score assigned are summarized in the Supplementary Table S1. The health check data of all time points and each calf were categorized in one summarizing health score as follows: 1 = checks without any health disturbances and thus entirely healthy, 2 = checks with one health disturbance, but not diseased, 3 = checks indicating disease and/or >40 °C rectal temperature, and 4 = checks with rectal temperature >39.5 °C without any other symptoms indicating disease or disorders. Health disturbances or appearing cases of diseases were further categorized as: 1 = rectal temperature > 39.5 °C, 2 = digestion, 3 = respiration, 4 = eye-related, and 5 = navel infection. In the case of health disturbances, such as diarrhea and infection of the navel, the calves were treated by a veterinarian and the incident was documented.

2.3. Calculation of Average Daily Gain (ADG) and Energy Intake

The ADG was calculated by dividing the weekly weight gain by the number of days between each weighing. The daily intake of metabolizable energy (ME) via MR and concentrate was calculated by multiplying the individual daily intake of each calf by the ME content (MJ/kg of DM) of MR (18.6), and starter concentrate (11.2). The ME content of MR was calculated using the equation: ME, MJ/kg of DM = (24.2 × CP + 36.6 × fat + 17.0 × total sugar)/100 × 0.97 gross energy (GE) × 0.96 digestible energy (DE), as detailed by Frieten et al. (2017) [11] and the National Research Council [21].

2.4. Blood Sampling and Analyses

Blood samples were taken by jugular vein puncture from 36 calves seven time points, i.e., 36 48 h after birth, from week 8 until week 16 of life in two-week intervals (week 8, 10, 12, 14, 16), and in week 20 of life. Out of a total of 37 calves, blood samples were taken from only 36, because one calf failed to complete the sampling schedule and was therefore excluded. This calf was neither diseased nor dead. Blood samples were also collected from the respective dams by coccygeal vein puncture 36–48 h after calving. Blood was collected in 10 mL serum and EDTA-plasma tubes (Sarstedt, Nümbrecht, Germany). The EDTA plasma samples were centrifuged at 4 °C for 20 min at 3000 × *g*, whereas serum tubes were incubated for 45 min at room temperature (RT) for clotting before centrifugation for 10 min at 3000 × *g* at room

temperature. Afterwards, serum and plasma were aliquoted and stored at -20 °C until analysis. The total protein content in the first serum samples (36–48 h after birth/*post natum* (p.n.)) was determined by an optical refractometer (Euromex Microscopen B.V., Arnhem, Netherlands). Haptoglobin (Hp), leptin and adiponectin in serum were measured using in-house developed ELISAs [22–24]. The mean intra and interassay coefficients of variation (CV) were 9.9% and 12.1% for Hp, 6.7% and 11.3% for leptin, and 9.6% and 11.2% for adiponectin, respectively.

2.5. Measurements of the Oxidative Status in Plasma

Reactive oxidative species were measured using the dROM test (detection of reactive oxygen metabolites) according to Alberti et al. (2000) [25] with modifications [26] using N, N-diethyl-para-phenylendiamine as chromogenic substrate. The dROM values are expressed as H_2O_2 equivalents. The mean intra-assay CV was 2.11% and the interassay CV was 10.6%. The antioxidative capacity was assessed as FRAP (ferric reducing ability of plasma) according to Benzie and Strain (1996) [27] with a mean intra-assay CV of 2.94% and an interassay CV of 5.20%. The values are given as µmol Fe^2+/L. The concentrations of AOPP (advanced oxidation products of proteins) were measured according to Witko-Sarsat et al. (1996) [28], with a mean intra-assay CV of 1.24% and the interassay CV of 3.16%. The AOPP values are expressed per volume unit (µmol/L) as well as per g of total protein (µmol/g). For this purpose, the total protein concentration in plasma was assessed by the Bradford method [29] using BSA as standard. The mean intra-assay CV was 1.83% and the mean interassay CV was 5.56%. In addition, the TBARS (thiobarbituric acid reactive substances) test [30] was used for measuring oxidized lipids in plasma with a mean intra-assay CV of 6.18% and a mean interassay CV of 9.08%. The concentrations are given as nM malonyldialdehyde (MDA) which was used as standard. The activity of the selenium-dependent glutathione peroxidase (GSH-Px) in plasma was determined spectrophotometrically according to original method proposed by Paglia and Valentin (1967) [31]. One unit (U/mL) equates to the amount of enzyme necessary to catalyse the oxidation of 1 µmol NADPH per min at 25 °C, pH 7.0. The mean intra-assay CV was 4.04% and the mean interassay CV was 4.40%.

2.6. Statistical Analyses

Data were statistically analyzed using SPSS (version 26; SPSS Inc., Chicago, IL, USA). The normal distribution was checked by the Kolmogorov-Smirnov-test and the homogeneity of variance with the Levene's test. If the data were not normally distributed, nonparametric tests were performed and data were log-transformed for further statistical analysis. For the identification of outliers in metabolic hormones, Hp, and oxidative parameters, box plots of

each raw data set were checked, and Z-standardization was performed. Outliers in total protein, AOPP, TBARS, dROM, FRAP, GSH-Px, Hp, and leptin were identified as values with SD > 2, in Adiponectin with SD > 3 (Z-standardization) and were excluded. Furthermore, data were analyzed by a linear mixed model with Bonferroni post hoc tests and with repeated measurements, considering the individual calf as a random effect and feeding group (group), and week of life (time) and group by time interaction as fixed effects. Differences between the groups within each time point were tested with Student´s t-test or Mann-Whitney-test if data were not normally distributed and homogeneity of variance was not given. Health check score categories in the two feeding groups were compared by the Chi-square test. Correlations (ρ) were calculated by Spearman analysis. Results were declared as significant when $p < 0.05$, and $0.05 \leq p < 0.10$ was considered as a trend. In all graphs, non-transformed data are shown as means ± SEM.

3. Results

3.1. Birth Weight and Colostrum Intake

Within the first 2 h after birth, each calf received at least 3 L of colostrum. In total, 28 calves drank the whole amount of offered colostrum by themselves, five calves had to be drenched and four calves have been additionally drenched the rest of the colostrum to reach the whole amount of 3 L. The following data are reported as means ± SD. Birth weight in group HIGH (10 L/d, n = 18; 39.7 kg ± 6.0) was not different ($p = 0.472$) as compared to group RES (5.7 L/day, n = 19; 41.0 kg ± 4.1). The first colostrum intake was similar in both groups (HIGH 3.3 kg ± 0.8; RES 3.3 kg ± 0.6; $p = 0.327$). Colostrum quality was not different between the groups: The mean Brix values in HIGH were 23.5% ± 3.9 and 25.3% ± 3.9 in RES, respectively ($p = 0.209$). Based on the measured Brix values of >22% and at least 3 L colostrum intake in all calves, a supply of at least 150 g IgG with the first meal was expected [32]. The second meal of dams' milk (5 L) was fed no later than 12 h after birth. The specific gravity (SG) as assessed by spindle was >1045 in both groups, and thus colostrum was classified as good quality according to the reference zone on the spindle provided by the manufacturer (Kruuse UK Ltd., Langeskov, Denmark). However, the spindle values in the HIGH group were slightly lower than in the RES group (HIGH 1047 SG ± 5.7 vs. RES 1052 SG ± 4.7; $p = 0.002$). The protein concentration (g protein/100 mL) in serum, 36 - 48 h after birth, measured by optical refractometer, was similar between both groups and thus confirmed an adequate intake of colostrum of good quality (HIGH 5.4 ± 0.5; RES 5.6 ± 0.7; $p = 0.377$).

3.2. Intake of Milk, Starter, and Metabolizable Energy (ME)

The daily milk and MR intake differed between the two groups throughout the differential liquid feeding period from day five until the end of weaning at 14 weeks of age (Figure 1a, Table 2). The data reported in this text section are means ± SD. During the first five days of life, milk intake was 7.1 L/d ± 2.4 in the HIGH group and 6.9 L/d ± 2.3 in the RES group, without differences between the groups (p = 0.536). From week 2 (beginning of differential feeding) until the end of week 12 (onset of weaning), the RES calves consumed 5.5 ± 0.4 L MR/d, whereas the HIGH calves drank 9.2 L/d ± 0.9 L MR/d (Figure 1a). The mean starter intake was not different between both groups over the entire milk-drinking period until week 14 (Figure 1b, Table 2) and was negligible in both feeding groups in the first 6 weeks of life. Thereafter it increased to 2.9 kg/d ± 1.2 kg/d in wk 14 at the end of weaning. Figure 1c shows the ME intake of both feeding groups. HIGH had greater daily ME intakes than RES over 12 weeks of life until the beginning of weaning. This difference leveled off during the weaning process (weeks 13 and 14 of life, Figure 1c).

Table 2. Preweaning intakes (means ± SEM) of transition milk (whole milk of the dam, first five days of life) or/and milk replacer (MR), of starter, and of metabolizable energy (ME), total number of visits per day (no./d) at the automatic milk feeding station per day, as well as the number of unrewarded, rewarded visits, and drinking speed of calves with preweaning high allowance to MR (HIGH, 10 L/d) or restrictive allowance (RES, 5.7 L/d). The MR intake was gradually reduced in week (wk) 13 and 14 to 2 L/d in both groups. Pre and postweaning until wk 20 (means SEM) body weight and average daily gain (ADG) of these calves.

Variable [1]	Feeding Group		SEM	*p*-Value		
	HIGH	RES		Group (G)	Week of Life (T)	G × T
Milk & MR intake, L/d	8.43	5.25	±0.09	**<0.01**	**<0.01**	**<0.01**
Starter intake, kg/d	0.77	0.86	±0.07	0.16	**<0.01**	0.75
Total ME intake, MJ/d	29.72	22.35	±0.64	**<0.01**	**<0.01**	**<0.01**
Total visits, no./d	12.07	20.91	±0.59	**<0.01**	**<0.01**	0.87
Unrewarded visits, no./d	5.28	15.72	±0.52	**<0.01**	**<0.01**	0.62
Rewarded visits, no./d	6.74	5.19	±0.14	**<0.01**	**<0.01**	0.27
Drinking speed, mL/min	488.68	479.00	±6.08	0.91	**<0.01**	0.68
Body weight, kg (wk 1–20)	107.86	99.14	±2.18	**<0.01**	**<0.01**	0.22
ADG, kg/d (wk 1–20)	0.98	0.89	±0.03	**0.02**	**<0.01**	<0.01

[1] Values are presented as means ± SEM.

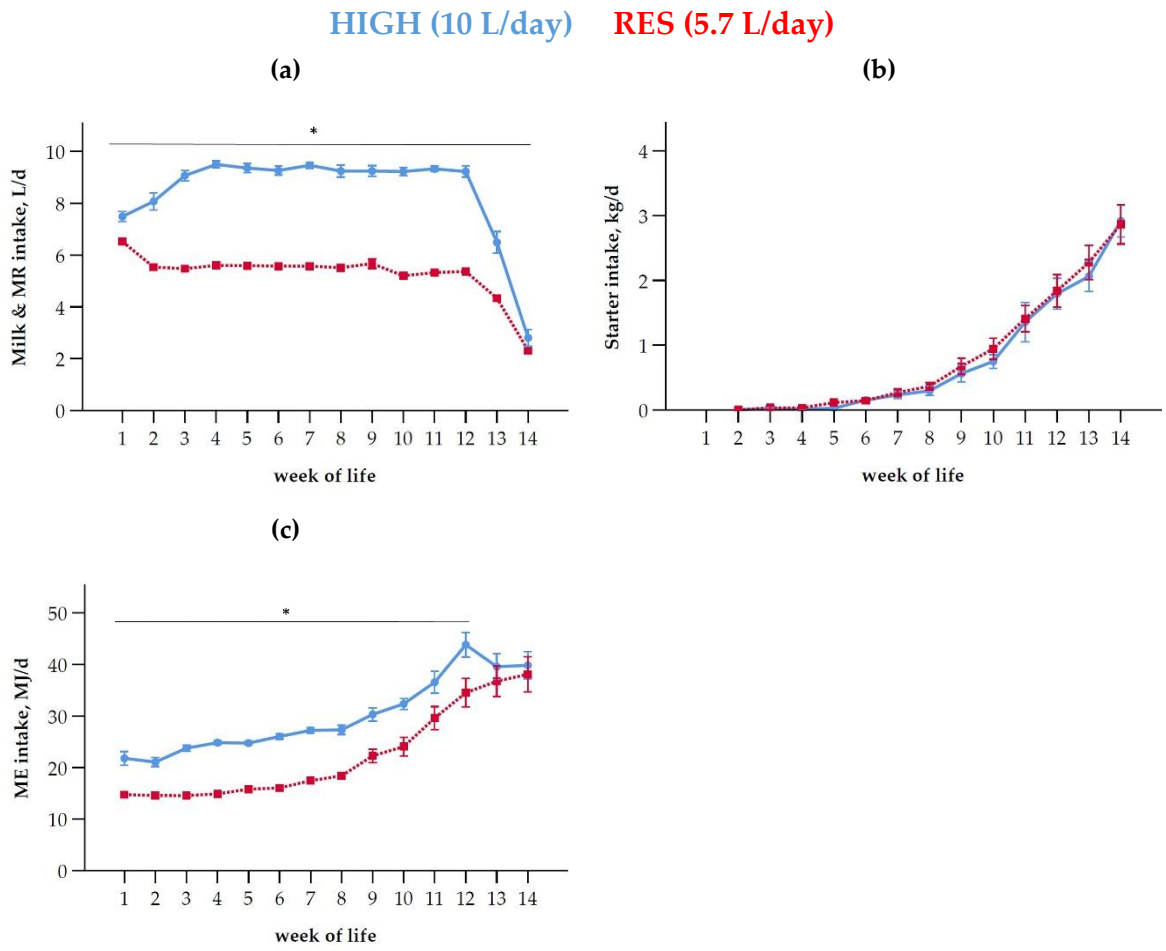


Figure 1. Preweaning intakes (means ± SEM) of (a) transition milk (whole milk of the dam, first 5 d of life) or/and milk replacer (MR), (b) starter, and (c) of metabolizable energy (ME) of calves with preweaning high allowance to MR (HIGH; blue line) or restrictive allowance (RES; red line). The MR intake was gradually reduced in week 13 and 14 to 2 L/d in both groups. Asterisks indicate differences ($p < 0.05$) between groups within individual time points (week of life).

3.3. Rewarded and Unrewarded Visits

Over the entire liquid feeding period, the RES group visited the automatic milk feeder more often per day than the HIGH group (Figure 2a, Table 2). In week 5, the number of visits/day (means ± SD) peaked in both groups, whereby RES had 1.6-fold more visits than HIGH (26.5 ± 10.1 vs. 16.4 ± 9.6; $p = 0.004$). The RES calves also had more unrewarded visits over the entire time until week 14 of life (Figure 2b, Table 2). From week 3 until week 11, HIGH calves had more rewarded visits than RES (Figure 2c). During the linear weaning, in week 13 ($p = 0.76$), but not in week 14 ($p = 0.023$), the number of rewarded visits per day was not different between HIGH and RES anymore (Figure 2c), while unrewarded visits were still higher in RES (Figure 2b). In both groups, the drinking speed was increasing with age until

week 12 and was not different between the groups (Figure 2d, Table 2). During weaning the situation changed and RES calves showed a higher drinking speed (mL/min, means ± SD) in week 13 than HIGH (551.7 ± 49.5 vs. 474.4 ± 122.8; $p = 0.01$), but not in week 14 ($p = 0.76$, Figure 2d).

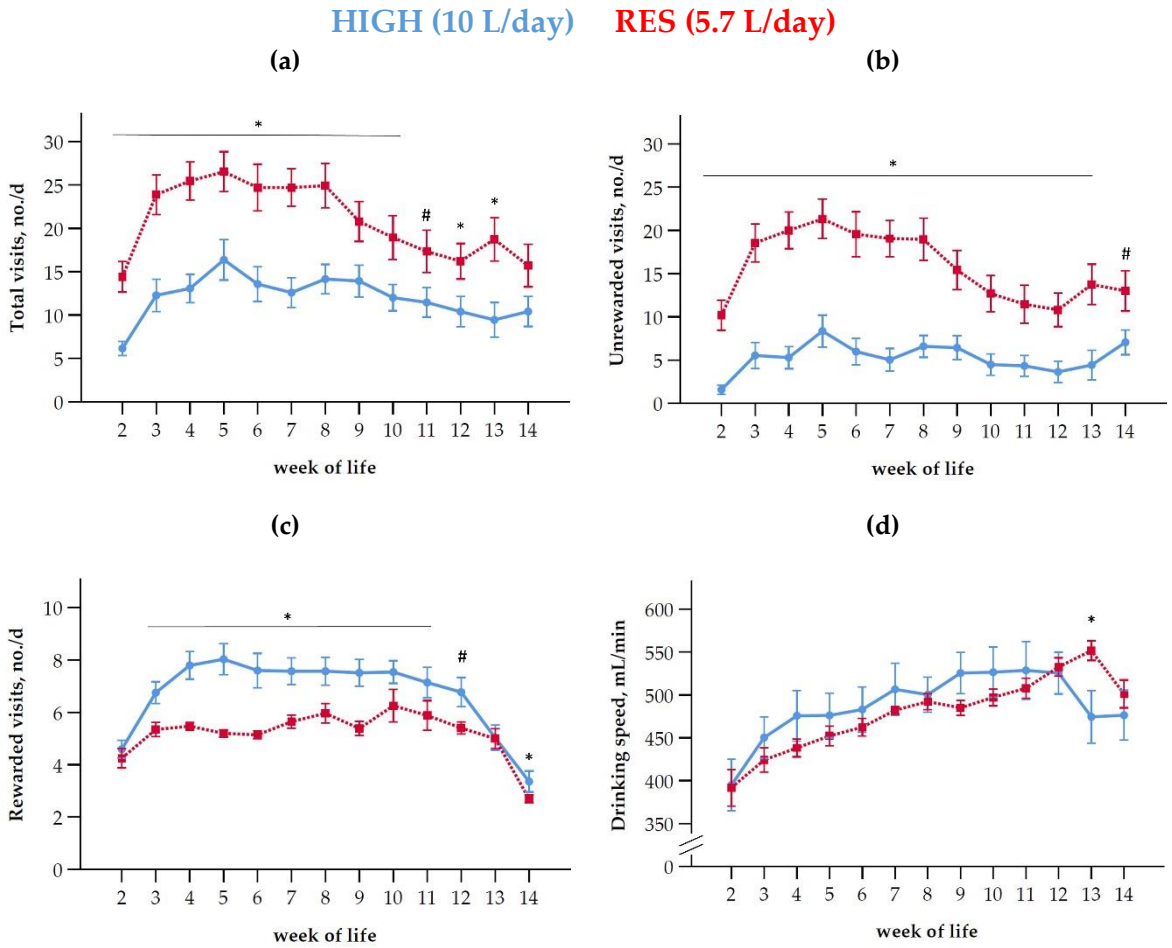


Figure 2. Preweaning number (means ± SEM) of (a) total visits at the automatic milk feeding station per day (no./d), (b) number of unrewarded, (c) rewarded visits, and (d) drinking speed in calves fed at a high level of MR (HIGH; blue line) or had a restricted MR allowance (RES; red line). For weaning, MR intake was gradually reduced in weeks 13 and 14 to 2 L/d in both groups. Asterisks indicate differences between groups within week of life ($p < 0.05$) and hashtags indicate trends ($p < 0.1$).

3.4. BW and ADG

On average, HIGH calves were heavier than RES over the entire observation period until 20 weeks (Figure 3a, Table 2). In none of the groups, weaning was associated with BW loss. Overall, both groups gained steadily in weight. The group differences in BW were significant from week 4 to 11, in week 13 during weaning, and in weeks 16–18 and 20 after weaning (Figure 3a).

The ADG fluctuated over the trial period and was affected by group, time, and the interaction thereof (Figure 3b, Table 2). On average, the ADG decreased until week 3 and increased until the end of weaning (week 14). Calves of HIGH gained more than RES from weeks 2–5 and in week 7 of life (Figure 3b). After weaning, calves were transferred to another stable with older calves and received a TMR (Table 1). After changing stables in week 15, a decline in ADG was observed in both feeding groups, which was more pronounced in the RES calves (1.5-fold lower) than in the HIGH ones in week 16, i.e., 2 weeks after weaning (Figure 3b).

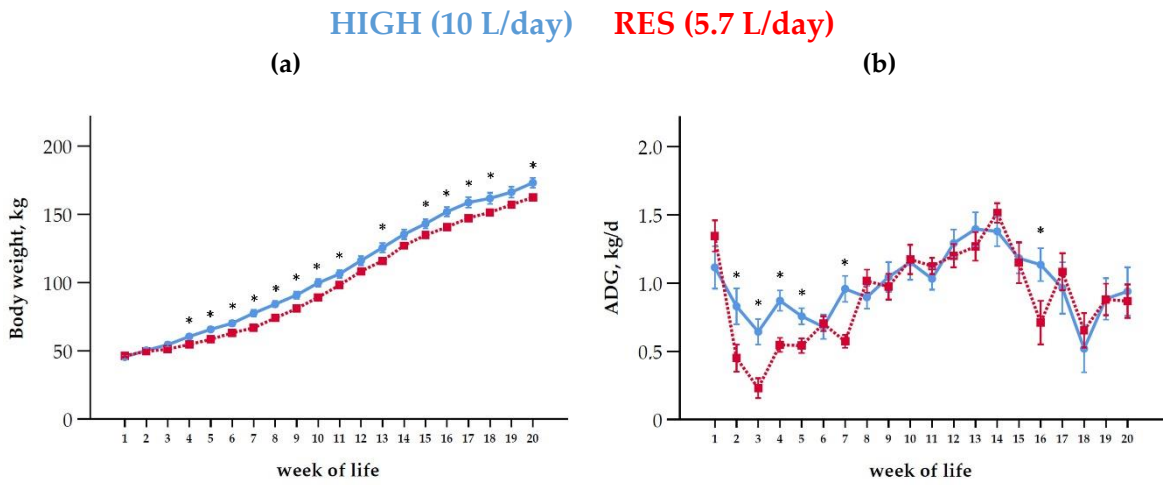


Figure 3. Pre and postweaning changes (means ± SEM) of (a) body weight and (b) average daily gain (ADG) of calves fed at a high level of MR (HIGH; blue line) or at a restricted level (RES; red line). Weaning was done by gradually reduced the MR allowance from week 13 to 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a TMR (Table 1). During the whole trial the animals had free access to water and hey. Asterisks indicate differences between groups within week of life ($p < 0.05$).

3.5. Health Records

Throughout the 20-week observation period, 23 calves (62.2%) remained entirely healthy without any clinical signs. In general, the incidence of health checks indicating a diseased calf (and/or >40 °C rectal temperature) within the entire 20 weeks of the trial was low: In 2.97% of all 740 health checks (22 findings in total), indications for a disease were observed: 14 animals (37.8%) were diseased once or more often, whereby the diseased animals were equally distributed between the two feeding groups, i.e., seven calves each. In week 6 of life after dehorning in week 5, the incidence of cases of fever or elevated body temperature (>39.5 °C) peaked. In the summarizing health score categories (Supplementary Table S1) of both groups, there was a trend ($p = 0.073$) for differences between groups, i.e., in HIGH 86.7% of all checks were in health score category 1 (completely healthy) and in RES calves 92.4% of all checks. In the further categorization of the occurring health disturbances and diseases no difference appeared between feeding groups.

3.6. Haptoglobin (Hp)

When considering all Hp values, there were neither time nor group effects, and also the interaction was nonsignificant. In total, 18 extreme Hp outliers (SD > 2; Z-standardization within each time point) were identified. By excluding these 18 outliers, there was a time effect ($p < 0.001$), but the feeding group had no effect (Figure 4a, Table 3). The extreme outliers (SD > 2 and Hp > 1000 µg/mL) were mainly observed in week 16 (n = 3, RES) and in week 20 of life (n = 4; equally in RES and HIGH). Additionally, in week 20, two outliers (one each in RES and HIGH) with 400 µg/mL < Hp < 1000 µg/mL were excluded. The Hp concentrations recorded in week 16 of these three calves (RES) were > 4000 µg/mL. These outliers, which were observed as elevated Hp values, could not consistently be attributed to clinical findings: Only one calf of the three calves with elevated Hp in week 16 had a fever and one calf of the six calves with elevated Hp in week 20 had an elevated rectal temperature (39.6 °C), whereas the remaining ones showed no signs of disease. Similarly, for the outliers identified in week 1 to week 14 (n = 9), concurrent clinical findings were limited to one calf, which showed a slight navel infection without fever or other health disturbances. It should be noted that health checks were done independently of the blood sampling. After birth (36–48 h), Hp concentration in the serum of calves were lower than in the serum of their respective dams after calving ($p < 0.001$), irrespective of the feeding groups (Table 3).

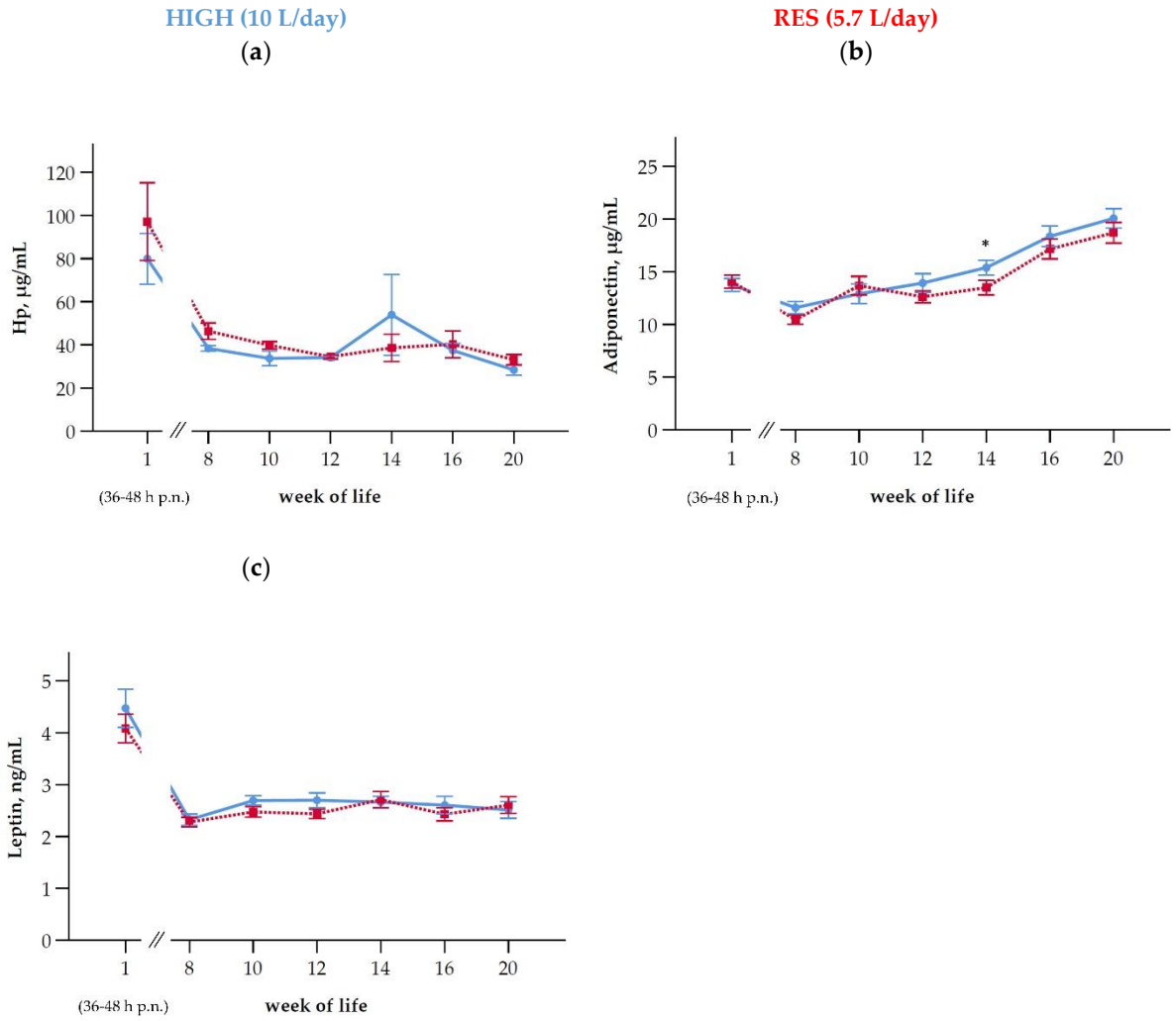


Figure 4. Time-dependent changes (means ± SEM) of (a) haptoglobin (Hp), (b) adiponectin, and (c) leptin in calves fed at a high level of MR (HIGH; blue line) or a restrictive level (RES; red line). The MR intake was gradually reduced in week 13 and 14 to 2 L/d in both groups. From week 15 until week 20 of life, the calves were moved to a new group pen in another stable receiving a TMR (Table 1). Asterisks indicate differences between groups within weeks of life ($p < 0.05$) and hashtags indicate trends ($p < 0.1$). After birth/*post natum* is abbreviated as p.n.

Table 3. Serum concentrations (means ± SEM) of haptoglobin (Hp), adiponectin, and leptin, and plasma concentrations (means ± SEM) of total protein content, advanced oxidation products of proteins (AOPP), thiobarbituric acid reactive substances (TBARS), reactive oxidative metabolites (dROM), ferric reducing ability of plasma (FRAP), and glutathione peroxidase activity (GSH-Px) in calves fed at a high level of MR (HIGH, 10 L/d) or a restricted level (RES, 5.7 L/d) and of their respective dams. The MR intake was gradually reduced in week (wk) 13 and 14 to 2 L/d in both groups. From week 15 until week 20 of life, the calves were moved to a new group pen in another stable receiving a TMR (Table 1). After birth/*post natum* is abbreviated as p.n. and after calving/*post partum* is abbreviated as p.p.

Variable [1]	Feeding Group		SEM	*p*-Value		
	HIGH	RES		Group (G)	Week of Life (T)	G × T
Haptoglobin (µg/mL)						
Dams (36-48 h p.p.)	780.69	813.99	±175.39	0.58	-	-
Calves (36–48 h p.n.)	79.94	97.09	±14.91	0.47	-	-
Calves (wk 8–20)	37.92	38.89	±2.53	0.14	**0.01**	0.39
Calves (wk 1–20)	44.09	47.34	±3.54	0.10	**<0.01**	0.48
Adiponectin (µg/mL)						
Dams (36–48 h p.p.)	5.72	5.94	±0.58	0.81	-	-
Calves (36–48 h p.n.)	13.75	14.06	±0.63	0.73	-	-
Calves (wk 8-20)	15.41	14.38	±0.43	**0.03**	**<0.01**	0.67
Calves (wk 1–20)	15.18	14.33	±0.38	**0.05**	**<0.01**	0.55
Leptin (ng/mL)						
Dams (36–48 h p.p.)	21.84	22.93	±0.99	0.44	-	-
Calves (36–48 h p.n.)	4.48	4.09	±0.32	0.39	-	-
Calves (wk 8–20)	2.58	2.49	±0.05	0.17	**0.03**	0.74
Calves (wk 1–20)	2.83	2.72	±0.08	0.11	**<0.01**	0.82
Total protein (g/L)					-	-
Dams (36–48 h p.p.)	55.32	56.06	±1.89	0.79	-	-
Calves (36-48 h p.n.)	44.1	45.43	±1.11	0.40	-	-
Calves (wk 8–20)	49.39	49.81	±0.50	0.51	**<0.01**	0.85
Calves (wk 1–20)	48.68	49.19	±0.48	0.34	**<0.01**	0.89
AOPP (µmol/g of Protein)						
Dams (36–48 h p.p.)	0.82	0.88	±0.05	0.40	-	-
Calves (36–48 h p.n.)	1.95	2.40	±0.22	0.16	-	-
Calves (wk 8–20)	0.91	0.99	±0.03	**0.03**	**<0.01**	0.41
Calves (wk 1–20)	1.05	1.20	±0.06	**0.01**	**<0.01**	0.42
TBARS (nM)						
Dams (36–48 h p.p.)	-	-	-	-	-	-
Calves (36–48 h p.n.)	406.81	393.74	±21.47	0.67	-	-
Calves (wk 8–20)	243.75	246.19	±4.36	0.90	**<0.01**	0.19
Calves (wk 1–20)	266.83	266.43	±2.88	0.91	**<0.01**	0.23
dROM (µg H_2O_2 equival./L)						
Dams (36–48 h p.p.)	-	-	-	-	-	-
Calves (36–48 h p.n.)	17.11	19.02	±1.51	0.29	-	-
Calves (wk 8–20)	33.92	31.61	±1.11	0.11	**<0.01**	0.24
Calves (wk 1–20)	31.65	29.93	±1.09	0.28	**<0.01**	0.15
FRAP (µM Fe^{2+}/L)						
Dams (36–48 h p.p.)	229.07	221.89	±8.55	0.55	-	-
Calves (36–48 h p.n.)	151.13	173.32	±7.43	**0.05**	-	-
Calves (wk 8–20)	161.77	168.32	±1.89	**0.01**	**<0.01**	0.12
Calves (wk 1–20)	160.35	169.05	±1.94	**0.001**	**<0.01**	0.10
GSH-Px (U/mL)						
Dams (36–48 h p.p.)	-	-	-	-	-	-
Calves (36–48 h p.n.)	0.011	0.012	±0.0006	0.42	-	-
Calves (wk 8–20)	0.01	0.01	±0.0002	0.69	**<0.01**	0.82
Calves (wk 1–20)	0.01	0.01	±0.0002	0.47	**<0.01**	0.86

[1] Values are presented as means ± SEM.

3.7. Metabolic Hormones

The concentrations of adiponectin in serum were affected by both group and time, without the respective interaction. Three outliers in adiponectin (SD > 3, Z-standardization) were observed in week 8, 10, and 12. Higher values were observed 36–48 h after birth (wk 1 of life) in both groups (13.9 ± 2.61 g/mL; mean ± SD) than in week 8 (11.0 ± 2.18 g/mL, $p < 0.001$), whereas there was no difference between week 1 and week 10, 12, and 14, respectively (Figure 4b). From week 14 onwards, the adiponectin concentrations were steadily increasing until week 20 (Figure 4b). In week 14 higher adiponectin concentrations in HIGH than RES appeared ($p = 0.048$; Figure 4b). In addition, the correlations between adiponectin and Hp and the parameters of the oxidative status were determined: Between adiponectin and Hp, there were no association detectable ($\rho = -0.044$, $p = 0.509$). Considering the indicators for oxidative status tested herein, there were weak correlations between adiponectin and AOPP (mol/g of protein; $\rho = –0.135$, $p = 0.037$) and dROM ($\rho = 0.376$, $p < 0.001$) detectable, but not between adiponectin, FRAP and TBARS, respectively ($\rho = –0.077$, $p = 0.237$; $\rho = –0.094$, $p = 0.152$).

The leptin concentrations decreased from week 1 until week 8 of life ($p = 0.001$) and remained rather stable from week 8 to 20 of life without any group differences (Figure 4c). One calf in the HIGH group had considerably greater leptin concentrations than the mean from all calves in five out of seven time points, which were further recognized as outliers (SD > 2) and therefore excluded from further statistical analysis. When comparing the hormone concentrations between the calves and their dams, about 5-fold higher leptin concentrations were observed in cows than in their calves ($p > 0.001$; Table 3). For serum adiponectin the situation was vice versa: Calves had nearly 3-fold higher adiponectin concentrations than their respective dams ($p < 0.001$; Table 3). Neither the Hp, nor the leptin and adiponectin concentrations of the dams were correlated with those of their calves (36–48 h after calving; Hp $\rho = 0.170$, $p = 0.361$; leptin $\rho = –0.206$, $p = 0.249$; adiponectin $\rho = 0.216$, $p = 0.253$).

3.8. Oxidative Status

The total protein concentration in plasma, assessed as reference for the AOPP values, but also as a general indicator of health, was not different between groups, but increased with time until week 16; in week 20 decreasing protein concentrations were observed (Figure 5a, b and Table 3). Regardless of being considered per volume or per g total protein, the AOPP values were mainly influenced by time, but also by feeding group (Figure 5b, Table 3). In the first sample collected (36–48 h after birth), the highest AOPP values were recorded with a steady decline towards the end of the observation period (Figure 5b). The AOPP concentrations in

calves were higher than those of their dams in the *post partum* samples (Table 3). The TBARS values decreased with time but were not different between feeding groups (Figure 5c).

The dROM values in plasma increased with time but were not affected by the feeding regime (Figure 5d, Table 3). Differences in dROM between both groups were limited to week 14 ($p = 0.015$) at the end of weaning and a trend for differences in week 20 of life ($p = 0.05$), respectively (Figure 5d). For FRAP, both group and time were significant; the RES calves had higher values than the HIGH calves in the preweaning period (Figure 5e, Table 3). From the end of the weaning procedure in week 14 until the end of the trial in week 20, the group differences were balanced out (Figure 5e).

In comparison to their respective dams after calving, the FRAP values in calves amounted to about 70% of those observed in their dams (Figure 5e, Table 3). The differences between the feeding groups of calves after birth when feed intakes were not yet different were not related to the dams' FRAP status, as indicated by the equal values in the dams of both groups (Table 3) and the lack of correlations between calves and dams (FRAP $\rho = -0.270$, $p = 0.135$; AOPP $\rho = 0.267$, $p = 0.133$).

The activity of the selenium-dependent GSH-Px, which catalyzes the reduction of hydrogen peroxide (H_2O_2) with a simultaneous oxidation of glutathione, was not influenced by feeding group (Figure 5f, Table 3).

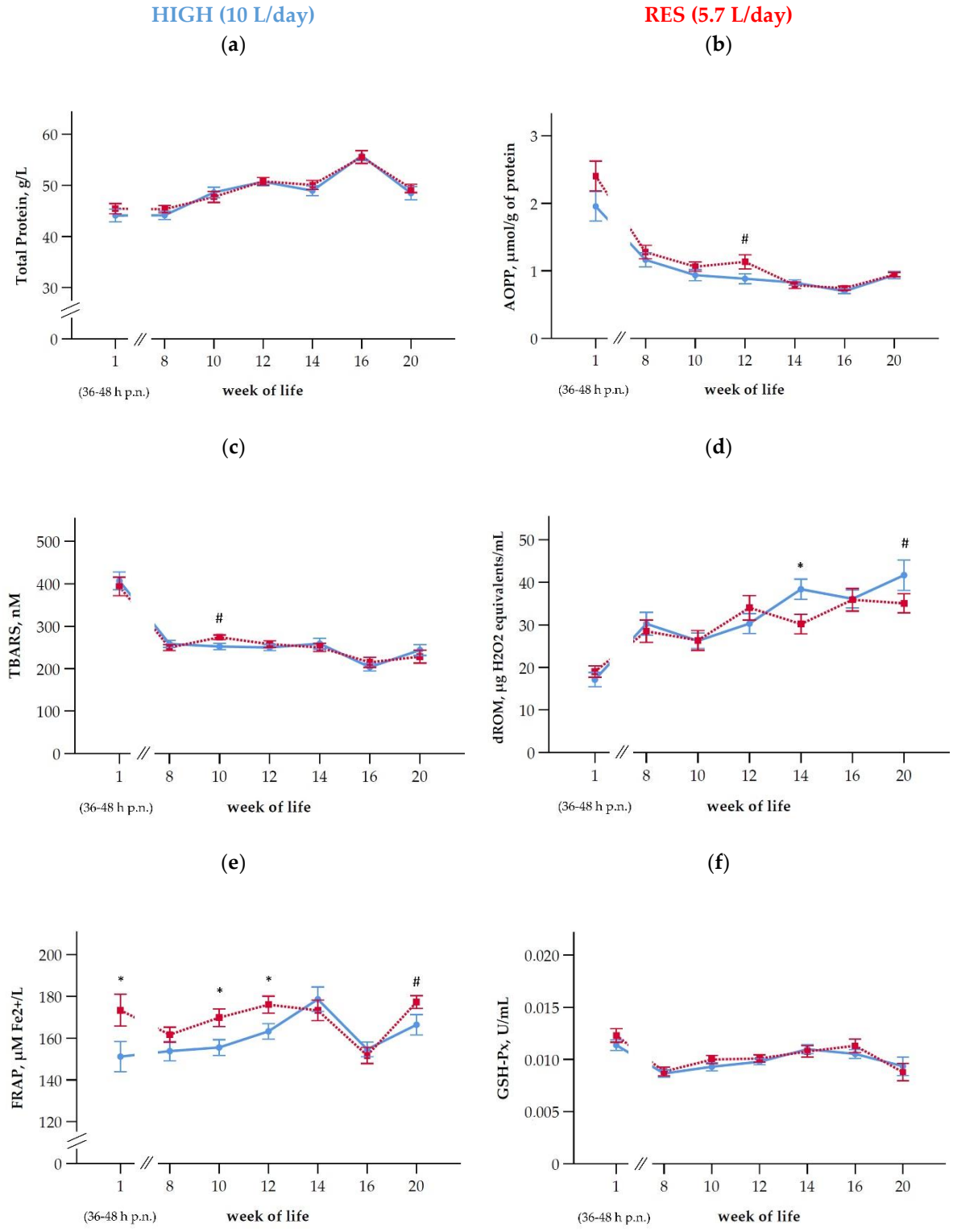


Figure 5. Changes in the systemic oxidative status during the first 20 weeks of life (means ± SEM): (a) Total protein content in plasma, (b) advanced oxidation products of proteins (AOPP), (c) thiobarbituric acid reactive substances (TBARS), (d) reactive oxidative metabolites (dROM), (e) ferric reducing ability of plasma (FRAP), and (f) glutathione peroxidase activity (GSH-Px) in calves fed at a high level of MR (HIGH; blue line) or at a restricted level (RES; red line). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. From week 15 until week 20 of life, the calves were moved to a new group pen in another stable receiving a TMR (Table 1). Asterisks indicate differences between groups within wk of life ($p < 0.05$) and hashtags indicate trends ($p < 0.1$). After birth/*post natum* is abbreviated as p.n.

4. Discussion

4.1. Feed and ME Intake

Expectedly, MR intake was greater in HIGH than in RES calves during the entire time of the differential feeding preweaning. The average daily MR intake of 9.18 L in HIGH was less than the daily allowance of 10 L, in particular, during the first weeks of life, when the HIGH calves were still increasing their intake until they reached a plateau of about 9–10 L/d around week four of life. These findings were similar to other reports [5,10,33]. Previous studies have demonstrated that MR-intake during weaning is less for late-weaned (12 or 16 weeks) calves compared to early-weaned (6 weeks) calves, confirming that with increasing age the demand for milk is less due to a substituting intake of energy via calf starter and hay [6,16]. De Passillé et al. (2011) [6] showed that later-weaned calves on a high milk feeding regime ate more starter and hay and had higher energy intakes and weight gains, and made fewer visits to the automated milk feeder than early-weaned calves fed on a high milk feeding level.

The lower number of visits to the automated feeding station in late weaned calves reflects the reduced need for milk with increasing age and less hunger. In our trial, a decrease in the number of visits at the automated feeder from week eight onwards was registered in both groups, in particular in the HIGH group. The greater number of unrewarded visits at the automatic feeding station in the RES group in our trial supports the notion that this level of restriction is not satisfying hunger, which is in line with previous findings [5,14]. Thus, the conventional restricted feeding violates one of the principal features of animal welfare, i.e., the freedom of hunger as one of the five freedoms formulated by Webster (1997) [12]. Increased numbers of unrewarded visits in restrictively fed calves were previously reported [5,14]. At preweaning, both groups in our trial showed a steady increase in drinking speed with age. Surprisingly, the situation changed during weaning, when RES calves showed still an increase whereas the HIGH calves reduced their drinking speed. This result is in contrast to previous findings of Gerbert et al. (2018) [14] showing that ad libitum fed calves had lower drinking speeds than restrictively fed calves. De Passillé et al. (2011) [6] showed that delaying the weaning age can reduce a decline in energy intake and behavioral signs of hunger. Furthermore, our trial showed, that even if the weaning age is delayed to 14 weeks of age, the desire for milk remains.

It is well known that a close relationship between milk feeding and solid feed consumption of calves exists [13]. Therefore, greater starter intakes in RES for compensating the lesser energy intake via MR and satisfying the feeling of hunger might be expected, as

reported also for calves weaned between 8 and 10 weeks of life [5,10,11,33,34]. However, both feeding groups, HIGH and RES, had equal starter intakes during the preweaning period. Accordingly, the ME intake was higher in HIGH calves than in RES until the beginning of weaning, when ME intake via MR was reduced, confirming results reported by Rosadiuk et al. (2020) [33]. Our findings are thus in support of the notion that a high plane of MR feeding does not counteract starter feed intake.

The consumption of starter is not only promoted by lesser milk or MR intake. Another main influencing factor is the developmental stage of the rumen and its ability to digest solid feed for an adequate energy supply. Therefore, weaning age also influences the consumption of starter and gradual weaning stimulates the starter intake during the preweaning period [13]. In our trial, the starter intake of calves increased mainly after week nine of life and this is the age in which most of the previous studies started or even finalized weaning. The higher number of unrewarded visits in the RES group even after week 9 of life showed that these calves were still in a developmental stage in which milk is an important and digestible source of energy and therefore better suited to satisfy hunger than starter concentrate and delivers energy as the basis for growth and development.

Previously, a high and early intake of starter was assumed to be associated with an enhanced rumen development, an earlier possible weaning age and lesser costs for milk replacer [2,5]. Korst et al. (2017) [5] calculated higher costs for ad libitum milk feeding than for restrictive feeding, but they concluded ad libitum feeding enhances animal welfare and later economic profit from milk. Therefore, the savings achieved by reducing MR costs should be balanced against the potential benefit of improved growth and welfare. Besides, this ratio is not constant and depends on the prices for rearing including MR and income from milk.

4.2. BW Gain and ADG

The HIGH feeding resulted in a greater pre and postweaning BW than in RES. The ADG declined in both groups within the first 3 weeks of life, whereas RES calves showed a stronger decline in ADG than HIGH calves. The restrictive feeding regime thwarted the ADG and caused a slower BW gain in comparison to HIGH calves preweaning. Postweaning, ADG in both groups decreased but HIGH fed calves showed even then still a higher BW. These findings are in line with previous studies [5,10,11], confirming improved preweaning growth with enhanced milk or MR intake. Additionally, findings from Rosadiuk et al. (2020) [33] showed that the preweaning ADG and BW were greater for heifers offered 10 L milk/d, but, in contrast to our results, these effects were not maintained after weaning. Geiger et al. (2016) [35] showed

that an enhanced preweaning MR feeding at 1.13 kg MR/d increased BW beyond weaning in week eight as compared to restrictively fed calves (0.45 kg MR/d). This corresponds to our results. Jasper and Weary (2002) [10] described that during and immediately after weaning the growth rate and BW gain slowed down independent of the MR feeding regime.

4.3. Health Status and Hp

Haptoglobin (Hp) is a major positive acute-phase protein in cattle [36]. In newborn calves, the concentrations decrease during the first days of life when colostrum is provided [37], but there is no indication that colostral Hp is directly transferred into the calf's circulation [37,38]. Both morbidity and mortality during the first 4 months of life were demonstrated to increase in calves with elevated Hp in the first week of life, but the predictive and diagnostic value of Hp measurements is limited [39]. In our study, we found little concordance between increased Hp concentrations and health disturbances.

It should be noted that the health checks were not necessarily performed at the same day of blood sampling. In our trial, we observed no differences in the number of diseased animals, the incidence of health disturbances, and the Hp concentrations between feeding groups. There were also no differences in the Hp values of the cows related to the group allocation of their calves. Nevertheless, the variation in Hp concentration in all calves was higher after birth than in the following weeks. The time course of the Hp concentration in the calves of both feeding groups of our trial, i.e., a decrease after the first wk of life to a constant level until the end of the trial, is in line with the results from Hulbert et al. (2011) [40].

Weaning may result in increased Hp concentrations as reported by Belli et al. (2018) [41] for calves fed ad libitum and weaned at 7 weeks of age. Hulbert et al. (2011) [40] showed that early-weaned calves (45 d) compared with later weaned calves (66 d) had increased plasma concentrations of cortisol and Hp, suggesting that weaning is less stressful when calves are older. In line with this, we did not see a distinct peak of Hp around weaning at 14 weeks of age in our trial. However, the sampling frequency might have been too low for detecting short-time changes.

4.4. Metabolic Hormones

Adiponectin and leptin are mainly secreted from adipose tissue and are thus termed adipokines [42,43]. Both hormones are involved in several physiological processes. Adiponectin is considered as an insulin sensitizer and is also involved in the regulation of lipid and glucose metabolism, as well as in inflammation [44]. In neonatal calves, the concentrations of both adiponectin and leptin were demonstrated to increase with colostrum intake within the

first day of life [5,34,45,46]. In the present study, the first blood sample collected from the calves was collected after colostrum intake (36–48 h after birth), and the concentrations of both adiponectin and leptin were in the same range as reported previously [34]. There was no correlation between the calves' leptin nor adiponectin serum concentrations with those of their dams.

Studies in humans have demonstrated inverse relations between circulating adiponectin and BW or body fat, in particular, visceral fat [47]. Decreasing concentrations of adiponectin were also reported in overfed dairy cows [48]. In the present study, the feeding level of MR affected the serum concentrations of adiponectin, which were greater in the HIGH than in the RES calves. A similar trend for higher adiponectin levels was reported in bull calves on a high preweaning level [49], as well as in ad libitum fed dairy heifer calves, accompanied by lower insulin concentrations in blood [34]. The greater concentrations of adiponectin in HIGH may point to higher insulin sensitivity in comparison to RES. However, we did not assess insulin sensitivity or responsiveness in the present study. Adiponectin was also suggested to have anti-inflammatory properties [50] and to exert a modulatory effect on oxidative stress [51]. However, when testing for correlations between the adiponectin and the Hp concentrations, there were no associations detectable. Considering the indicators for oxidative status tested herein, there were weak correlations between adiponectin and AOPP (mol/g of protein; $\rho = -0.135$, $p = 0.037$) and dROM ($\rho = 0.376$, $p < 0.001$) detectable, but not between adiponectin, FRAP, and TBARS, respectively.

Circulating leptin parallels the body fat content and acts as a negative feedback regulator on feed intake [52]. However, for leptin neither the increase reported by Schwarzkopf et al. (2019) [16] between week 6 to week 20 of life for calves weaned in week 16 of life, nor a difference between the high and the restricted feeding level, as shown by Bruinjé et al. (2020) [53], was observed in our trial. The feeding group did not affect leptin values.

4.5. Oxidative Status

Oxidative stress is caused by an imbalance between pro and antioxidatives [54]. Due to the abrupt environmental change at birth, from hypoxic to hyperoxic, the cells of neonates generate reactive oxygen species (ROS) which can cause oxidative stress in neonates [17]. In the first sample collected from the calves herein (36–48 h p.n.), the relatively greatest values were observed for those variables that are indicative for oxidative damage of lipids (TBARS) and proteins (AOPP), whereas dROM increased with time reaching the greatest value around and after weaning. For FRAP, representing the antioxidant activity, no such patterns were

clearly discernible. The activity of GSH-Px, which couples the reduction of H_2O_2 with the oxidation of reduced glutathione as part of the enzymatic antioxidative defense, showed also the greatest values at first sampling (36–48 h p.n.) and a decline in the following weeks. Similar results were described by Ranade et al. (2014) [17], i.e., a decline of AOPP values from birth until week 18 of life, but the concentrations of dROM did not change in this study. Albera and Kankofer (2011) [18] reported that the total antioxidant capacity, based on FRAP measurements, at birth, 48 h later and on day 12 of life were on a similar level and higher than in their dams. The relatively higher values of TBARS and AOPP after birth might be related to the increased release of iron from fetal haemoglobin (Hb) [55] that is gradually replaced by adult Hb [56]. Iron is known for generating harmful oxygen species by promoting the Fenton reaction which produces the potent oxidant hydroxyl radical [55]. However, from this, also greater values of dROM at birth would be expected. AOPP values showing a decrease with increasing age supporting previous findings from Ranade et al. (2014) [17].

The underlying kinetics of the reactions related to the maturation of fetal Hb over time are unknown, and thus the results remain at the descriptive level. Group differences were limited to FRAP and AOPP. In case of FRAP, these differences were already apparent when the calves were not yet differing in feed intake (36–48 h p.n.). Potential differences in both the FRAP and the AOPP status of the dams were ruled out, and the respective values of the calves and their dams were also not correlated. Another reason might have been random differences in the redox balance of the colostrum provided, which may affect the oxidative status of the newborns and also their IgG uptake [57]. However, colostrum was not specifically analyzed in this regard. The reasons for this initial group difference thus remain unknown and the question, whether the continuation of the difference between groups is related to the feeding level or is a trajectory of the starting condition, cannot be answered. Concerning FRAP, the intake of antioxidatives, such as vitamin E, with MR was likely greater in the HIGH than in the RES group but it was the RES group that had elevated FRAP values. For AOPP, greater values in RES indicated higher portions of oxidized proteins, which are often functionally inactive and prone to hydrolysis but may also accumulate and cause further damages [58]. Regarding the activity of GSH-Px, the shift over time was comparable to AOPP and TBARS, but different to that of FRAP.

The weaning process can cause transient neutrophilia and suppress neutrophil phagocytic and oxidative burst responses in calves [40]. Weaning was mostly not associated with clear deflections in the curves of the indicators of oxidative status assessed herein. Only for FRAP, a distinct drop in concentrations was observed from week 14 to 16; thereafter the

difference with greater values in RES tended to be re-established. The AOPP values remained at the same level in both groups at that time. In a previous study of Ranade et al. (2014) [17], the dROM values in plasma did not change over the six-week-interval from birth until weaning at the age of 18 weeks, whereas in our study dROM increased with age.

5. Conclusions

High planes of MR feeding for 14 weeks improved the pre and the postweaning BW and ADG, without impairing the intake of solid starter feed. The reduced number of unrewarded visits at the feeding station in calves receiving 10 L/d compared to the restrictive feeding level (5.7 L/d) indicates that hunger as a sign of impaired welfare was alleviated by greater daily MR offers. Neither health status nor the Hp concentrations in serum were different between the feeding groups. The greater concentration of adiponectin, an insulin-sensitizing hormone, in HIGH-fed calves may reflect increasing insulin sensitivity, while leptin was not influenced by the feeding group. The patterns of various indicators of oxidative status were characterized throughout the first 20 weeks of life, but the results yielded no clear benefit or disadvantage for either group in terms of oxidative loads. Differences between feeding groups in FRAP were already apparent in newborns and thus might not be related to the MR feeding level. Greater AOPP concentrations in RES may be related to a loss of function of the oxidized proteins which in turn point to a need for degrading these proteins and synthesizing new ones, possibly resulting in greater protein turnover. It remains open to what extent the positive effects of HIGH feeding on BW and ADG would determine the future performance as a cow and influence the oxidative status.

Supplementary Materials: The following are available online at https://zenodo.org/record/4351318#.YA7rRhYxlPY, https://www.mdpi.com/2076-3921/10/2/260/s1, Table S1: Health parameters of weekly health checks.

Author Contributions: Conceptualization, H.S., C.K., T.S., M.H.G., and K.D.S.; methodology, K.D.S.; implementation and sampling, K.D.S., T.S., and C.K.; facility management, K.D.S.; investigation, K.D.S., T.S., and C.K.; digitalization K.D.S.; raw data analysis, K.D.S.; statistical analysis, K.D.S.; resources, H.S., C.K., and T.S.; data curation, K.D.S.; writing—original draft preparation, K.D.S.; writing—review and editing, H.S.; visualization, K.D.S.; supervision, H.S., C.K., and T.S.; project administration, K.D.S., H.S.,

and C.K.; funding acquisition, H.S. and C.K. All authors have read and agreed to the published version of the manuscript.

Funding: Katharina Diana Seibt (K.D.S.) is recipient of a scholarship from the H.Wilhelm Schaumann Foundation (Hamburg, Germany). Otherwise, this research received no external funding.

Institutional Review Board Statement: The present study was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a. d. Alsenz, Germany, following the guidelines of the German Law for Animal Welfare by permission of the local authority in charge (G 17-20-071; Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data that support the findings and which are presented in this study are available on request from the corresponding author, Helga Sauerwein, sauerwein@unibonn.de. The data is not publicly available as not all data of the study has been published yet.

Acknowledgments: The authors thank the staff of the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle in Muenchweiler a. d. Alsenz in Rhineland-Palatinate (Germany), especially A. Brubacher, E. Therre, A. Schoenborn, S. Jobelius, K. Korst, D. Karch, E. Kröger, G. Backhaus, and the veterinary D. Lang for their support and effort. This project would not have been possible without their daily commitment, workforce and help. For their excellent technical assistance and support in the laboratory analyses, we thank B. Heitkönig, T. Blees and K. Strack.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Soberon, F.; Raffrenato, E.; Everett, R.W.; van Amburgh, M.E. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. J. Dairy Sci. 2012, 95, 783–793. [CrossRef]

2. Lohakare, J.D.; Südekum, K.-H.; Pattanaik, A.K. Nutrition-induced Changes of Growth from Birth to First Calving and Its Impact on Mammary Development and First-lactation Milk Yield in Dairy Heifers: A Review. Asian-australas. J. Anim. Sci. 2012, 25, 1338–1350. [CrossRef]

3. Khan, M.A.; Bach, A.; Weary, D.M.; Keyserlingk, M.A.G. von. Invited review: Transitioning from milk to solid feed in dairy heifers. J. Dairy Sci. 2016, 99, 885–902. [CrossRef]

4. Steele, M.A.; Penner, G.B.; Chaucheyras-Durand, F.; Le Guan, L. Development and physiology of the rumen and the lower gut: Targets for improving gut health. J. Dairy Sci. 2016, 99, 4955–4966. [CrossRef]

5. Korst, M.; Koch, C.; Kesser, J.; Müller, U.; Romberg, F.-J.; Rehage, J.; Eder, K.; Sauerwein, H. Different milk feeding intensities during the first 4 weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation. J. Dairy Sci. 2017, 100, 3096–3108. [CrossRef]

6. de Passillé, A.M.; Borderas, F.; Rushen, J. Cross-sucking by dairy calves may become a habit or reflect characteristics of individual calves more than milk allowance or weaning. Appl. Anim. Behav. Sci. 2011, 133, 137–143. [CrossRef]

7. de Passillé, A.M.; Rushen, J. Adjusting the weaning age of calves fed by automated feeders according to individual intakes of solid feed. J. Dairy Sci. 2012, 95, 5292–5298. [CrossRef] [PubMed]

8. Reinhardt, V.; Reinhardt, A. Natural sucking performance and age of weaning in zebu cattle (Bos indicus). J. Agric. Sci. 1981, 96, 309–312. [CrossRef]

9. Eckert, E.; Brown, H.E.; Leslie, K.E.; DeVries, T.J.; Steele, M.A. Weaning age affects growth, feed intake, gastrointestinal development, and behavior in Holstein calves fed an elevated plane of nutrition during the preweaning stage. J. Dairy Sci. 2015, 98, 6315–6326. [CrossRef] [PubMed]

10. Jasper, J.;Weary, D.M. Effects of Ad Libitum Milk Intake on Dairy Calves. J. Dairy Sci. 2002, 85, 3054–3058. [CrossRef]

11. Frieten, D.; Gerbert, C.; Koch, C.; Dusel, G.; Eder, K.; Kanitz, E.;Weitzel, J.M.; Hammon, H.M. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. J. Dairy Sci. 2017, 100, 6648–6661. [CrossRef]

12. Webster, J. Animal Welfare. A Cool Eye towards Eden; Blackwell Science: Oxford, UK, 1997; ISBN 0632039280.

13. Khan, M.A.; Weary, D.M.; von Keyserlingk, M.A.G. Invited review: Effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. J. Dairy Sci. 2011, 94, 1071 1081. [CrossRef]

14. Gerbert, C.; Frieten, D.; Koch, C.; Dusel, G.; Eder, K.; Stefaniak, T.; Bajzert, J.; Jawor, P.; Tuchscherer, A.; Hammon, H.M. Effects of ad libitum milk replacer feeding and butyrate supplementation on behavior, immune status, and health of Holstein calves in the postnatal period. J. Dairy Sci. 2018, 101, 7348–7360. [CrossRef]

15. Mirzaei, M.; Dadkhah, N.; Baghbanzadeh-Nobari, B.; Agha-Tehrani, A.; Eshraghi, M.; Imani, M.; Shiasi-Sardoabi, R.; Ghaffari, M.H. Effects of preweaning total plane of milk intake and weaning age on intake, growth performance, and blood metabolites of dairy calves. J. Dairy Sci. 2018, 101, 4212–4220. [CrossRef]

16. Schwarzkopf, S.; Kinoshita, A.; Kluess, J.; Kersten, S.; Meyer, U.; Huber, K.; Dänicke, S.; Frahm, J. Weaning Holstein Calves at 17 Weeks of Age Enables Smooth Transition from Liquid to Solid Feed. Animals 2019, 9, 1132. [CrossRef] [PubMed]

17. Ranade, R.; Talukder, S.; Muscatello, G.; Celi, P. Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of dairy heifer calves from birth to weaning. Vet. J. 2014, 202, 583–587. [CrossRef] [PubMed]

18. Albera, E.; Kankofer, M. The comparison of antioxidative/oxidative profile in blood, colostrum and milk of early post-partum cows and their newborns. Reprod. Domest. Anim. 2011, 46. [CrossRef] [PubMed]

19. Vannucchi, C.I.; Silva, L.G.; Lúcio, C.F.; Veiga, G.A.L. Oxidative stress and acid-base balance during the transition period of neonatal Holstein calves submitted to different calving times and obstetric assistance. J. Dairy Sci. 2019, 102, 1542–1550. [CrossRef]

20. Bordignon, R.; Volpato, A.; Glombowsky, P.; Souza, C.F.; Baldissera, M.D.; Secco, R.; Pereira, W.A.B.; Leal, M.L.R.; Vedovatto, M.; Da Silva, A.S. Nutraceutical effect of vitamins and minerals on performance and immune and antioxidant systems in dairy calves during the nutritional transition period in summer. J. Therm. Biol. 2019, 84, 451–459. [CrossRef]

21. National Research Council. Nutrient Requirements of Dairy Cattle, 7th ed.; National Academies Press: Washington, DC, USA, 2001; ISBN 978-0-309-06997-7.

22. Hiss, S.; Mielenz, M.; Bruckmaier, R.M.; Sauerwein, H. Haptoglobin Concentrations in Blood and Milk After Endotoxin Challenge and Quantification of Mammary Hp mRNA Expression. J. Dairy Sci. 2004, 87, 3778–3784. [CrossRef]

23. Sauerwein, H.; Heintges, U.; Hennies, M.; Selhorst, T.; Daxenberger, A. Growth hormone induced alterations of leptin serum concentrations in dairy cows as measured by a novel enzyme immunoassay. Livest. Prod. Sci. 2004, 87, 189–195. [CrossRef]

24. Mielenz, M.; Mielenz, B.; Singh, S.P.; Kopp, C.; Heinz, J.; Häussler, S.; Sauerwein, H. Development, validation, and pilot application of a semiquantitative Western blot analysis and an ELISA for bovine adiponectin. Domest. Anim. Endocrinol. 2013, 44, 121–130. [CrossRef]

25. Alberti, A.; Bolognini, L.; Macciantelli, D.; Caratelli, M. The radical cation of N,N-diethyl-para-phenylendiamine: A possible indicator of oxidative stress in biological samples. Res. Chem. 2000, 253–267. [CrossRef]

26. Regenhard, P.; Nakov, D.; Sauerwein, H. Applicability of a Spectrophotometric Method for Assessment of Oxidative Stress in Poultry. Mac. Vet. Rev. 2014, 37, 43–47. [CrossRef]

27. Benzie, I.F.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. Anal. Biochem. 1996, 239, 70–76. [CrossRef]

28. Witko-Sarsat, V.; Friedlander, M.; Capeillère-Blandin, C.; Nguyen-Khoa, T.; Nguyen, A.T.; Zingraff, J.; Jungers, P.; Descamps- Latscha, B. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int. 1996, 49, 1304–1313. [CrossRef] [PubMed]

29. Bradford, M.M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 1976, 72, 248–254. [CrossRef]

30. Gutteridge, J.M.; Quinlan, G.J. Malondialdehyde formation from lipid peroxides in the thiobarbituric acid test: The role of lipid radicals, iron salts, and metal chelators. J. Appl. Biochem. 1983, 5, 293–299. [PubMed]

31. Paglia, D.E.; Valentine, W.N. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 1967, 70, 158–169. [PubMed]

32. Buczinski, S.; Vandeweerd, J.M. Diagnostic accuracy of refractometry for assessing bovine colostrum quality: A systematic review and meta-analysis. J. Dairy Sci. 2016, 99, 7381–7394. [CrossRef] [PubMed]

33. Rosadiuk, J.P.; Bruinjé, T.C.; Moslemipur, F.; Fischer-Tlustos, A.J.; Renaud, D.L.; Ambrose, D.J.; Steele, M.A. Differing planes of pre- and postweaning phase nutrition in Holstein heifers: I. Effects on feed intake, growth efficiency, and metabolic and development indicators. J. Dairy Sci. 2020. [CrossRef] [PubMed]

34. Kesser, J.; Korst, M.; Koch, C.; Romberg, F.-J.; Rehage, J.; Müller, U.; Schmicke, M.; Eder, K.; Hammon, H.M.; Sadri, H.; et al. Different milk feeding intensities during the first 4 weeks of rearing dairy calves: Part 2: Effects on the metabolic and endocrine status during calfhood and around the first lactation. J. Dairy Sci. 2017, 100, 3109–3125. [CrossRef] [PubMed]

35. Geiger, A.J.; Parsons, C.L.M.; James, R.E.; Akers, R.M. Growth, intake, and health of Holstein heifer calves fed an enhanced preweaning diet with or without postweaning exogenous estrogen. J. Dairy Sci. 2016, 99, 3995–4004. [CrossRef]

36. Ceciliani, F.; Ceron, J.J.; Eckersall, P.D.; Sauerwein, H. Acute phase proteins in ruminants. J. Proteom. 2012, 75. [CrossRef] [PubMed]

37. Sadri, H.; Getachew, B.; Ghaffari, M.H.; Hammon, H.M.; Steinhoff-Wagner, J.; Sauerwein, H. Short communication: Plasma concentration and tissue mRNA expression of haptoglobin in neonatal calves. J. Dairy Sci. 2020, 103. [CrossRef] [PubMed]

38. Liermann, W.; Schäff, C.T.; Gruse, J.; Derno, M.; Weitzel, J.M.; Kanitz, E.; Otten, W.; Hoeflich, A.; Stefaniak, T.; Sauerwein, H.; et al. Effects of colostrum instead of formula feeding for the first 2 days postnatum on whole-body energy metabolism and its endocrine control in neonatal calves. J. Dairy Sci. 2020, 103, 3577–3598. [CrossRef] [PubMed]

39. Murray, C.F.; Windeyer, M.C.; Duffield, T.F.; Haley, D.B.; Pearl, D.L.; Waalderbos, K.M.; Leslie, K.E. Associations of serum haptoglobin in newborn dairy calves with health, growth, and mortality up to 4 months of age. J. Dairy Sci. 2014, 97, 7844–7855. [CrossRef] [PubMed]

40. Hulbert, L.E.; Cobb, C.J.; Carroll, J.A.; Ballou, M.A. The effects of early weaning on innate immune responses of Holstein calves. J. Dairy Sci. 2011, 94, 2545–2556. [CrossRef] [PubMed]

41. Belli, A.L.; Reis, R.B.; Veronese, A.; Moreira, R.; Flanagan, K.; Driver, J.; Nelson, C.D.; Clapper, J.A.; Ballou, M.A.; Jeong, K.C.; et al. Effects of treatment of preweaning dairy calves with recombinant bovine somatotropin on immune responses and somatotropic axis. J. Dairy Sci. 2018, 101, 6602–6615. [CrossRef] [PubMed]

42. Kajantie, E.; Hytinantti, T.; Hovi, P.; Andersson, S. Cord plasma adiponectin: A 20-fold rise between 24 weeks gestation and term. J. Clin. Endocrinol. Metab. 2004, 89, 4031–4036. [CrossRef] [PubMed]

43. Corbetta, S.; Bulfamante, G.; Cortelazzi, D.; Barresi, V.; Cetin, I.; Mantovani, G.; Bondioni, S.; Beck-Peccoz, P.; Spada, A. Adiponectin expression in human fetal tissues during mid- and late gestation. J. Clin. Endocrinol. Metab. 2005, 90, 2397–2402. [CrossRef]

44. Sauerwein, H.; Häußler, S. Endogenous and exogenous factors influencing the concentrations of adiponectin in body fluids and tissues in the bovine. Domest. Anim. Endocrinol. 2016, 56, S33–S43. [CrossRef]

45. Casabiell, X.; Piñeiro, V.; Tomé, M.A.; Peinó, R.; Diéguez, C.; Casanueva, F.F. Presence of leptin in colostrum and/or breast milk from lactating mothers: A potential role in the regulation of neonatal food intake. J. Clin. Endocrinol. Metab. 1997, 82, 4270–4273. [CrossRef]

46. Kesser, J.; Hill, M.; Heinz, J.F.L.; Koch, C.; Rehage, J.; Steinhoff-Wagner, J.; Hammon, H.M.; Mielenz, B.; Sauerwein, H.; Sadri, H. The rapid increase of circulating adiponectin in neonatal calves depends on colostrum intake. J. Dairy Sci. 2015, 98, 7044–7051. [CrossRef] [PubMed]

47. Gariballa, S.; Alkaabi, J.; Yasin, J.; Al Essa, A. Total adiponectin in overweight and obese subjects and its response to visceral fat loss. BMC Endocr. Disord. 2019, 19, 55. [CrossRef]

48. Locher, L.; Häussler, S.; Laubenthal, L.; Singh, S.P.; Winkler, J.; Kinoshita, A.; Kenéz, Á.; Rehage, J.; Huber, K.; Sauerwein, H.; et al. Effect of increasing body condition on key regulators of fat metabolism in subcutaneous adipose tissue depot and circulation of nonlactating dairy cows. J. Dairy Sci. 2015, 98, 1057–1068. [CrossRef] [PubMed]

49. Byrne, C.J.; Fair, S.; English, A.M.; Urh, C.; Sauerwein, H.; Crowe, M.A.; Lonergan, P.; Kenny, D.A. Effect of breed, plane of nutrition and age on growth, scrotal development, metabolite concentrations and on systemic gonadotropin and testosterone concentrations following a GnRH challenge in young dairy bulls. Theriogenology 2017, 96, 58–68. [CrossRef] [PubMed]

50. Zhang, K.; Guo, Y.; Ge, Z.; Zhang, Z.; Da, Y.; Li,W.; Zhang, Z.; Xue, Z.; Li, Y.; Ren, Y.; et al. Adiponectin Suppresses T Helper 17 Cell Differentiation and Limits Autoimmune CNS Inflammation via the SIRT1/PPARϒ/RORϒt Pathway. Mol. Neurobiol. 2017, 54, 4908–4920. [CrossRef]

51. Essick, E.E.; Wilson, R.M.; Pimentel, D.R.; Shimano, M.; Baid, S.; Ouchi, N.; Sam, F. Adiponectin modulates oxidative stress induced autophagy in cardiomyocytes. PLoS One 2013, 8, e68697. [CrossRef]

52. Wylie, A.R.G. Leptin in farm animals: Where are we and where can we go? Animal 2011, 5, 246–267. [CrossRef] [PubMed]

53. Bruinjé, T.C.; Rosadiuk, J.P.; Moslemipur, F.; Sauerwein, H.; Steele, M.A.; Ambrose, D.J. Differing planes of pre- and postweaning phase nutrition in Holstein heifers: II. Effects on circulating leptin, luteinizing hormone, and age at puberty. J. Dairy Sci. 2020. [CrossRef]

54. Sies, H. Oxidative Stress; Elsevier Science: Burlington, VT, USA, 1985; ISBN 9780126427608.

55. Halliwell, B.; Gutteridge, J.M. Oxygen toxicity, oxygen radicals, transition metals and disease. Biochem. J. 1984, 219, 1–14. [CrossRef] [PubMed]

56. Comporti, M.; Signorini, C.; Buonocore, G.; Ciccoli, L. Iron release, oxidative stress and erythrocyte ageing. Free Radic. Biol. Med. 2002, 32, 568–576. [CrossRef]

57. Abuelo, Á.; Pérez-Santos, M.; Hernández, J.; Castillo, C. Effect of colostrum redox balance on the oxidative status of calves during the first 3months of life and the relationship with passive immune acquisition. Vet. J. 2014, 199, 295–299. [CrossRef] [PubMed] 58. Dean, R.T.; Fu, S.; Stocker, R.; Davies, M.J. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 1997, 324 (Pt 1), 1–18. [CrossRef]

58. Dean, R.T.; Fu, S.; Stocker, R.; Davies, M.J. Biochemistry and pathology of radical-mediated protein oxidation. Biochem. J. 1997, 324 (Pt 1), 1–18. [CrossRef]

K.D.S. et al. - Supplementary Table 1. Health parameters of weekly health checks.

Health score	1 = Completely healthy 2 = One health disturbance within one check and week 3 = Diseased and / or > 40°C rectal temperature (rectally) 4 > 39.5°C without any other symptoms / health disturbances
Health disturbances	1 = Rectal temperature > 39.5°C 2 = Digestion 3 = Respiration 4 = Eye-related 5 = Navel
Body posture	1 = lively, jumping around 2 = slightly subdued, standing 3 = subdued, chest position 4 = subdued, side position
Body temperature (rectal)	1 ≤ 38.0°C 2 = 38.1 – 39.5°C 3 = 39.6 – 40.0°C 4 ≥ 40.0°C
Navel inflammation	1 = Yes, 2 = No
Faecal consistency	1 = pasty 2 = thinly mushy 3 = soupy 4 = watery 5 = ruminant *faeces*
Abdominal rigidity	1 = soft 2 = tense, hardened
Apdominal spaltter / ringing	1 = negative on both sides 2 = positive on the left side 3 = positive on the right side
Depth of *bulbus oculi* in *orbita*	1 = 0 mm 2 = 1-2 mm 3 = 3-4 mm 4 ≥ 5 mm
Episcleral vessels filling	1 = moderately injected 2 = slightly injected 3 = moderate injected 4 = highly injected
Episcleral vessels sharpness	1 = sharp 2 = blurred
Heart rate	1 = 80 - 110/min 2 = 111 - 140/min 3 ≥ 140/min 4 ≤ 80/min
Colour mucosa (mouth)	1 = pink 2 = pale pink 3 = cyanotic 4 = white
Respiratory frequency	1 = 20 - 36/min 2 = 37 - 60/min 3 ≥ 60/min
Dyspnoea (shortness of breath)	1 = none 2 = slight 3 = moderate 4 = high
Nares blown	1 = Yes, 2 = No
Mouth breathing	1 = Yes, 2 = No
Straight head-neck-posture	1 = Yes, 2 = No

4 Manuscript II

Interpretive Summary: Effects of different feeding levels during a 14-week preweaning phase in dairy heifers: Part 1: Framing of an ultrasonographic atlas of the developing bovine mammary gland. *By Seibt et al.*, The development of the mammary gland was captured in ultrasonic images from 37 calves from week 3 to 20 of life. For assigning the images to defined stages, schematic exemplary drawings per stage were elaborated framing an ultrasonographic atlas of the developing bovine mammary gland. This atlas provides a tool for qualitatively categorizing the tissue types from ultrasound images and for classifying the developmental stage. Furthermore, quantitative measurements by measuring the pixel brightness can be standardized based on this atlas, because well-defined tissue structures of interest can be selected and subjected to assess the brightness as pixel values.

RUNNING HEAD: ATLAS OF THE DEVELOPING BOVINE MAMMARY GLAND

Effects of different feeding levels during a 14-week preweaning phase in dairy heifers: Part 1: Framing of an ultrasonographic atlas of the developing bovine mammary gland

K. D. Seibt,[1] T. Scheu,[2] C. Koch,[2] M. H. Ghaffari,[1] and H. Sauerwein[1]

[1]Institute of Animal Science, Physiology Unit, University of Bonn, Katzenburgweg 7, 53115 Bonn, Germany

[2]Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, 67728 Münchweiler an der Alsenz, Germany

*Corresponding author: sauerwein@uni-bonn.de (Helga Sauerwein)

ABSTRACT

Ultrasound technologies allow for non-invasive observation of mammary gland development in vivo. The differentiation between tissues of the mammary gland (MG) and the evaluation of changes in MG tissue composition is based on the tissues´ different abilities to reflect or absorb ultrasonic waves. A detailed description of different visually determined stages of MG development and a template to describe these in a targeted manner, for classifying structures, and finally to measure them quantitatively was still missing. In this study, ultrasonic images were acquired of 37 German Holstein dairy heifer calves during the first 5 months of life. Calves either received a high plane of milk replacer (MR; 14% solids) at 10 L/d (1.4 kg MR/d; HIGH, n = 18) or a restrictive plane of 5.7 L/d (0.8 kg MR/d; RES, n = 19) until the linear weaning in wk 13 and wk 14 of life. Ultrasound MG scans were performed first in wk 3 of life, fortnightly from wk 8–16, and in wk 20 of life, from each quarter, in standing position, using a B-mode ultrasound device equipped with a linear probe at a frequency of 18 MHz, in a standardized position of 45° inclination to the teat. Before the ultrasonic scans, the MG quarters were palpated for assessing the size of the mammary parenchyma (PAR), and teat length was measured. The developmental stages of the PAR, visible in ultrasound images, obtained over 20 wks of life, were categorized, described, and drawn by hand. On this basis, a template for the classification of visible categories of mammary PAR development and for its surrounding tissue (SURR) was created. Ultrasound images were further classified by PAR structure and SURR. The template for classifying the visible developmental stages of PAR is differentiating and describing 11 categories, thus providing an Ultrasonographic Atlas of the developing bovine MG. With increasing age of the calves, higher Atlas Categories of PAR developmental stages were shown. While in wk 3 of life, 84% of MG quarter images could be assigned to category 1, in wk 8, 59.1% reached category 2, and in wk 20 the categories 10 and 11 were represented in 25.0% and 33.8% of all images, respectively. Furthermore, the mean visible category number (MVC) was calculated as the average of visible MG category number from images of 4 quarters of the whole udder, as well as for the mean palpation score. The preweaning daily milk allowance had no effect on the MVC of mammary PAR development. The mean palpation score of all quarters increased until wk 14 of life when most calves reached score 8, where a flat, arching structure like a coin was palpable and there was only a trend for an influence of MR feeding regimen on the mean palpation score. The mean teat length increased over time but without an effect of preweaning MR feeding. The atlas can serve as a template for the categorization and qualitative assessment of MG structures and further for the quantitative measurement of PAR´s development by measuring the pixel brightness.

Keywords: mammary gland, dairy heifer, calf, sonography, ultrasound

INTRODUCTION

The development of the mammary gland (MG) contributes to the final performance of dairy cows. Gaining a deeper insight into MG development in early life is important not only for basic science but also for testing different feeding and management strategies in early life in terms of future MG development, productivity, and udder health. Earlier studies mainly focused on MG development from puberty to the first lactation, but interest in the earlier phases increased with the first report about the rapid growth of the mammary parenchyma (PAR) in the first wks of life while the total MG mass remained largely stable (Capuco and Akers, 2010). As reviewed by Geiger (2019), the pre-pubertal MG development became a focus topic in lactation biology. It is becoming evident that the period between wk 8 and 10 of life during rearing is critical for imprinting the MG capacity for milk production (Brown et al., 2005; Meyer et al., 2006a, 2006b); during this time, i.e., around 8 wk of age, calves are commonly weaned (Lohakare et al., 2012). Previous studies already showed that MG development begins in the early life of calves and can be influenced by preweaning nutrition (Brown et al., 2005; Meyer et al., 2006a). An intensive preweaning feeding regimen until wk 8 of life, i.e. increased energy and protein intake, can positively influence MG development, especially the growth of PAR mass (Brown et al., 2005; Meyer et al., 2006a). A trend for an increase in mammary epithelial cell proliferation in PAR in response to higher preweaning (wk 6) energy intake was also observed (Meyer et al., 2006a).

The MG itself is a highly differentiated apocrine gland of the epidermis developing from the germ layer ectoderm in the embryonic stage to form a first bud (Hovey et al., 1999; Franz et al., 2009; Rowson et al., 2012). During the fetal stage, the developing MG contains the mammary PAR and early stages of the adjacent mammary fat pad (MFP; Hovey et al., 1999; Rowson et al., 2012). These tissue fractions largely determine the postnatal growth of the MG. In neonatal ruminants, a single primary duct extends from the teat to the gland cistern and ends with the epithelial ducts (Rowson et al., 2012). The PAR at this stage is described by Meyer et al. (2006b) as a threadlike mass that extends dorsally above each teat.

The PAR is the part where milk synthesis takes place in lactation (Franz et al., 2009; Akers, 2017). The milk is synthesized by the secretory cells of the PAR, the alveoli (Franz et al., 2009; Akers, 2017). During mammary gland development, the ducts grow and spread into the surrounding tissue (SURR) which contains adipose and connective tissue and is referred to as

the MFP (Hovey et al., 1999; Albino et al., 2015). Hovey et al. (1999) refer to the MFP as a central factor in regulating mammary PAR development. It is assumed that the higher the amount of mammary fat tissue is, the more the duct and PAR have to penetrate and grow into the MFP, which might lead to a reduced total mammary gland development depending on its dimension (Albino et al., 2017).

There are different possibilities to get insights and to assess the MG development. Within the last decades mainly MG weight (Swanson and Poffenbarger, 1979; Geiger et al., 2016), and the tissue composition of the gland was investigated by requiring post mortem dissection (Brown et al., 2005; Daniels et al., 2009). Additional previous studies mainly focused on mammary growth by measuring the weight of tissue components of the growing gland, i.e. the mammary PAR, the MFP, and the teat length (Albino et al., 2015; Esselburn et al., 2015; Albino et al., 2017; Furini et al., 2018; Silva et al., 2018).

In the last years, ultrasound sonography became more attractive to evaluate tissue development or structures of specific parts of the body. The main advantage of ultrasound measurements is that it is a non-invasive technique and allows for longitudinal assessments following the developmental process in the same animal. Furthermore, it offers the possibility to gain a detailed insight into the different structures and characteristics of tissues in order to differentiate and define them, without the need for dissection. One of the first B-mode ultrasound measurements of the bovine mammary gland was performed by Cartee et al. in (1986). Over the years, various studies which used ultrasonographic examinations mainly focused on the structure of the teat and gland cistern in context with milking-induced alterations (Bruckmaier and Blum, 1992). Other studies already used ultrasound techniques (Albino et al., 2015; Esselburn et al., 2015; Albino et al., 2017; Furini et al., 2018; Silva et al., 2018), but a detailed description of different visual stages of mammary development and a template to describe these in a targeted manner, to classify structures and finally to measure them quantitatively is still missing.

Therefore, in our study, we wanted to elucidate more precisely the early pre-pubertal MG development. Therefore, the time frame of mammary PAR ultrasonic measurements were extended to wk 20 of life covering the nutritional transition phase around weaning to gain a detailed view of different stages and their possible characteristics of PAR. Furthermore, different PAR structures visible in ultrasound images occurring with increasing age are described and categorized in this paper. For assigning the ultrasonic images to defined stages, schematic exemplary drawings per stage were elaborated framing an ultrasonographic atlas of

the developing bovine mammary gland. Additionally, the effects of different milk replacer (MR) feeding levels during a 14 wk preweaning phase on the visible categories of mammary PAR development, as well as on palpation score and teat length was investigated. We hypothesized that a higher MR allowance within an elongated milk-drinking period of 14 wks may promote mammary gland development in comparison to restrictive allowance. In the end, we were able to create the first template of categorizing visible structures of MG PAR development, the so-called *Ultrasonographic Atlas of the developing bovine MG* (USAtlasMG), as a tool for qualitative and further as a basis for quantitative brightness analysis of defined structures in ultrasound images of MG which is presented in this paper Part 1. In the accompanying paper Part 2 the results of quantitative brightness analysis of ultrasound MG images using the USAtlasMG are described.

MATERIALS AND METHODS

The present study was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Germany, following the guidelines of the German Law for Animal Welfare by permission of the corresponding authority (G 17-20-071; Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Animals, Diets, Feeding, and Management

Details of the experimental design with feeding regimens, weaning, blood sampling, and results about feed intake, feeding behavior, and various indicators of the oxidative status were previously described (Seibt et al., 2021). In brief: 37 German Holstein heifer calves were studied from birth until wk 20 of life, from February 2018 until March 2019. All calves received at least 3 L colostrum of their dam within the first 3 h of life. Only healthy calves born without dystocia cows were included in this study. From birth until d 10 of life, they were kept in straw-bedded single hutches and were fed twice a day with teat buckets. Until the 9th meal (d 5 of life), all calves received 5 L of their dam's acidified transition milk (2 mL/L of Schaumacid acidifier, H. Wilhelm Schaumann GmbH, Pinneberg, Germany) each in the morning and evening (10 L/d), respectively. Transition milk was also supplemented with a mix of trace elements and vitamins (1 mL/L Milkivit Quick- Mix, Trouw Nutrition Deutschland GmbH, Burgheim, Germany). Thereafter, the calves were stratified into two different feeding groups according to birth weight, sire, and parity of the dam to create equal groups. Half siblings were also considered and were equally distributed to the feeding groups: Group HIGH (n = 18) received 10 L/day of milk replacer (MR; 14% solids; Milkivit Titan, Trouw Nutrition

Deutschland GmbH), and groups RES (n = 19) was restricted to 5.7 L MR/d. The intake of transition milk and MR was documented by weighing the buckets before feeding and the residues in the bucket before offering the next meal. After the 20th meal, at d 10 of life, the calves were moved to an open straw-bedded stable where they were kept in a group. Newly incoming calves were assigned to a "baby group", in a separated pen within the stable for the first 5 d for allowing them to learn the use of the automated feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany) without having to compete with older calves. Calf starter concentrate (Blattin Kälberstart Gold, Höveler Spezialfutterwerke GmbH & Co. KG, Dormagen, Germany) and MR were dispensed according to respective feeding regimen and the intake of both, MR and pelleted calf starter concentrate as well as the number of rewarded and unrewarded visits were recorded individually by the feeding system (Förster-Technik GmbH). Water, hay, and calf starter were offered ad libitum. The ingredients and the chemical composition of the MR and starter were reported previously (Seibt et al., 2021). At 15 d of life, the calves were moved from the "baby group" to larger group pens that were also equipped with the automated feeding system (Förster-Technik GmbH) until weaning starting at d 84 (end of wk 12 of life). Over two wks the allowance for MR intake was linearly reduced until d 98 (wk 14) of life by 0.3 L/d in the RES and 0.6 L/d in the HIGH group, respectively. From d 99 onwards the MR supply was stopped and calves were moved to a new group pen in another stable where they had free access to water and a total mixed ration (TMR) for heifers as described previously (Seibt et al., 2021). The health status was checked weekly by veterinarians over the entire 20 wk of the trial (Seibt et al., 2021). On the day of the weekly health check, BW was recorded until wk 20 of life by using a mobile electronic scale for small farm animals (KWM GmbH Waagen- und Metallbau, Thiersheim, Germany).

Ultrasound scanning of the Mammary gland

Ultrasound scanning was performed at 7 time points, first in wk 3 of life, and then fortnightly from wk 8 until wk 16 of life, and in wk 20 of life. All ultrasound records were performed by the same person. The ultrasound images were obtained using a B-mode ultrasound device MyLab Five Vet scanner (Esaote Biomedica GmbH, Cologne, Germany) equipped with a linear probe. A fixed preset as a defined template was created to ensure equal conditions and their repeatability for all measurements. The presetting was in B-mode, 18 MHz, 4 cm measurement depth, and a greyscale between 40% and 60%. Two ultrasound images each were taken from each mammary quarter at each time point. For the evaluation of the MG, the animals were in

standing position with the probe placed in a standardized position at a 45° inclination to the teat, always in caudal-cranial direction according to Nishimura et al. (2011), as adapted by Albino et al. (2015) and Silva et al. (2018). To increase the contact of the probe with the skin and to improve the visualization of the images, the udder was wetted with clear ultrasound gel (Cb Healthcare Intl. S.r.l., Predappio, Italy). Together with the frequent handling for weighing and health checks, the calves became rapidly familiar with the procedure and were calm during the measurements that were performed on an electronic scale for small farm animals (KWM GmbH Waagen- und Metallbau) with frontal and lateral boundaries.

Description of structure and the visible categories of mammary development

In total, 2054 ultrasound images were collected during the experiment: 37 calves with 7 time points, 4 quarters each, and 2 records per quarter and time point (18 records were not measured). The different shapes and structures of PAR, which were visually discernible over time, were used as a basis for classifying the different developmental stages: schematic drawings were hand-crafted, differentiating and describing 11 different categories. The visible structures in the developing MG in ultrasound images were matched with descriptions of dissected MG in previous ultrasound measurements by Esselburn et al. (2015) and by Rowson et al. (2012), serving as a basis to differentiate and define structures and tissues in ultrasound images. The drawn templates of the different developmental categories classified framed an *Ultrasonographic Atlas of the developing bovine MG* (USAtlasMG). For comparative assessments, the developmental category number to which the images obtained per quarter were assigned, were averaged for the whole udder. The thereby calculated mean visible category of PAR development (MVC) was used to assess the effects of differential MR feeding over time. For assessing the variation between visible Categories of the 4 quarters per udder within each animal and wk of life, we calculated the difference between the highest visible category and the lowest visible category within the 4 quarters of one udder at each time point, the so-called delta visible category.

Palpation Scoring and teat length

Every quarter of the MG was examined by palpation before each ultrasound evaluation by one person according to Esselburn et al. (2015) and Furini et al. (2018). The palpation score and methodology of scoring were adapted from Esselburn et al. (2015), and extended by two steps of scoring thus comprising the following 9 categories: (0) nothing palpable, (1) small and

threadlike mass of PAR, comparable to a small grain of rice, (2) a larger grain of rice, (3) a small pea, (4) a chickpea, (5) almond-sized, (6) larger than an almond, (7) a whole pecan nut, and (8) a flat, arching structure like a coin. The palpation scores of both front and both rear glands were averaged as well as for the whole udder. In addition, the teat length on every quarter was measured with a ruler before the ultrasound examinations and the average per udder was calculated.

Statistical Analyses

Data were analyzed using SPSS (version 26; SPSS Inc., Chicago, IL). The normal distribution was checked by the Kolmogorov-Smirnov-test and the homogeneity of variance by using the Levene´s test. If data were not normally distributed, nonparametric tests were performed and data were log-transformed for further statistical analysis. Outliers were identified by Z-standardization and excluded as values with SD > 2. Furthermore, metric data from teat length, MVC, delta visible category, and mean palpation score were analyzed by a linear mixed model with Bonferroni post-hoc test and with repeated measurements, considering the individual calf as a random effect and feeding group (group), wk of life (time) as well as the interaction therefrom as fixed effects. Differences between the groups within each time point were tested with Student´s t-test or Mann-Whitney-test, in case of not normally distributed data and no homogeneity of variance. In all graphs, non-transformed data are shown as means ± SEM. Correlations (ρ) were calculated by Spearman analysis. Categorical data like visible categories of PAR development of MG quarters were analyzed by cross tables and Chi-square test. Results were declared as significant when $P < 0.05$, and $0.05 \leq P < 0.10$ was considered as a trend.

RESULTS AND DISCUSSION

Growth performance and feed intake

Detailed information about growth performance, BW gain, average daily gain (ADG), as well as MR, starter feed and ME intake, and health status was previously described by Seibt et al. (2021). In brief, birth weight, colostrum quality, and intake were not different between the two feeding groups, receiving either 10 L (HIGH) or 5.7 L of MR per day (RES). Expectedly, the MR intakes were greater in the HIGH than in the RES calves throughout the entire liquid feeding period until the beginning of weaning. The intake of starter feed was not compromised by the higher MR feeding level and both groups increased their starter intake at the same rate

reaching 2.9 ± 1.2 kg/d in wk 14 at the end of weaning, which is in accordance with the study of Korst et al. (2017), but in contrast to other previous findings (Passillé et al., 2011; Gerbert et al., 2018). The greater daily ME intake in the HIGH calves until the beginning of weaning (in wk 13 of life) was thus exclusively attributable to their MR intake. On average, HIGH calves were heavier than RES calves until 20 wk of age. Weaning at 14 wks of age in our study was not associated with BW loss in either group. The ADG were greater in HIGH than in RES calves until wk 7 of life. In the postweaning phase, ADG in both feeding groups declined whereby the nadir was reached earlier (in wk 16) in RES calves than in HIGH calves (wk 18). In general, the HIGH group had a developmental advantage over the RES group which might also be evident in terms of MG development which is in focus of this study.

Structures of the developing mammary gland

The representation of tissue structures on a B-mode ultrasound image is based on the different echogenicity, i.e., how strong ultrasonic waves are reflected, weakened, or absorbed by a specific tissue structure. The echogenicity becomes visible in different levels of brightness as black, white, or grey levels, depending on the different tissue characteristics such as firmness and the specific cell types within the tissues (Delorme and Debus, 2005), and is thus the basis for assigning, recognizing and describing tissue structures in ultrasound images.

In the study of Esselburn et al. (2015) the structure of the MG was described after a dissection directly after the ultrasonic measurement. Hence, they could relate the different parts of MG visible in the ultrasonic image to the different tissues they identified. In the current study, we did not slaughter and dissect the calves and could thus keep the number of the calves for following up their development and later milk production. However, the description of PAR and the visible structures in the developing MG by Esselburn et al. (2015) and Rowson et al. (2012) served as a reference to differentiate and define the structures, layers, and tissues observed in our ultrasonic images.

During the ultrasound measurements in this study, a linear probe was attached to the skin of each mammary gland, as described above. Hence, the first tissue layers visible in the upper part of the ultrasonic image are the skin layers including the *cutis* consisting of the *epidermis* and the *dermis s. corium* (Salomon et al., 2005). In ultrasonic images, the *cutis* appears as a homogeneous layer with a medium echogenicity (Figure 1, Area 1) as described by Trasch et al. (2007). Under the *cutis* in dorsal-cranial direction follows the *subcutis s. hypodermis*, differentiated in *stratum adiposum*, which is directly attached to the *dermis s. corium*, and in

stratum fibrosum, consisting of coarse-grained connecting tissue and also of dermal musculature (Figure 1, Area 1-3). Due to the high number of adipose cells, the *stratum adiposum* appears as a fine-grained structure which is visible in Figure 1, delimitated by the *stratum fibrosum* (Salomon et al., 2005). In most of the images obtained in our study, the skin layers were not clearly and separately visible in later time points of ultrasonic measurements.

Underneath the skin layers, the developing PAR appeared as a hypoechoic dark structure, which was mostly circular and clearly separated from the surrounding tissues (SURR) in wk 8 of life (Figure 1, Area 4). Underneath the structure of PAR, there is a hyperechogenic area visible delimiting by the abdominal layers (Figure 1, Area 6). The black, highly hypoechogenic structure of PAR, which is more or less anechoic, can provoke an artifact, the so-called distal enhancement, which appears underneath the PAR and is visible in Figure 1, Area 6 (Delorme and Debus, 2005; Penninck and d'Anjou, 2015). In this case, ultrasonic waves which pass an anechoic structure like PAR are less attenuated when they reach the tissue underneath (Delorme and Debus, 2005). A so-called sound window is created (Delorme and Debus, 2005). Therefore, the SURR underneath PAR is appearing bright due to this distal enhancement (Figure 1, Area 6). Normally, this distal enhancement is appearing under fluid-filled curved structures like a renal cyst or urine bladder (Delorme and Debus, 2005; Penninck and d'Anjou, 2015). On the right and left side of the PAR, visible shadowy streaks can occur (Figure. 1, Area 5). This so-called edge shadowing borders the distal enhancement artifact of a curved liquid filled structure (Penninck and d'Anjou, 2015).

These phenomena should be taken into account when aiming at quantitative assessments of tissue brightness (pixel value) of SURR: the regions of interest in which the pixel values are assessed should be within the brighter area adjacent to the PAR´s border, if a distal enhancement appears, and not into the shadow zones of edge shadowing (Seibt et al., xxxx Part 2). This new approach for the brightness measurements of SURR can be utilized mainly in the developmental categories 2, 3, 4, and eventually, 5 defined in the USAtlasMG (Figure 2 and in Seibt et al., xxxx Part 2: Supplementary Table 1 Templates 2 - 5) when these phenomena were occurring. The results about pixel values are provided in the companion paper (Seibt et al., xxxx Part 2). The SURR is demarcated to the *cavum abdominis* in the *regio mammaria abdominalis* (Figure 1, Area 9) by the abdominal wall (Figure 1, Areas 7 and 8), which consists of fascia and muscle layers (Salomon et al., 2005).

The brightness of SURR in ultrasound images likely reflects its lipid and liquid content (Delorme and Debus, 2005; Albino et al., 2015). Similar to the liver, greater lipid contents result

in a brighter and less homogenous appearance (Delorme and Debus, 2005). Hence, we concluded that the brighter SURR is, the higher is the content of lipid-filled adipocytes. When compared against photographs of sagittal sections of MG quarters from 8-wk-old heifer calves from Esselburn et al. (2015) and Rowson et al. (2012), SURR represented the MFP into which a circular and pigmented PAR was embedded. Esselburn et al. (2015) made ultrasonic measurements before dissection and described the PAR in ultrasonic images as "a dark contrast (hypoechoic feature) compared with more dorsally located hyperechoic MFP". Albino et al. (2015) also described the sub-ductal region as the region of MFP in the MG. The MFP develops around day 80 of fetal life and is well-established at birth (Sheffield, 1988; Ellis et al., 2012). Comparative results about pixel values are provided in the companion paper (Seibt et al., xxxx Part 2).

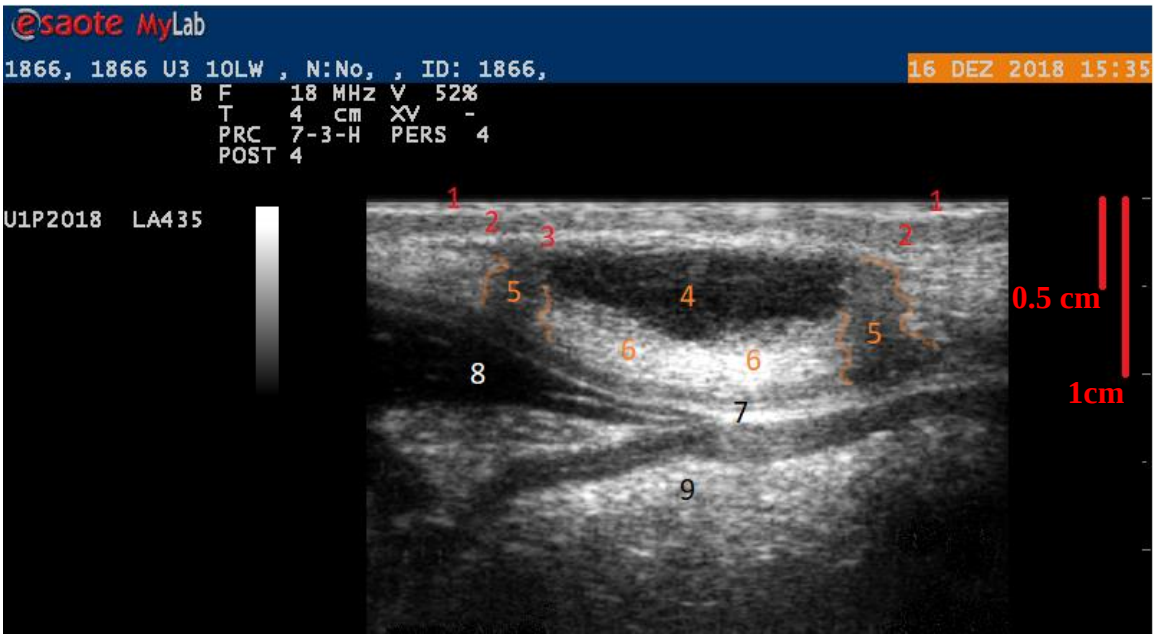


Figure 1. Representative ultrasonographic image of a mammary gland quarter from a 10-wk old heifer calf. On top of the ultrasonic image (18 MHz, Esaote MyLab Five Vet scanner) are (1-3) the different skin layers, followed down in dorsal-cranial direction by (4) the mammary parenchyma (PAR) structure (hypoechogenic = black), which is lateral right, lateral left, framed by (5) shadow areas (ultrasonic artefacts). Underneath attached to the PAR structure is (6) the bright (hyperechogenic) surrounding tissue (SURR), appearing bright due to an ultrasonic wave amplification. At the bottom area of the image, mammary gland tissue structures are bordered dorsal-cranial (7) the abdominal layers, (8) the abdominal muscle, and (9) the *cavum abdominis*. White marks on the right vertical axis of the ultrasound image are spaced 0.5 cm apart.

Classifying the visible Categories of mammary PAR development

In total, 1001 ultrasound images of all 37 calves and time points could be assigned to the categories in the USAtlasMG (Figure 2) which represents 96.6% of a total of 1036 images of scans: 0.7% were not assessable, because images were blurred, in 1.8% of all images the category was not identifiable and in 0.9% were not assessed. The distribution of visible categories in accordance to the USAtlasMG within each wk of measurement (time point) during the trial until wk 20 of life is shown in Figure 3.

In wk 3 of life, the first assessment we did, in most images (84% of the records), PAR was classified as Category 1 (Figure 3) according to the atlas (USAtlasMG) developed herein (Figure 2). In this Category 1, the visible structures were limited to the teat base under the skin layers with a speckled, white and grey mottled tissue underneath, which was bordered by abdominal layers. No further signs of MG formation were observed. Meyer et al. (2006b) described the stage of PAR development after birth as a threadlike mass that extends dorsally above each teat, which was comparable with the structures we obtained in Category 1. However, in contrast to Esselburn et al. (2015) who reported an average PAR area in a range of 10.7-15.3 mm^2/gland from ultrasound imaging, we found clearly discernible PAR structures, i.e. in Categories 2 and 3 only in 15.3% and 0.7% of all assessments in wk 3 of life (Figure 3).

In wk 8 of life, most calves reached Category 2, i.e. 59,1% of the images were assigned to Category 2, in which a circular clear bordered black (hypoechoic) PAR was identifiable (Figure 2 and 3) similar to the findings reported by Esselburn et al. (2015) and Rowson et al. (2012) in 8-wk old calves. Since the PAR was identifiable as a black structure, it was possible to create the USAtlasMG as a template to arrange visible PAR structures of different stages of PAR´s development, for the categorization and qualitative assessment of mammary gland structures.

The order of the USAtlasMG categories (1 – 11) about the PAR's shape and structure (Figure 2) did not entirely correspond to a chronological order of PAR-developmental stages in one single animal and udder. Some categories were observed to occur at the same age in different quarters of the udder: In Category 3 (Figure 2), the round circularity of PAR showed some roundish spreads at the dorsal side; in Category 4 the PAR appeared as a flat structure with signs of a cauliflower shape (transition; Figure 2); and in Category 5, the transition to the typical shape of cauliflower was more clear yet but still not fully reached (Figure 2). Therefore the ultrasound image evaluation showed, that those categories defined likely gave some overlap, possibly representing similar stages of development at the same age, or may occur successively at different wks of age in one single calf.

In Categories 3-5, the PAR was still visible as a dark (black), hypoechoic mass clearly separated by clearly visible borderlines from SURR (Figure 2). Categories 3 to 5 were mainly represented in wks 8–12 (Figure 3). The shape of PAR in Category 3 was characterized by the dorsal dissolution of the roundish structure oriented towards the inner body (Figure 2). A flat elongated oval structure of PAR was typical for PAR of category 4 (Figure 2). From Category 3 towards Category 5, the circular shape of PAR was increasingly dissolved, i.e. the roundish structures disappeared. Different structures can be assigned to Category 5 (Figure 2) and designated the transition from round structures to single, thickened buds that are connected to each other to form a larger structure, which we termed cauliflower structure of Category 6. PAR´s structure is still comparable with those of categories 3 and 4, but the structure of cauliflower is already visible in most parts (Figure 2).

Categories 3–5 were observed in wk 8 (17.5%, 5.1%, 6.6% respectively) and were mainly represented in wk 10 of life in our trial (26.8%, 8.0%, 17.4% respectively; Figure 3). Similarly, Furini et al. (2018) described that the oval-shaped structure of PAR was altered after 2 mo (wk 8) of age in Holstein-Gyr crossbreed heifers, whereas feeding of different total solid concentrations by adding MR powder to whole milk during the preweaning period had no effect on PAR. The PAR area in 8-wk–old heifers was reported to has a size in a range of 32.1- 53.4 mm^2/gland in one study (Esselburn et al., 2015), and to lesser values, i.e., 26.9-31.1 mm^2/gland in another study (Furini et al., 2018).

Category 6 represents the cauliflower structure of PAR (Figure 2). There is no round formation and no circular bordered structure visible anymore. The shape and structure of the hypoechoic area resembled a cauliflower of varying size and depth, but with well-discernible characteristics (Figure 2). Category 6 was represented first in wk 8 of life with 5.1% of images and mainly in wk 12 with 34.3% of all images (Figure 3).

Category 7 is characterized by a coral structure, whereby the PAR´s area is still identifiable. The typical budding structure of the cauliflower is dissolving, instead, primary slender branches are formed (Figure 2). Images that could be assigned to Category 7 appeared for the first time in wk 10 (5.1%) and were mainly represented in wk 14 (35.9%, Figure 3). Swanson and Poffenbarger (1979) described that mammary PAR spreads into the MFP displaced the adipose tissue and different types of udder tissue were distributed within PAR and therefore the total udder weight did not reveal the extent of the mammary gland development. That supports the impression of the developmental stage which was reached and represented as Category 8 (Figure 2).

In Category 8, the PAR is more and more distributed into single dark hypoechoic pieces within the SURR (Figure 2). The Category 8 occurred mainly in wk 12-16 with 13.3%, 18.6%, and 19.2% respectively (Figure 3). The structures of PAR were increasingly dissolving in the following categories 9-11: In Category 9 only one main ductus was clearly visible (Figure 2), confirming the findings of Franz et al. (2009) who described that the teat canal appears as a central echoic line which is bordered on each side by two parallel hypoechoic bands. Category 9 was mainly according in wk 16 and 20 (31.5% and 34.5% respectively). In Category 10, a grey flecked tissue structure without a clearly visible main ductus was seen and an increasingly homogeneous interweaving of tissue structures (Figure 2).

When comparing Categories 10 and especially 11 (Figure 2) which we found mainly in 20-wk old heifers (25.0% and 33.8% of all images in wk 20 respectively, Figure 3) to photographs of a dissected stained sagittal section of mammary glands and the teat cistern area from an 18 months-old heifer at the beginning of pregnancy (Swanson and Poffenbarger, 1979), we interpret the developmental stage in Category 11 as the first formation of a cistern near the teat base (Figure 2). Franz et al. (2009) described the healthy mammary PAR structure of adult lactating cows as an uniform echogenic structure with a granular structure, connective tissue as a structure with a higher echoic density (bright = white), and gland PAR with less echoic density. Occurring visibly dark structures and formations in adult bovine PAR are anechoic which can either correspond to blood vessels or lactiferous ducts and the cistern is anechoic as well (Franz et al., 2009). Hence larger lactiferous ducts coming out of the PAR going into the gland cistern were clearly visible (Franz et al., 2009). This description is also comparable to that of Category 11 (USAtlasMG, Figure 2).

The overall mean differences in visible categories (delta visible category) of the 4 quarters per udder considering each wk of life were not exceeding 2 (delta visible category ≤ 2) except for the RES calves in wk 20 of life, indicating a rather homogenous appearance in one visible category of development in the quarters of one udder. From wk 3 to wk 16 of life, there were also no group differences in delta visible category, except for wk 20, where RES calves showed mean delta visible categories values of > 2 (RES 2.05 ± 0.52 SD) indicating a higher variability in quarters categories within udder than in HIGH calves (1.28 ± 0.75 SD; $P = 0.004$).

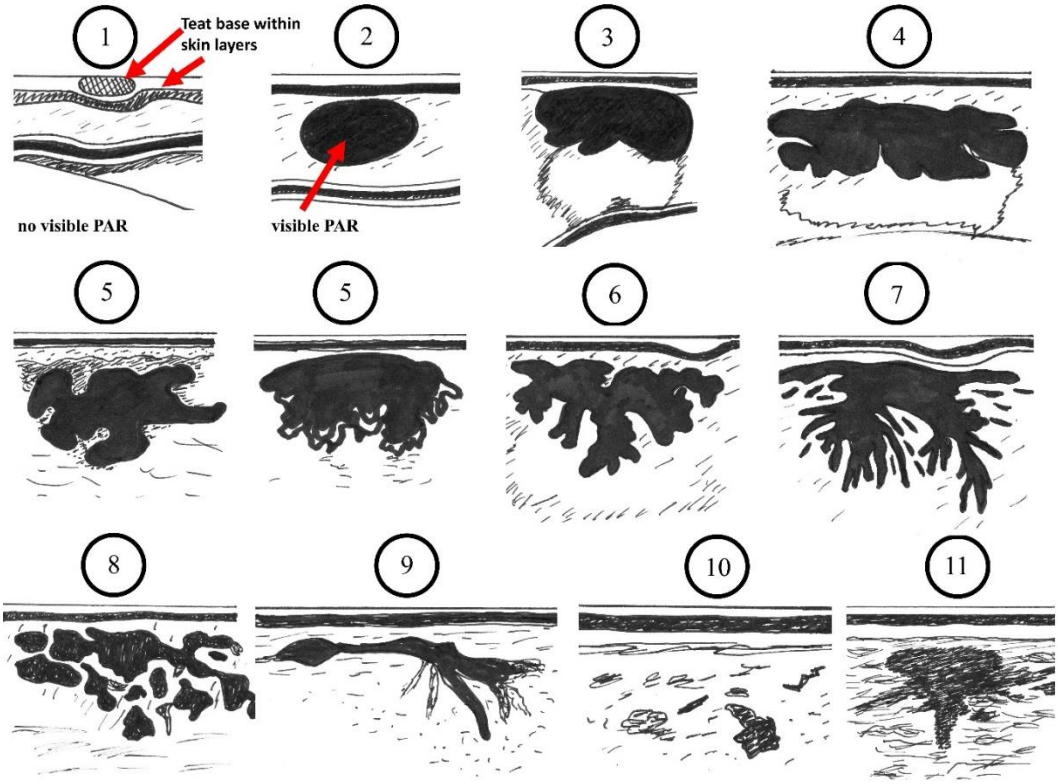


Figure 2. The Atlas of visible structures (categories) of the developing bovine mammary gland. These visible structures were observed in ultrasound images of 37 calves with preweaning high allowance to milk replacer (MR; HIGH; 10 L/d; n = 18) and restrictive allowance (RES; 5.7 L/d; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). The order of the categories (1 – 11) of the parenchyma´s (PAR) shape and structure did not correspond to the complete chronological order of PAR-developmental stages in one single animal. The categories represent the different PAR structures, visible in ultrasonic images (18 MHz, Esaote MyLab Five Vet scanner), in wk 3, then fortnightly from wk 8 – 16, and in wk 20 of calves´ life. In category 1 the teat base under the skin layers and underneath a white and grey flecked tissue bordered by abdominal layers and tissue was visible (red arrows), but not a mammary gland formation. In category 2-8, the PAR structures are outlined in black according to visible structures in the ultrasound image. In category 6 the cauliflower structure and in category 7 the coral structure is represented. In category 8 the PAR´s black structure is dissolving/distributing into single dark hypoechoic pieces within the surrounding tissue. In category 9 the formation of *ducti* is visible. In category 10 and 11 a grey flecked tissue structure without clearly visible *ductus* is visible, while in 11 a cistern might appear.

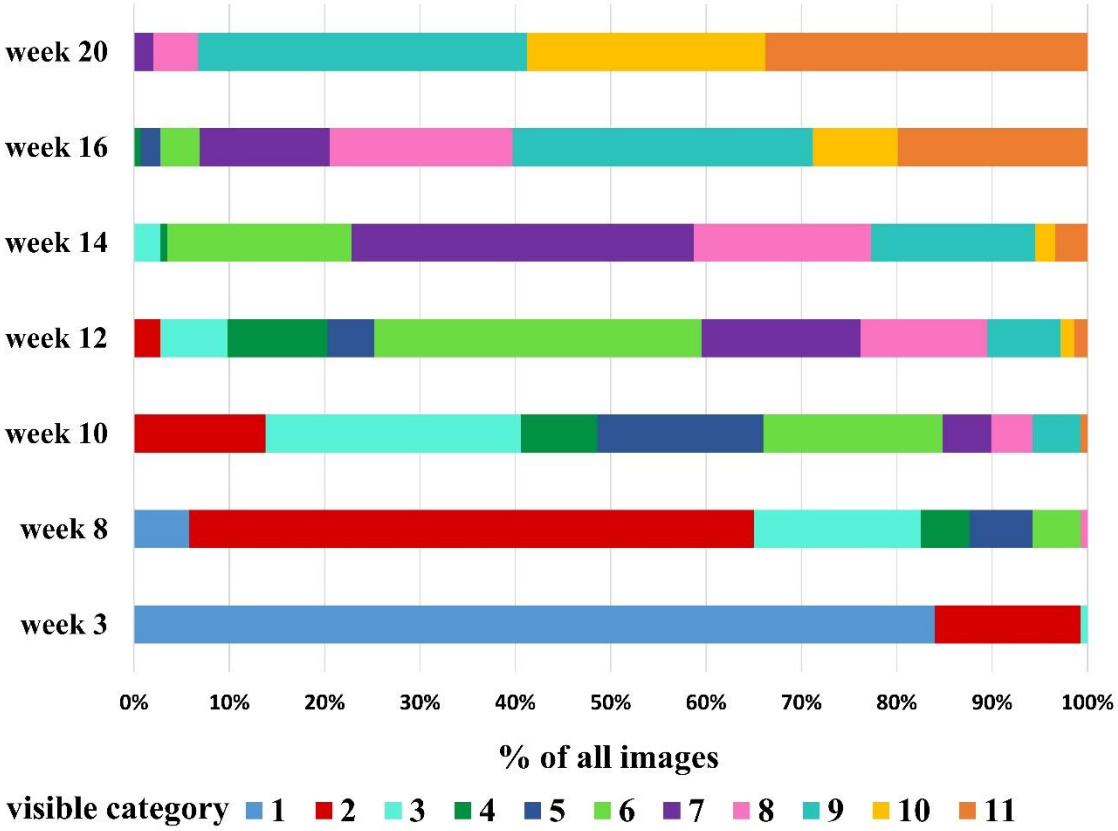


Figure 3. Distribution of visible Categories of mammary gland (MG) development. The visible structures of the parenchyma (PAR) in 1001 ultrasound images of all 37 calves and time points (week of life) could be assigned to the Categories in the Atlas of visible structures (categories) of the developing bovine mammary gland (Figure 2) which represents 96.6% of a total of 1036 images of scans: 0.7% were not assessable, because images were blurred, in 1.8% of all images the category was not identifiable and in 0.9% were not assessed.

Effects of time and feeding group on the occurrence of the mean visible category of PAR development

The mean visible category (MVC) of mammary gland PAR was calculated as the average of the category numbers calculated across all 4 quarters assessed per time point and animal. The MVC increased with time, without group differences and group x time interactions (Figure 4). In wk 16, HIGH tended to reach greater MVC than RES (P = 0.071). One animal had from wk 12 to 20 remarkably low MVC that were identified as outliers SD > 2.5 by Z-standardization and this calf was therefore excluded from further statistics.

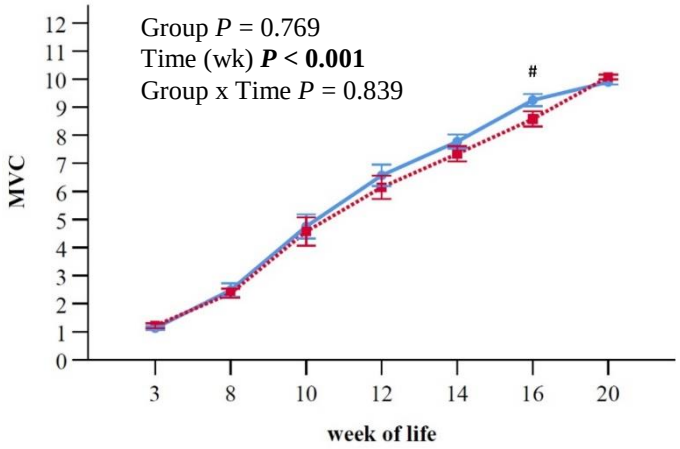


Figure 4. The mean visible category (MVC) of mammary PAR development. The MVC (means ± SEM) represents the average category of all 4 quarters within individual calves with preweaning high allowance to milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) and restrictive allowance (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

When comparing the visible categories, over all 4 mammary quarters, directly between groups by Chi-square analysis within each wk of life, a trend for a difference in appearing visible categories between feeding groups in wk 10 ($P = 0.059$) and a difference in wk 20 ($P = 0.02$) was observed (Figure 5): In HIGH only the higher Categories 8-11 were represented in wk 20, whereas Category 7 was still observed in 3.9% over all quarters of the RES calves. In wk 20, only 2.8% of all quarters of the HIGH calves were assigned to Category 8, whereas 10.3% of the quarters in the RES group were still classified in Categories 7 and 8 (Figure 5). Figure 5 shows the distribution of numbers/cases of the appearance of the visible category in different fed animals' quarters and the shift of development from lower to higher visible categories. It should be considered that in some calves more than one visible category could appear in the different quarters at the same time point.

The USAtlasMG developed herein with the different categories of the developmental stages of the bovine mammary gland, enabled the comparison and distinction between developmental stages and structures of the mammary gland. The USAtlasMG served as a template enabling us

to identify and clearly assign structures. Furthermore, the USAtlasMG can be used as a template for further quantitative measurements of PAR development by measuring the pixel brightness in US images, because the categories of the atlas can enable the precise placement of regions of interest within defined tissue structures in US images for measuring the brightness as pixel values. This US atlas can allow for the categorization and quantification of the tissue development and the concomitant shifts in the composition of the mammary gland.

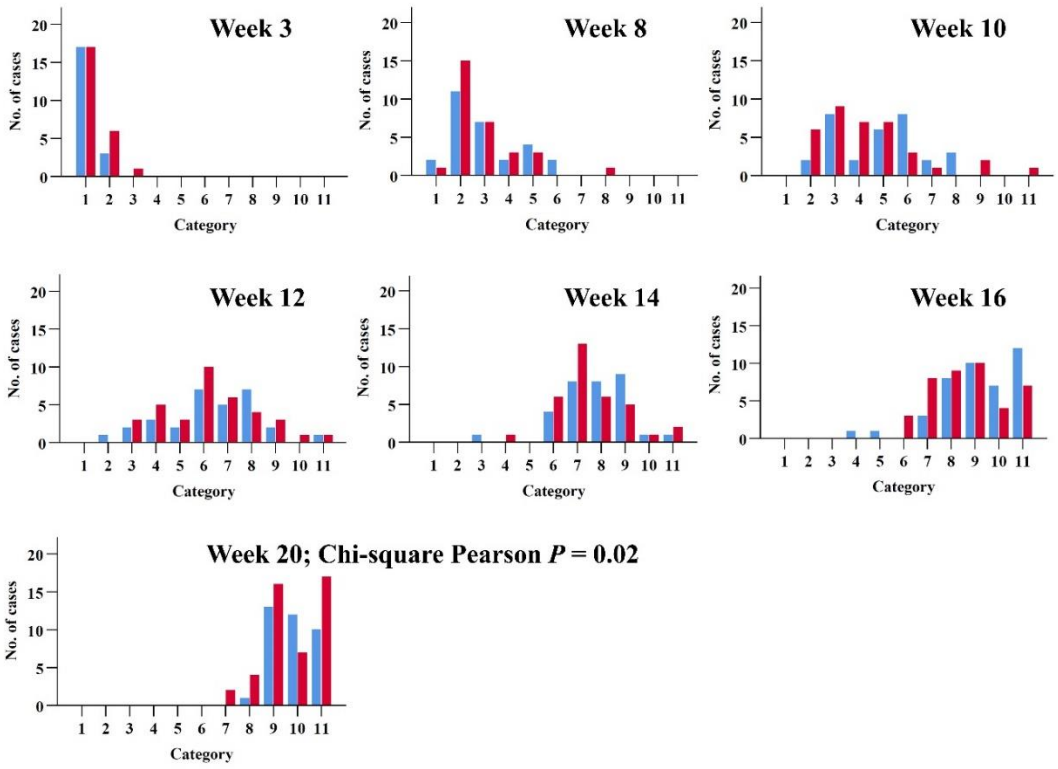


Figure 5. Weekly distribution of numbers of cases of appearance of the visible category in calves over all mammary quarters. The shift of development from lower to higher visible categories over wks is visible in calves with preweaning high allowance to milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) and restrictive allowance (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). It should be considered that in some animals more than one visible category could appear in the same animal in different quarters.

Palpation score and teat length

The palpation score of mammary glands is a cheap and non-invasive, but a subjective method to evaluate PAR development (Esselburn et al., 2015; Furini et al., 2018). There was only a tendency for an effect of feeding group on the mean palpation score (of all 4 quarters) visible in the linear mixed model, albeit there were a trend for higher mean palpations scores in wk 8 and a significant higher mean palpations score in wk 10 in HIGH group respectively. In both groups the mean palpation score showed an increase with age; from wk 3 (mean Score 0.34 ± 0.6 SD) until wk 14 (mean Score 7.38 ± 0.5 SD), when most calves reached Score 8 (Figure 6 A). Score 8 was added to the original palpation score of Esselburn et al. (2015), because in wk 12 and mainly in wk 20 of life we could palpate a flat, arching structure like a coin, which was not described so far, likely because the previous studies ended in wk 8 or 11 of life (Esselburn et al., 2015; Furini et al., 2018). Furthermore, Score 0 was added, because in contrast to previous studies in wk 3 of life we could only palpate the skin and teat in 26 calves (70.3%). In wk 3 of life, a PAR formation was not discernible in most ultrasound images except for 6 calves (HIGH n = 2, RES = 4). Therefore, wk 3 was excluded when testing for correlations. The mean palpation score was highly correlated with BW ($\rho = 0.83$; $P < 0.001$), which is not surprising and may support isometric MG growth in terms of mass at that time. The mean palpation score and the MVC from wk 8 – 20 were highly correlated ($\rho = 0.82$, $P < 0.001$). Hence with this study, we can also confirm that palpation is a suitable tool as an indicator for mammary PAR growth as suggested previously (Esselburn et al., 2015; Furini et al., 2018).

When analyzing the raw data on teat length occasionally decreases rather than increases with age were observed. Due to the implausibility of such findings, these cases were excluded from further statistical analyses. Albeit the calves were mostly calm during the measurements, they were not completely immobilized and thus errors might have occurred. However, 85.3% of all mean teat length measurements and values were plausible and were included in the further analyses. The teat length was mainly influenced by time, showing a linear increase from wk 3 until wk 20 of life, from 0.97 cm ± 0.5 SD to 2.87 cm ± 0.43 SD respectively. There were no group differences in teat length (Figure 6 B). Several studies considered the teat length as an indicator for circulating estrogen, which is a factor for prepubertal development of PAR (Lammers et al., 1999; Capuco et al., 2002). Hence, teat length was also considered to represent PAR growth (Esselburn et al., 2015).

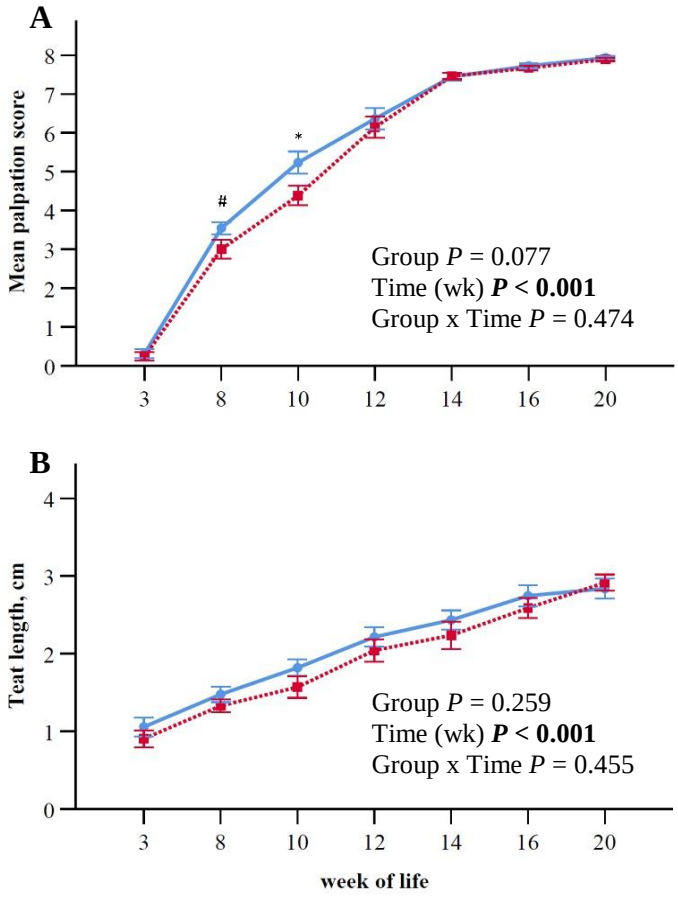


Figure 6. Pre- and postweaning changes (means ± SEM) in (A) mean palpation score and (B) teat length of four udder quarters of calves. The calves were fed at a high level of milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) or at a restricted level (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

CONCLUSION

Ultrasonically based categorization of PAR and SURR, as well as teat length measurements and palpation scoring, can be non-invasive ways to assess and monitor the mammary gland growth and its structural development. The Ultrasonographic Atlas of the developing bovine mammary gland (USAtlasMG) is a tool for the categorization and qualitative assessment of mammary gland structures. On this basis, the USAtlasMG was created as a template to arrange visible PAR structures of different stages of PAR´s development, for categorization and qualitative assessment of mammary gland structures. Furthermore, the USAtlasMG should be used for further defined quantitative measurements of PAR development by measuring the pixel brightness in US images as pixel values, because the categories of the USAtlasMG allow for the precise placement of regions of interest within defined tissue structures with regard to the respective category of development. Since the preweaning MR feeding regimen had no effect on the MVC and the mean teat length or only a tendency for influencing the mean palpation score it remains to be evaluated whether differences in performance become apparent in the first lactation.

ACKNOWLEDGMENTS

The authors declare no conflicts of interest. K. D. S. is a recipient of a scholarship from the H. Wilhelm Schaumann Foundation (Hamburg, Germany). The authors thank the staff of the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle in Münchweiler a. d. Alsenz in Rhineland-Palatinate (Germany), especially A. Brubacher, E. Therre, A. Schoenborn, S. Jobelius, K. Korst, D. Karch, E. Kröger, and the veterinary D. Lang for their support and effort. This project would not have been possible without their daily commitment, workforce, and help. The data that support the findings and which are presented in this study are available on request from the corresponding author, H. Sauerwein, sauerwein@unibonn.de.

REFERENCES

Akers, R. M. 2017. A 100-Year Review: Mammary development and lactation. J. Dairy Sci. 100(12):10332–10352. https://doi.org/10.3168/jds.2017-12983.

Albino, R. L., S. E. F. Guimarães, K. M. Daniels, M. M. S. Fontes, A. F. Machado, G. B. Dos Santos, and M. I. Marcondes. 2017. Technical note: Mammary gland ultrasonography to evaluate mammary parenchymal composition in prepubertal heifers. J. Dairy Sci. 100(2):1588–1591. https://doi.org/10.3168/jds.2016-11668.

Albino, R. L., M. I. Marcondes, R. M. Akers, E. Detmann, B. C. Carvalho, and T. E. Silva. 2015. Mammary gland development of dairy heifers fed diets containing increasing levels of metabolisable protein: Metabolisable energy. J. Dairy Res. 82(1):113–120. https://doi.org/10.1017/S0022029914000697.

Brown, E. G., M. J. VandeHaar, K. M. Daniels, J. S. Liesman, L. T. Chapin, J. W. Forrest, R. M. Akers, R. E. Pearson, and M. W. Nielsen. 2005. Effect of Increasing Energy and Protein Intake on Mammary Development in Heifer Calves. J. Dairy Sci. 88(2):595–603. https://doi.org/10.3168/jds.S0022-0302(05)72723-5.

Bruckmaier, R. M., and J. W. Blum. 1992. B-mode ultrasonography of mammary glands of cows, goats and sheep during α- and β-adrenergic agonist and oxytocin administration. J. Dairy Res. 59(02):151. https://doi.org/10.1017/S0022029900030399.

Capuco, A. V., and M. Akers. 2010. Management and environmental influences on mammary gland develoment and milk production. p. 259–292. In P. L. Greenwood, A. W. Bell, P. E. Vercoe, and G. J. Viljoen (eds.). Managing the Prenatal Environment to Enhance Livestock Productivity. Springer Netherlands, Dordrecht.

Capuco, A. V., S. Ellis, D. L. Wood, R. M. Akers, and W. Garrett. 2002. Postnatal mammary ductal growth: three-dimensional imaging of cell proliferation, effects of estrogen treatment, and expression of steroid receptors in prepubertal calves. Tissue Cell 34(3):143–154. https://doi.org/10.1016/s0040-8166(02)00024-1.

Cartee, R. E., A. K. Ibrahim, and D. McLeary. 1986. B-mode ultrasonography of the bovine udder and teat. J. Am. Vet. Med. Assoc. 188(11):1284–1287.

Daniels, K. M., A. V. Capuco, M. L. McGilliard, R. E. James, and R. M. Akers. 2009. Effects of milk replacer formulation on measures of mammary growth and composition in Holstein heifers. J. Dairy Sci. 92(12):5937–5950. https://doi.org/10.3168/jds.2008-1959.

Delorme, S., and J. Debus. 2005. Sonographie. 2., vollst. überarb. und erw. Aufl. Duale Reihe. Thieme, Stuttgart, Germany.

Ellis, S., R. M. Akers, A. V. Capuco, and S. Safayi. 2012. Triennial Lactation Symposium: Bovine mammary epithelial cell lineages and parenchymal development. J. Anim. Sci. 90(5):1666–1673. https://doi.org/10.2527/jas.2011-4671.

Esselburn, K. M., T. M. Hill, H. G. Bateman, F. L. Fluharty, S. J. Moeller, K. M. O'Diam, and K. M. Daniels. 2015. Examination of weekly mammary parenchymal area by ultrasound, mammary mass, and composition in Holstein heifers reared on 1 of 3 diets from birth to 2 months of age. J. Dairy Sci. 98(8):5280–5293. https://doi.org/10.3168/jds.2014-9061.

Franz, S., M. Floek, and M. Hofmann-Parisot. 2009. Ultrasonography of the bovine udder and teat. Vet. Clin. N. Am-Food Animal Prac. 25(3):669-85, Table of Contents. https://doi.org/10.1016/j.cvfa.2009.07.007.

Furini, P. M., R. A. Azevedo, S. R. A. Rufino, F. S. Machado, M. M. Campos, L. G. R. Pereira, T. R. Tomich, B. C. Carvalho, G. B. Santos, and S. G. Coelho. 2018. The effects of increasing amounts of milk replacer powder added to whole milk on mammary gland measurements using ultrasound in dairy heifers. J. Dairy Sci. 101(1):767–773. https://doi.org/10.3168/jds.2017-12798.

Geiger, A. J. 2019. Review: The pre-pubertal bovine mammary gland: unlocking the potential of the future herd. Animal 13(S1):s4-s10. https://doi.org/10.1017/S1751731119001204.

Geiger, A. J., C. L. M. Parsons, and R. M. Akers. 2016. Feeding a higher plane of nutrition and providing exogenous estrogen increases mammary gland development in Holstein heifer calves. J. Dairy Sci. 99(9):7642–7653. https://doi.org/10.3168/jds.2016-11283.

Gerbert, C., D. Frieten, C. Koch, G. Dusel, K. Eder, T. Stefaniak, J. Bajzert, P. Jawor, A. Tuchscherer, and H. M. Hammon. 2018. Effects of ad libitum milk replacer feeding and butyrate supplementation on behavior, immune status, and health of Holstein calves in the postnatal period. J. Dairy Sci.. https://doi.org/10.3168/jds.2018-14542.

Hovey, R. C., T. B. McFadden, and R. M. Akers. 1999. Regulation of Mammary Gland Growth and Morphogenesis by the Mammary Fat Pad: A Species Comparison. J. Mammary Gland Biol. Neoplasia, Vol. 4, No. 1, 1999. https://doi.org/10.1023/A:1018704603426.

Korst, M., C. Koch, J. Kesser, U. Müller, F.-J. Romberg, J. Rehage, K. Eder, and H. Sauerwein. 2017. Different milk feeding intensities during the first 4 weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation. J. Dairy Sci. 100(4):3096–3108. https://doi.org/10.3168/jds.2016-11594.

Lammers, B. P., A. J. Heinrichs, and R. S. Kensinger. 1999. The Effects of Accelerated Growth Rates and Estrogen Implants in Prepubertal Holstein Heifers on Estimates of Mammary Development and Subsequent Reproduction and Milk Production. J. Dairy Sci. 82(8):1753–1764. https://doi.org/10.3168/jds.S0022-0302(99)75406-8.

Lohakare, J. D., K.-H. Südekum, and A. K. Pattanaik. 2012. Nutrition-induced Changes of Growth from Birth to First Calving and Its Impact on Mammary Development and First-lactation Milk Yield in Dairy Heifers: A Review. Asian Australas. J. Anim. Sci. 25(9):1338–1350. https://doi.org/10.5713/ajas.2012.12282.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006a. Developmental and Nutritional Regulation of the Prepubertal Bovine Mammary Gland: II. Epithelial Cell Proliferation, Parenchymal Accretion Rate, and Allometric Growth. J. Dairy Sci. 89(11):4298–4304. https://doi.org/10.3168/jds.S0022-0302(06)72476-6.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006b. Developmental and Nutritional Regulation of the Prepubertal Heifer Mammary Gland: I. Parenchyma and Fat Pad Mass and Composition. J. Dairy Sci. 89(11):4289–4297. https://doi.org/10.3168/jds.S0022-0302(06)72475-4.

Nishimura, M., T. Yoshida, S. El-Khodery, M. Miyoshi, H. Furuoka, J. Yasuda, and K. Miyahara. 2011. Ultrasound imaging of mammary glands in dairy heifers at different stages of growth. J. Veterinary Med. Sci. 73(1):19–24. https://doi.org/10.1292/jvms.09-0503.

Passillé, A. M. de, T. F. Borderas, and J. Rushen. 2011. Weaning age of calves fed a high milk allowance by automated feeders: Effects on feed, water, and energy intake, behavioral signs of hunger, and weight gains. J. Dairy Sci. 94(3):1401–1408. https://doi.org/10.3168/jds.2010-3441.

Penninck, D., and M.-A. d'Anjou. 2015. Atlas of Small Animal Ultrasonography. 2nd ed. Wiley, Somerset.

Rowson, A. R., K. M. Daniels, S. E. Ellis, and R. C. Hovey. 2012. Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. Sem. Cell Dev. Biol. 23(5):557–566. https://doi.org/10.1016/j.semcdb.2012.03.018.

Salomon, F.-V., H. Geyer, and U. Gille. 2005. Anatomie für die Tiermedizin. Enke, Stuttgart.

Seibt, K. D., M. H. Ghaffari, T. Scheu, C. Koch, and H. Sauerwein. 2021. Characteristics of the Oxidative Status in Dairy Calves Fed at Different Milk Replacer Levels and Weaned at 14 Weeks of Age. Antioxidants 10(2):260. https://doi.org/10.3390/antiox10020260.

Sheffield, L. G. 1988. Organization and growth of mammary epithelia in the mammary gland fat pad. J. Dairy Sci. 71(10):2855–2874. https://doi.org/10.3168/jds.S0022-0302(88)79881-1.

Silva, A. L., E. Detmann, J. Dijkstra, A. M. Pedroso, L. H. P. Silva, A. F. Machado, F. C. Sousa, G. B. Dos Santos, and M. I. Marcondes. 2018. Effects of rumen-undegradable protein on intake, performance, and mammary gland development in prepubertal and pubertal dairy heifers. J. Dairy Sci.. https://doi.org/10.3168/jds.2017-13230.

Swanson, E. A., and J. I. Poffenbarger. 1979. Mammary Gland Development of Dairy Heifers during Their First Gestation. J. Dairy Sci. 62(5):702–714.

Trasch, K., A. Wehrend, and H. Bostedt. 2007. Ultrasonographic description of canine mastitis. Vet. Rad. Ultrasound 48(6):580–584. https://doi.org/10.1111/j.1740-8261.2007.00301.x.

5 Manuscript III

Interpretive Summary: Effects of different feeding levels during a 14-week preweaning phase in dairy heifers: Part 2: Mammary gland development evaluated by ultrasound and brightness measurements, body growth, and first lactation performance. *By Seibt et al.,* Mammary gland development from week 3 to 20 of calves' life was evaluated by ultrasonography measuring the pixel brightness of distinct mammary gland structures according to a previously developed Ultrasonographic Atlas of the developing bovine mammary gland. Feeding milk replacer at a high or a restrictive level for 14 weeks did not alter the brightness of mammary tissue structures, but a high milk replacer level had advantages in body growth in length and width and potentially also for udder health in first lactation.

RUNNING HEAD: EXTENDED MILK FEEDING FOR 14 WEEKS IN DAIRY CALVES

Effects of different feeding levels during a 14-week preweaning phase in dairy heifers: Part 2: Mammary gland development evaluated by ultrasound and brightness measurements, body growth, and first lactation performance

K. D. Seibt,[1] T. Scheu,[2] C. Koch,[2] M. H. Ghaffari,[1] and H. Sauerwein[1]

[1]Institute of Animal Science, Physiology Unit, University of Bonn, Katzenburgweg 7, 53115 Bonn, Germany

[2]Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, 67728 Münchweiler an der Alsenz, Germany

*Corresponding author: sauerwein@uni-bonn.de (Helga Sauerwein)

ABSTRACT

Ultrasound technologies allow for non-invasive observation of mammary gland (MG) development and the differentiation between the tissue types of the MG. In addition, measurements of body dimensions may provide better insights into the general growth patterns of calves. In this study, ultrasonic images were acquired of 37 German Holstein dairy heifer calves at 7 time points during the first 5 months of life. Calves either received a high plane of milk replacer (MR; 14% solids) at 10 L/d (1.4 kg MR/d; HIGH, n = 18) or a restrictive plane of 5.7 L/d (0.8 kg MR/d; RES, n = 19) until the linear weaning in wk 13 and in wk 14 of life. Ultrasound evaluations of the MG as well as of back-fat thickness (BFT) and rib-fat thickness (RFT) were performed in wk 3 of life, fortnightly from wk 8–16, and in wk 20 of life, using a B-mode ultrasound device equipped with a linear transducer (18 MHz). In 30 calves (HIGH n = 16; RES n = 14) of pluriparous cows only, the morphometric traits for body height, body length, thorax, and hip were determined. According to the previously developed Ultrasonographic Atlas of the developing bovine mammary gland (USAtlasMG), which allows for the categorization of 11 visible developmental stages of mammary parenchyma (PAR) and its surrounding tissue (SURR), ultrasound images were further classified by PAR structure, and pixel brightness was measured in PAR and SURR by using ImageJ Fiji. Additionally, the difference in pixel brightness between PAR and SURR was calculated, the delta pixel value (Δ = SURR - PAR). The preweaning daily MR allowance had no effect on the pixel values of PAR, SURR, and Δ pixel value. The PAR brightness and pixel value increased from wk 8 to wk 20 of life, whereas SURR and Δ pixel values were decreasing with the beginning of weaning in the end of wk 12 of life. Both BFT and RFT increased with age, whereby HIGH calves had more RFT and tended to have greater BFT values than RES calves from wk 8 until the end of weaning. In 30 calves of pluriparous cows, all variables of the body dimensions were increasing with age. Back length, diagonal body length, chest girth, shoulder bone distance, distances between hip joints, hip bones, and ischial tuberosity were greater in HIGH than in RES calves. Records of lactational performance were limited to 23 heifers (HIGH n = 11; RES n = 12) and their first 7 wks of lactation; age at first calving was not different between the groups, but cows from the former HIGH group produced in average 4.6 kg/d more milk than those from the RES group. The difference was mainly attributed to two low-performing animals in the RES group that also had elevated somatic cell counts (> 200.000 cells/mL). Hence, feeding milk replacer at a high or a restrictive level for 14 wks did not alter the brightness of mammary tissue structures, but a high MR feeding level had advantages in body growth in length and width, and potentially also for udder health in first lactation.

Keywords: mammary gland, dairy heifer, sonography, ultrasound, rib-fat

INTRODUCTION

Ultrasonography became an attractive tool for evaluating tissue development or structures of specific parts of the body. It is a non-invasive technique and allows for repeated examining of an individual during phases of life that are of specific interest. Ultrasonography can also provide a detailed insight into the different structures and characteristics of tissues in order to differentiate and define them, without the need for dissection. The development of the mammary gland (MG) in dairy cattle is one of the determinants for future performance in lactation. Traditionally, MG growth has been considered as relatively unimportant or to be decelerated and quiescent in early life (Geiger, 2019), but the notion that mammary gland development is already established in early life and that nutritional and management conditions during the preweaning phase can influence future development and function of the MG has been substantiated in several studies (Brown et al., 2005; Meyer et al., 2006b; Akers and Denbow, 2013) including ultrasound-based studies (Albino et al., 2015; Esselburn et al., 2015; Albino et al., 2017).

The mammary gland develops from the germ layer ectoderm in the embryonic stage building a first bud (Hovey et al., 1999; Franz et al., 2009; Rowson et al., 2012). In the fetal stage, two different tissue fractions are formed; the mammary parenchyma (PAR) and the early formation of an adjacent mammary fat pad (MFP; Hovey et al., 1999; Rowson et al., 2012). After birth, these two tissue fractions largely determine the postnatal growth of the MG (Rowson et al., 2012) and a single primary duct is already formed, extends from the teat to the gland cistern and ends with the epithelial ducts (Rowson et al., 2012; Akers and Denbow, 2013).

Mammogenesis, the development of MG parenchymal structures, during the following wks of life comprises growth of both the duct system and the surrounding tissue containing adipose and connective tissue, termed as MFP, and the spreading of the ducts into the MFP (Hovey et al., 1999; Albino et al., 2015). The lobuloalveolar development is largely limited to later ages, with substantial increases starting only with conception (Lawrence and Fowler, 2002).

Esselburn et al. 2015 postulated that in heifers from birth up to 2 mo (8 wk) of age, ultrasound provides an effective quantitative tool for measuring changes in the development of the PAR in vivo by assessing the pixel values of defined areas of the ultrasound images using the software program Image J. Several studies followed this example of a non-invasive evaluation and further improved the methodology (Silva et al., 2018; Albino et al., 2015; Furini et al., 2018):

The whole mammary PAR area (Esselburn et al., 2015), the circularity of PAR (Furini et al., 2018) and at least the brightness of different tissues in ultrasound images (pixel value) were measured (Albino et al., 2015; Albino et al., 2017; Furini et al., 2018; Silva et al., 2018).

Previous studies already showed that MG development in early life of calves can be modulated by preweaning nutrition (Brown et al., 2005; Meyer et al., 2006b; Geiger, 2019). An intensive preweaning feeding regimen until wk 8 of life, i.e. increased energy and protein intake, can positively influence MG development, especially of PAR mass growth (Brown et al., 2005; Meyer et al., 2006a). A trend for an increase in mammary epithelial cell proliferation in PAR in response to higher preweaning (wk 6) energy intake was also observed (Meyer et al., 2006b). It was also becoming evident that the period between wk 8 and 10 of life during rearing is a critical time for imprinting mammary gland capacity for milk production (Brown et al. 2005, Meyer et al., 2006a).

In the present study, we aimed at expanding the current knowledge about the different developmental stages of PAR and MFP structural formation as discernible by ultrasound. For quantitative assessments, the brightness or pixel value of structures that were defined taking an Ultrasonographic Atlas of the developing bovine MG (Seibt et al., xxxx Part 1 companion paper) as a basis. A further goal was to characterize the development with body growth in general (assessed by measuring body dimensions and subcutaneous fat layers at two locations) by comparing two different feeding intensities during an extended preweaning phase of 14 wks. Finally, we aimed at assessing the performance of the heifers in their first lactation. We hypothesized that a higher allowance of MR in this elongated preweaning phase will promote MG development along with body and subcutaneous fat layer growth in comparison to a restricted feeding regimen.

MATERIALS AND METHODS

The present study was conducted at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle, Germany, following the guidelines of the German Law for Animal Welfare by permission of the corresponding authority (G 17-20-071; Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany).

Animals, Diets, Feeding, and Management

Details of the experimental design with feeding regimens, weaning, blood sampling, and results about feed intake, feeding behavior, and various indicators of the oxidative status were

previously described (Seibt et al., 2021). In brief: 37 German Holstein heifer calves were studied from birth until wk 20 of life, from February 2018 until March 2019. Only healthy calves born without dystocia were included in this study. After the colostrum feeding (at least 3 L within 3 h post partum), all calves received 5 L twice per day of their dam's transition milk via teat buckets in two meals per day until d 5 of life. Thereafter the calves were stratified into two different feeding groups according to birth weight, sire, and parity of the dam to create equal groups. Half siblings were also considered and were equally distributed to the feeding groups: Group HIGH (n = 18) received 10 L MR/d (14% solids; Milkivit Titan, Trouw Nutrition Deutschland GmbH, Burgheim, Germany), and groups RES (n = 19) was restricted to 5.7 L MR/d. The intake of transition milk and MR was documented by weighing the buckets before feeding and the residues in the bucket before offering the next meal. At d 10 of life, the calves were moved from single hutches to an open straw-bedded stable with an automated feeding system (Vario Kombi, Förster-Technik GmbH, Engen, Germany). Calf starter concentrate (Blattin Kälberstart Gold, Höveler Spezialfutterwerke GmbH & Co. KG, Dormagen, Germany) and MR were dispensed according to the group's respective feeding regimen. The intake of both, MR and pelleted calf starter concentrate, as well as the number of rewarded and unrewarded visits were recorded individually by the feeding system (Förster-Technik GmbH). Water, hay, and calf starter were offered ad libitum. The ingredients and the chemical composition of the MR and starter were reported previously (Seibt et al., 2021). Weaning started at d 84 (end of wk 12) of life, by reducing the allowance for MR intake linearly until d 98 (wk 14) of life by 0.3 L/d in the RES and 0.6 L/d in the HIGH group, respectively. From d 99 onwards the MR supply was stopped and calves were moved to a new group pen in another stable where they had free access to water and a TMR for heifers as described previously (Seibt et al., 2021). The health status was checked weekly by veterinarians over the entire 20 wk of the trial (Seibt et al., 2021). On the day of the weekly health check, BW was recorded until wk 20 of life by using a mobile electronic scale for small farm animals (KWM GmbH Waagen- und Metallbau, Thiersheim, Germany).

Ultrasound measurements of the Mammary gland, of Back-fat, and Rib Fat Thickness (BFT and RFT)

Ultrasound measurements were performed at 7 time points, first in wk 3 of life, then fortnightly from wk 8 until wk 16 of life (wk 8, 10, 12, 14, 16) and in wk 20 of life. All ultrasound measurements were performed by the same person. The ultrasound images were obtained using

a B-mode ultrasound device MyLab Five Vet scanner (Esaote Biomedica GmbH, Cologne, Germany) equipped with a linear transducer. A fixed preset as a defined template was created to ensure equal conditions and their repeatability for all measurements. The presetting were in B-mode, 4 cm measurement depth, and a greyscale from 40% to 60%. Two ultrasound images each were taken per mammary quarter. For the evaluation of the mammary glands, the animals were in standing position with the transducer placed in a standardized position at a 45° inclination in relation to the teat position, in caudal-cranial direction according to (Nishimura et al., 2011). The udder was wetted with clear ultrasound gel (Cb Healthcare Intl. S.r.l., Predappio, Italy). Together with the frequent handling for weighing and health checks, the calves became quickly familiar with the procedure and were calm during the measurements that were performed on an electronic scale for small farm animals (KWM GmbH Waagen- und Metallbau, Thiersheim, Germany) with frontal and lateral boundaries. Based on the different shapes and structures visible in the ultrasound images over time, a template for classifying the developmental stages was drawn by hand, differentiating and describing 11 stages as detailed in the Ultrasonographic Atlas of the developing bovine mammary gland detailed in the companion paper (Seibt et al., xxxx Part 1).

In addition to the mammary records, back-fat thickness (BFT) in the sacral region and rib-fat thickness (RFT) at the last rib were evaluated using the same ultrasound conditions as described above for MG measurements. The BFT records were performed on 35 calves only due to an organizational error, and RFT assessments were limited to 30 calves from pluriparous dams (HIGH: n = 16; RES: n = 14).

Evaluation of Pixel values

The brightness of the tissues visible in the ultrasound images was evaluated by assessing the so-called pixel value of PAR and of the surrounding tissue (SURR). For this purpose, ultrasound images were saved in bitmap (BMP) format and then transferred to the Fiji ImageJ program (National Institutes of Health, Bethesda, MD; https://imagej.net/Fiji) for further analyses. All ultrasound images were evaluated for pixel value in an 8-bit format. In 8-bit images, each pixel was numerically represented on a scale of 256 shades of grey (0 = black; 256 = white) according to their brightness (Ferreira and Rasband, 2012).

A macro was programmed in Fiji ImageJ to open the ultrasound images, transform them to 8-bit images, and to create a size normed square of 0.25 cm^2 = 900 pixels/square. Each square represented a so-called “region of interest” (ROI) within the Fiji ImageJ measurements. The

scale of pixels per centimeter (1 cm = 120 pixels) was calibrated using the straight tracer of Fiji ImageJ according to Furini et al. (2018). Thus, the number of pixels per centimeter always depended on the cm-scale of the ultrasound image. Each defined 0.25 cm^2 square was positioned as a ROI with the mouse cursor on PAR areas and within surrounding (SURR) based on the specifications of the USAtlasMG (Seibt et al., xxxx Part 1 and Supplemental Table 1). The area of each square was fixed afterwards, added to the Fiji ROI (region of interest) manager and its pixel value (brightness) was measured. Within the PAR area and separately in SURR, we used the pixel value to calculate the average pixel brightness. The pixel values of each mammary gland quarter were calculated as the mean of 3 squares randomly laid into the PAR area and the mean of 2 squares (0.25 cm^2 each) which were laid into the SURR selected via the USAtlasMG (Seibt et al., xxxx Part 1 and Supplemental Table 1, Category 1-11). The difference between the mean pixel value of PAR of all 4 quarters and the mean SURR pixel value was calculated as Δ pixel value = mean pixel value SURR – mean pixel value PAR. Less differences in brightness and pixel values of those tissue structures are characterized by lower Δ pixel values.

Body dimensions

The assessment of the body dimensions was limited to those 30 calves (HIGH n = 16; RES n = 14) that were born by pluriparous cows (see above for RFT measurements) in two-week intervals from wk 8 until wk 16, and a final measurement in wk 20 of life. The dimensions recorded were (1) body height, including withers height and hip height, of (2) body length, including back length, and diagonal body length, of (3) the thorax, including chest girth, abdominal girth at the last rib, and shoulder joint bones distance, and (4) the hip, including hip joint distance, hip bone distance, and ischial tuberosity distance. A detailed description of measurement of these variables is shown in Table 1.

First lactation performance

In 2020 and 2021, from an initial of 37 heifers calves the first lactation, performance in the first 7 wks after calving could be recorded in 23 heifers; HIGH n = 11; RES n = 12. Due to managerial reasons on the farm, several heifers were sold before or during the first lactation and could thus not be followed for evaluating their later performance. Cows in the first lactation were milked twice daily at 0500 h and 1530 h. Daily milk yield was recorded electronically via the herd management system Dairy Plan C21 (GEA Farm Technologies GmbH). Milk fat, milk

protein, and somatic cell counts (SCC) in milk were assessed using an infrared milk analyzer (MilkoScan FT-6000; Fossomatic FC, Foss Analytical A/S, Hillerød, Denmark) at the laboratory of the milk recording organization, Landeskontrollverband Rheinland-Pfalz-Saar e. V. Bad Kreuznach, Germany. The energy-corrected milk (ECM) was calculated as ECM (kg/day) = milk yield (kg/day) × [1.05+(milk fat (%) × 0.38 + milk protein (%) × 0.21)]/3.28, according to the equation of the German Agricultural Society (Deutsche Landwirtschaftsgesellschaft, 2000).

Table 1. Measurements of body dimensions and growth.

	Variable[1]	Measuring tool	Point of measurement
Body height	Withers height (cm)	Yardstick	Height from the floor to the spinous / dorsal process of the first thoracic vertebra
	Hip height (cm)	Yardstick	Height from the floor to the sacrum
Body length	Back length (cm)	Yardstick	From the spinous / dorsal process of the first thoracic vertebra to the sacrum
	Diagonal Body length (cm)	Yardstick	From the shoulder/bow joint to the ischium
Thorax	Chest girth (cm)	Tape measure	behind the front leg
	Abdominal girth (cm)	Tape measure	at the level of the 12th rib
	Shoulder/bow joint bones distance (cm)	Measuring Forceps / pliers[1]	Distance between the centers of the shoulder/bow joints
Hip	hip joint distance (cm)	Measuring Forceps / pliers[1]	Distance between the centers of the hip joints
	hip bone distance (cm)	Measuring Forceps / pliers[1]	Distance between the centers of the hip bones
	ischial tuberosity distance (cm)	caliper	Distance between the centers of the ischial tuberosity

[1]Hipometer, Quidee GmbH, Homberg (Ohm), Germany.

Statistical Analysis

Adjustment of raw data on body dimension raw data for statistical analyses

The raw data of the body dimensions were first analyzed by using Microsoft Excel (Version 2016; Microsoft Corporation, Redmond, WA) and IBM SPSS (Version 26; SPSS Inc., Chicago, IL). The raw data of each variable were tested for normal distribution using the Kolmogorov-

Smirnov test. Boxplots were then generated for each variable to identify outliers (SD > 1.5 x interquartile distance) and extreme outliers (SD > 3 x interquartile distance). These outliers were checked for biological plausibility by calculating the differences between two subsequent measurement points, i.e., delta values, for each animal to check the biological plausibility of outliers. Negative delta values concerning height, body length, and width were not plausible and were thus excluded. Additionally, the delta values of each variable were Z-standardized using SPSS, i.e., the standard deviation (SD) of each value was calculated in SPSS. Extreme outliers (SD > 2) were excluded.

General statistical analyses

Data were analyzed using SPSS (version 26; SPSS Inc., Chicago, IL). The normal distribution was checked by the Kolmogorov-Smirnov-test and the homogeneity of variance by using the Levene´s test. If the data were not normally distributed, nonparametric tests were performed and data were log-transformed for further statistical analysis. Outliers were identified by Z-standardization and values with SD > 2 were excluded. Furthermore, data from pixel values, BFT, RFT, variables of body dimensions, and from milk yield and ECM in the first lactation were analyzed by a linear mixed model with Bonferroni post-hoc test and with repeated measurements, considering the individual calf as a random effect and feeding group (group), wk of life (time) as well as the interaction therefrom as fixed effects. Differences between the groups within each time point and of the age at first calving were tested with Student´s t-test or Mann-Whitney-U-test, in case of not normally distributed data and no homogeneity of variance. In all graphs, non-transformed data are shown as means ± SEM. Correlations (ρ) were calculated by Spearman analysis. Results were declared as significant when $P < 0.05$, and $0.05 \leq P < 0.10$ was considered as a trend.

RESULTS AND DISCUSSION

Growth performance and feed intake

Detailed information about growth performance, BW gain, average daily gain (ADG), as well as of MR, starter feed, and ME intakes, and health status is presented in (Seibt et al., 2021). In brief, birth weight, colostrum quality, and intake were not different between the two feeding groups. Expectedly, the MR intakes were greater in the HIGH than in the RES calves throughout the entire liquid feeding period until the beginning of weaning. The greater MR

intake in the HIGH calves did not result in decreased intake of starter intake. Thus the greater daily ME intake in the HIGH calves until the beginning of weaning (wk 13 – wk 14 of life) was exclusively attributable to their MR intake. On average, HIGH calves were heavier than RES calves until 20 wk of age. Weaning was not associated with BW loss in either group. The ADG were greater in HIGH than in RES calves until wk 7 of life. In the postweaning phase, ADG in both feeding groups declined whereby the nadir was reached earlier (in wk 16) in RES calves than in HIGH calves (wk 18). Overall, the HIGH group had an advantage in development when compared to the RES group, which may also be evident in the development of the mammary gland, in context with body dimensions which is the focus of this study.

Pixel values of PAR, SURR, and Delta pixel values

A pixel is the smallest discernible element in a digital image. Each image has a defined number of those; the bits per pixel (bpp), defining the so-called bit-depth. For the ultrasonographic image analysis in this study, we used 8 bpp, an 8-bit scale, allowing for the differentiation of 28 = 256 shades of grey (Ferreira and Rasband, 2012). This enables quantitative calculations and comparison of the structures of a digital image, due to their different echogenicity for ultrasonic waves.

The more the ultrasonic waves are reflected by tissue structures (hyperechoic), the brighter the structure appears on the image and thus shows a higher average pixel value. Fat tissue is hyperechoic and thus appears brighter (white), i.e. the pixel values are higher. Conversely, the more the ultrasonic waves are absorbed and less reflected (hypoechoic), the darker the structure appears along with a lower pixel value (Albino et al., 2015 and Figure 1). The PAR is such a hypoechoic tissue, has therefore a lower pixel value and appears darker (Albino et al., 2015).

A detailed description of the structures of the developing mammary gland that are discernible in ultrasonic images is presented in the companion paper Part 1 (Seibt et al., xxxx Part 1). In brief: the developing PAR appears as a hypoechoic dark structure and the surrounding tissue SURR is demarcated to the cavum abdominis by the abdominal wall (Salomon et al., 2005). The PAR is highly hypoechogenic, and thus an artifact, the so-called distal enhancement can arise underneath the PAR, causing a brighter appearance of the SURR underneath the PAR (Delorme and Debus, 2005; Penninck and d'Anjou, 2015). In addition, visible shadowy streaks, the so-called edge shadowing, can occur on the lateral PAR, caused by the lesser attenuation of ultrasonic waves when passing the PAR as compared to a direct passing of the PAR´s SURR (Delorme and Debus, 2005; Penninck and d'Anjou, 2015). Both ultrasonographic artifacts were

considered during the evaluation of ultrasound images, i.e. the ROI squares by Fiji ImageJ for evaluating the pixel value of SURR were laid within the brighter area adjacent to the PAR´s border, thus avoiding eventual artifact zones. On this basis, we evaluated the brightness of tissues visible in ultrasound images by assessing the so-called pixel value separately in PAR and SURR, respectively (Seibt et al., xxxx Part 1 and supplementary Table 1).

The PAR pixel values changed with time but were neither affected by the feeding group nor the group x time interaction (Figure 1A). In wk 3 of life, PAR formation was not detectable, hence the Fiji ImageJ squares (ROI) were laid onto the grey-flecked tissue layer between dermis and abdominal (Supplementary Table 1 and USAtlasMG Category 1), where PAR will be developed in further categories of development (USAtlasMG, Seibt et al., xxxx Part 1). This grey flecked structure was comparable to the tissue structure of spreading PAR in Category 9–10 (USAtlasMG) occurring mostly in wk 20 of life and the brightness of tissue structures of both time points was alike. Therefore the PAR pixel values in wk 3 and wk 20 were on a comparable level.

With progressing PAR development (USAtlasMG, Supplementary Table 1), the PAR pixel brightness increased from wk 10 to 20 of life (Figure 1A), i.e., the dark hypoechoic PAR structure was dissolving since the PAR spread into its SURR (USAtlasMG). Furini et al. (2018) observed that adding milk replacer powder to whole milk fed to heifers weaned at the age of 60 d of life (wk 9) increased their preweaning body weight, but neither the preweaning PAR growth, the average pixel value within PAR, nor the ultrasonically measured deposition of adipose tissue within the gland was affected by the diet in that period. Furthermore, Furini et al. (2018) evaluated the growth of the mammary PAR from wk 5–11 by measuring its circularity on ultrasound images. The oval-shaped structure of PAR was altered after 2 months of age (wk 8 of life) (Furini et al., 2018). These authors also reported that PAR growth was isometric with body growth from wk 5 - 11 of life in heifers (Furini et al., 2018). In our study, the average PAR pixel value increased from wk 10 of life onwards (Figure 1A). The PAR pixel values from wk 8 – 20 were positively correlated with BW ($\rho = 0.734$; $P < 0.001$), whereas by including wk 3 there was no correlation ($\rho = 0.052$; $P = 0.437$) anymore. Concerning the palpation score results, presented in the companion Seibt et al., xxxx (Part 1): positive correlations with the average PAR pixel values were observed for all 4 quarters from wk 8 – 20 ($\rho = 0.56$; $P < 0.001$). Values of wk 3 of life had to be excluded from testing for correlations, because palpable tissues were limited to skin and teat in 26 calves (70.3% Score 0); in most ultrasound images except for 6 calves (HIGH n = 2, RES = 4) a formation of PAR was not discernible (Seibt et al., xxxx Part 1). Our results thus point to an isometric phase of PAR development from wk 8 - 20 and

are thus in line with previous studies of Furini et al. (2018) and Esselburn et al. (2015). These authors showed that a correlation between palpation scoring and ultrasound image evaluation of PAR exists within a timeframe of wk 5 – 11 of life (Esselburn et al., 2015; Furini et al., 2018). Our study supports that in calves older than 11 wk, palpation is a suitable tool as an indicator for mammary PAR growth. Furthermore, the mean palpation score was highly correlated with BW ($\rho = 0.83$; $P < 0.001$, Seibt et al., xxxx Part 1), promoting further that the MG grows isometrically at that time.

An increase of protein and energy intake between wk 2 and 8 of life was shown to increase the PAR growth rate of heifers (Brown et al., 2005). In contrast, Esselburn et al. (2015) did not observe any dietary effect on PAR growth rate, PAR mass, or PAR composition at the day of slaughter at 2 months of age (8 wk). Albino et al. (2015) observed that MG of heifers with diets that contain protein:energy (MP:ME) ratios less than 38 g/Mcal became more reflective of ultrasound waves, indicating greater accumulation of hyperechongenic fat within the PAR area. They proclaimed that a significant change of pixel value during the prepubertal development from wk 8 of life until puberty suggests that ultrasound measurements can provide good insights into the dynamic development of the MG. Silva et al. (2018) reported that mammary ultrasonography indicated no effects of rumen-undegradable protein amounts in the diet on MG composition.

The assessment of the SURR pixel value was largely limited to wk 8 – wk 16 of life, because in wk 3 there were fewer cases with a defined SURR area on the ultrasound images due to their PAR developmental stage (USAtlasMG; Category 1, Seibt et al., xxxx Part 1) in wk 3 (n = 6; HIGH n = 2, RES = 4) and also in wk 20 (n = 3; HIGH n = 1, RES = 2; Figure 1B). When including all wks (3 – 20) into the statistical analyses, the SURR pixel value was neither influenced by time nor by feeding group or interactions thereof. When excluding wk 3 and 20, and taking only wk 8 -16 for further analysis into account, SURR was influenced by time, but not by feeding group and without interactions thereof (Figure 1B).

The reduction of the average pixel value in SURR (getting darker) is caused by the growth of hypoechogenic, secretory, parenchymal tissue into the MFP (Albino et al., 2015). Our findings confirm this observation. Moreover, this was also the explanation why it was not possible in most of wk 20 images laying Image J Fiji squares (ROI) onto the SURR area, since it was an increasing homogenous interweaving of PAR and SURR (USAtlasMG, supplementary Table 1). Considering, that in Categories 9 to 11 (USAtlasMG), the ImageJ Fiji squares were laid into the ultrasonic image area next to the visible ducti as described in USAtlasMG (supplemental

Table 1), a clear differentiation between PAR and SURR was not possible ad thus also not by pixel value measurements anymore.

Franz et al. (2009) described the healthy mammary parenchyma (PAR) structure of adult lactating cows as a uniform echogenic structure with a granular texture in which the connective tissue appears brighter than the PAR with less echoic density. The dark structures and formations visible in adult bovine PAR can either correspond to blood vessels or lactiferous ducts, and the cistern is anechoic as well (Franz et al., 2009). This observation corresponds to Category 11 (USAtlasMG, supplementary Table 1) and confirms the change in brightness that we also observed in the pixel measurements.

For the change in brightness in SURR pixel values, the MFP plays several important roles: Its structure supports and spaces the elongating ducts which brace eventually the proliferation of the mammary lobulo-alveolar tissue and the fat pad might be essential for the proliferation of the mammary epithelium and its enlargement (Lawrence and Fowler, 2002). In addition, studies in mice indicated that white adipocytes may transdifferentiate directly to milk-producing alveolar cells, so-called “pink adipocytes” (Cinti, 2018).

In our study, three squares in ImageJ were placed randomly into the visible hypoechogenic structure of the PAR based on the description in the USAtlasMG (supplemental Table 1). In 2015, Albino et al. only measured pixel values in the SURR area by using ImageJ. Two years later, (Albino et al., 2017) also used ImageJ and placed ROI squares into the PAR area, but in contrast to their previous study in 2015, the surrounding of PAR was not considered in ultrasonic images anymore. Thus a combined method for measuring both PAR and SURR area within the same image separately was still missing. In our current study both, the areas of PAR and SURR, were regarded and compared, i.e. the difference Δ pixel value (SURR – PAR) was calculated.

The novelty of our study is that the PAR and SURR pixel values were evaluated separately and simultaneously on the same ultrasonic image for the first time. There was no correlation between PAR and SURR pixel values (wk 8-16; $\rho = 0.141$; $P = 0.058$), possibly because the growing PAR was enlarging and spreading into SURR. The SURR tissue did not change its structure and pixel value until it was infiltrated by spreading PAR.

The Δ pixel value (SURR – PAR) was influenced by time, irrespective of the feeding group, and interaction thereof (Figure 1C). In wk 12 of life, RES calves showed a trend for higher Δ pixel values than HIGH calves ($P = 0.068$; Figure 1C). This might be explained by the less intensive spreading of PAR into SURR in the RES calves. The Δ pixel value (SURR – PAR)

showed the differences in brightness and pixel value between the two main structures of the growing MG and may indicate this dynamic. If PAR indeed spreads more and more into SURR, as described by Albino et al. (2015), then the MFP, corresponding to SURR, will change its structure, reduce its ultrasound echogenity (hypoechogenic) and the brightness of SURR, and the Δ pixel value would also decrease. In support of this notion, the Δ pixel value declined in both feeding groups from wk 10 onwards (Figure 1C). Albino et al. (2015) concluded that the reduction in pixels´ brightness in the subductal region (lower region of the ultrasonic image, "away to the buds growth") may indicate the growth of secretory tissue into the MFP. In our study, we observed the process of increasing pixel value and brightness of PAR from wk 10 onwards.

A previous study comparing PAR pixel value and composition showed that within the dissected PAR the percent of ether extract, i.e., lipid content, in PAR and the weight of MFP were positively correlated with PAR pixel values, whereas the percent of crude protein in PAR and the epithelial area in PAR were negatively correlated (Albino et al., 2017). The weight of dissected PAR did not correlate with measured pixel values within PAR (Albino et al., 2017). Nevertheless, Albino et al. (2017) concluded that ultrasonography of PAR can accurately measure and predict PARs composition in prepubertal heifers with different average daily body weight gains. In our trial, the PAR pixel value from wk 8 – 20 was positively correlated with BW ($\rho = 0.734$; $P < 0.001$).

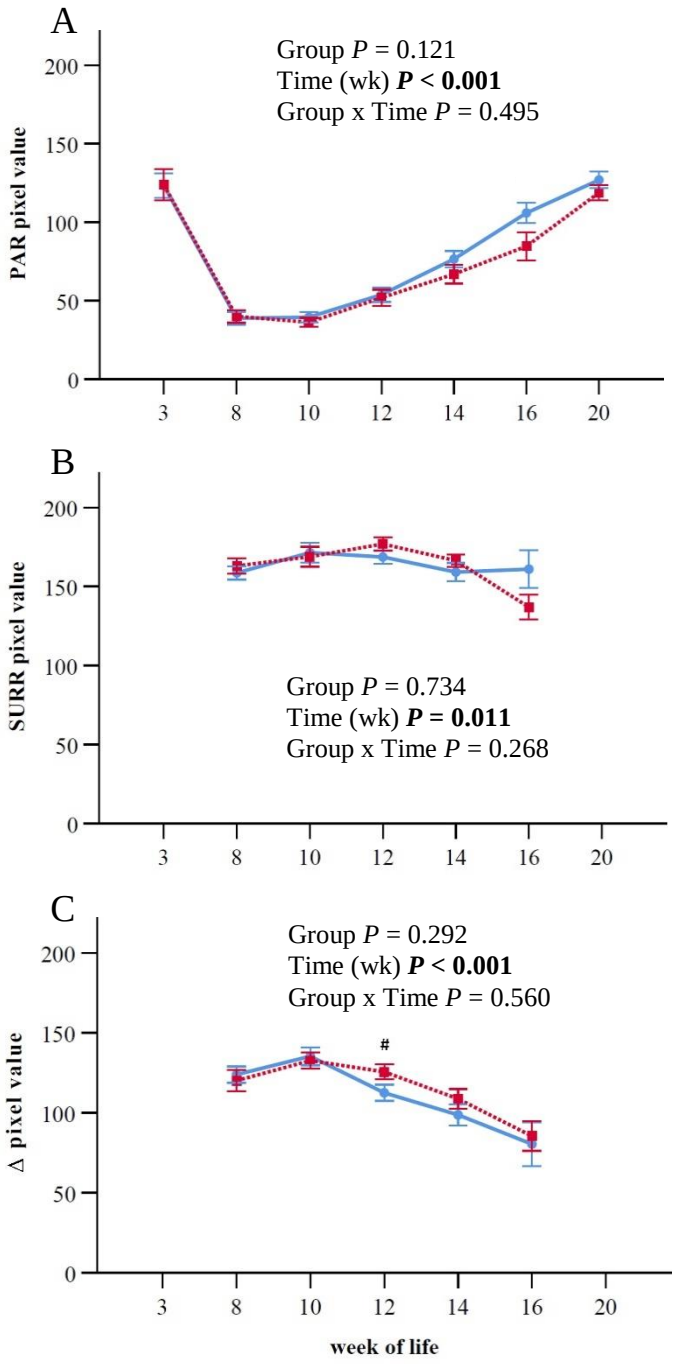


Figure 1. Preweaning and postweaning mean pixel values (means ± SEM) of four udder quarters in the developing mammary gland (A) parenchyma (PAR), (B) its surrounding (SURR), and (C) the difference between SURR – PAR pixel values (Δ pixel value) of calves with preweaning high allowance to milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) and restrictive allowance (RES; 7.5 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

BFT, RFT, and Body weight (BW)

In the previous study (Seibt et al., 2021) an overall higher BW and ADG in HIGH calves than in RES was detected ($P < 0.001$). The BW was also influenced by time showing a constant increase also around weaning ($P < 0.001$). The BFT (n = 35) was influenced by time, but not by feeding group or their interactions (Figure 2A), whereas the RFT (n = 30; calves from pluriparous cows only) was influenced by feeding group and time, without interaction (Figure 2B). The BFT increased from wk 3 to wk 14 of life, whereas after weaning (wk 14) until wk 20 the BFT remained around 11.4 – 11.8 ± 2 mm (mean ± SD) in both groups (Figure 2A). The RES group caught up with group HIGH in BFT growth in wk 10 (Figure 2A). In wk 3 of life, RFT was measurable only in 4 calves (HIGH n = 2; RES n = 2), because in most cases there were only skin layers visible in ultrasound images, and the contours were blurred. Therefore, the RFT data from wk 3 of life were excluded from the statistical analysis. The HIGH calves had greater RFT than the RES ones preweaning in wk 8 and wk 10 of life ($P = 0.023$; $P = 0.045$), and postweaning in wk 20 there was a trend for greater values in HIGH ($P = 0.076$; Figure 2B). In general, RFT increased from wk 8 to 14 of life ($P = 0.005$) in RES calves. In contrast, the HIGH group had no significant fluctuations in RFT until wk 20 (Figure 2B).

Overall, correlations ($P < 0.001$) were found between BW, BFT, and RFT respectively. BW and BFT were positively correlated ($\rho = 0.55$; $P < 0.001$), and also BFT and RFT ($\rho = 0.53$; $P < 0.001$). The correlation between BW and RFT was only weak ($\rho = 0.27$; $P < 0.001$). These results underline that the building of fat reserves begins preweaning and depends on the supply of energy. Besides having some buffering capacity for times of increased need for energy, e.g. in disease states, or reduced supply, adipose tissue through its endocrine functions, is also considered as important regulator for the immune system and the maturation of the reproductive functions (Nguyen et al., 2018). Thus, HIGH calves likely had an advantage as their RFT reserves were higher than in RES.

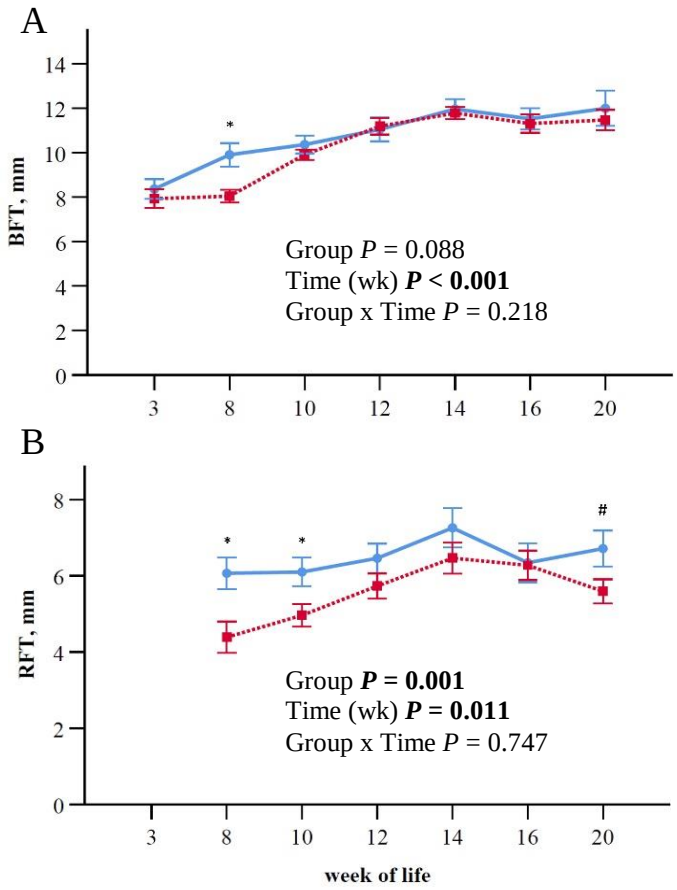


Figure 2. Pre- and postweaning changes (means ± SEM) of (A) back-fat thickness (BFT) and (B) rib-fat thickness (RFT) of calves fed at a high level of milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) or at a restricted level (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

Body dimensions

The development of all assessed variables of body dimensions of calves is shown in Figures 3 A-D and in Figure 4 A-F. In summary, all variables of body dimensions showed a linear increase with increasing age and body length (Figure 3 C, D), the thoracic growth except of the abdominal girth (Figure 4 A-C), and the hip growth (Figure 4 D-F) were affected by the MR feeding regimen, and without any interactions between feeding group and time respectively. Only the body height growth was not influenced by feeding regimen (Figure 3 A, B). Overall the HIGH-fed calves showed an growth advantage over the RES calves in all variables except for the body height.

These results of body height growth are in contrast to previous studies which observed higher withers and hip heights pre- and post-weaning in calves with elevated MR allowance in comparison to a restrictive MR allowance (Kiezebrink et al., 2015; Omidi-Mirzaei et al., 2015; Geiger et al., 2016). In previous studies, higher hip heights in cows have been associated with high milk yields in the first lactation (Shanks and Spahr, 1982; Sawa et al., 2013). In our study, higher values in back length, diagonal body length, and in the growth of thorax of HIGH calves in comparison to RES were observed and are consistent with results of previous studies (Omidi-Mirzaei et al., 2015; Geiger, 2019; Schwarzkopf et al., 2019).

If the growth advantage in chest girth in HIGH calves over RES from the milk feeding period would be carried over into adulthood, higher milk yields in the first lactation would be promoted (Sawa et al., 2013). This higher performance would be favored by the better expression of the shoulder joint bones distance, which indicates a large thorax providing sufficient space for powerful heart and lungs. A larger abdominal girth, which allows a large feed intake capacity, is advantageous for high-yielding cows and is also still included as a breeding goal of cows´ conformation traits for example in Germany (Deutscher Holstein Verband e.V., 2020).

In previous studies, individual variables of hip growth were not specified. In most cases, only the hip width, the distance between the outermost points of the hip perpendicular to the back, was measured (Shanks and Spahr, 1982; Omidi-Mirzaei et al., 2015; Hill et al., 2016). The result of wider hips in calves of a more intensive milk feeding regimen of our study is consistent with the study by Omidi-Mirzaei et al. (2015). In contrast to our results, Hill et al. (2016) measured a wider hip-width in restrictively fed calves than in highly fed calves. According to Shanks and Spahr (1982), hip-width indicates the cow's body frame, which is available to support the animal, udder and milk production.

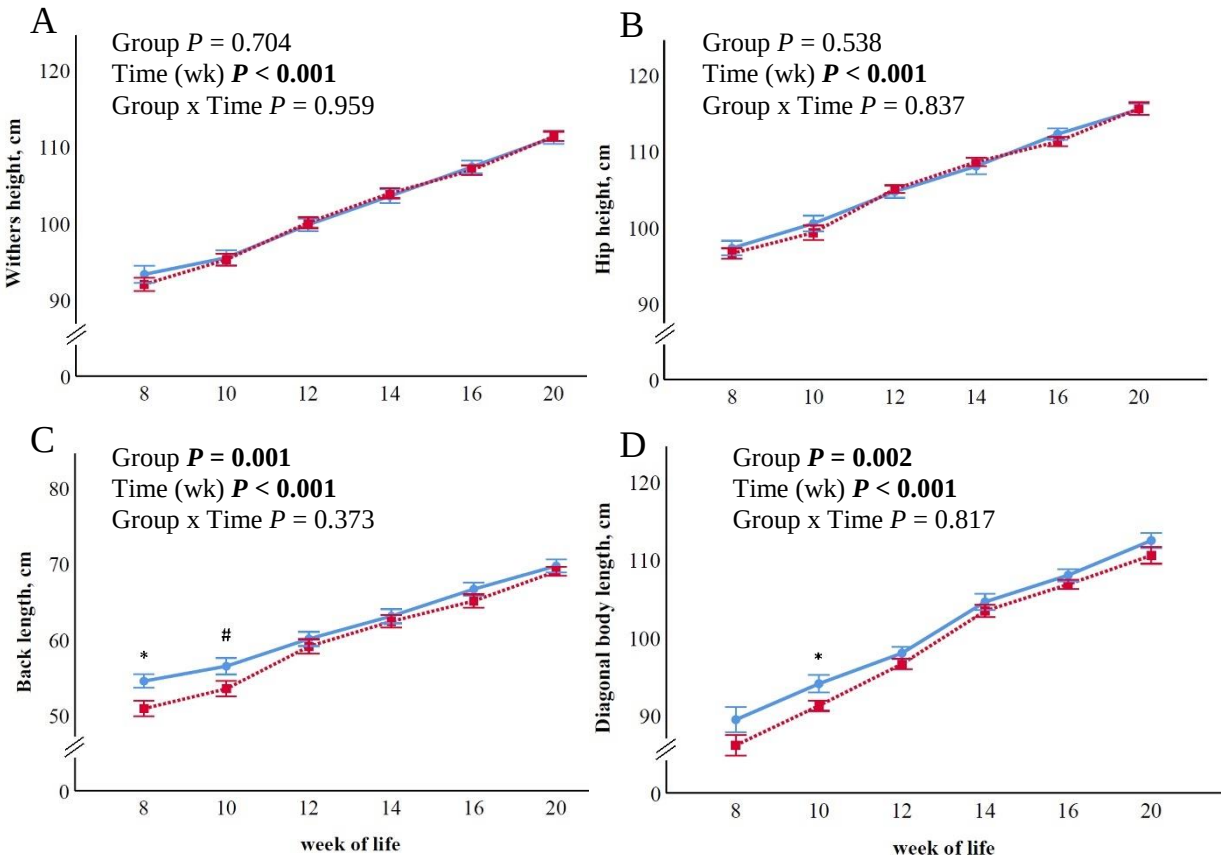


Figure 3. Pre- and postweaning changes (means ± SEM) of calves´ morphometry, including (A) withers height, (B) hip height, (C) back length, and (D) diagonal body length, of calves fed at a high level of milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) or at a restricted level (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

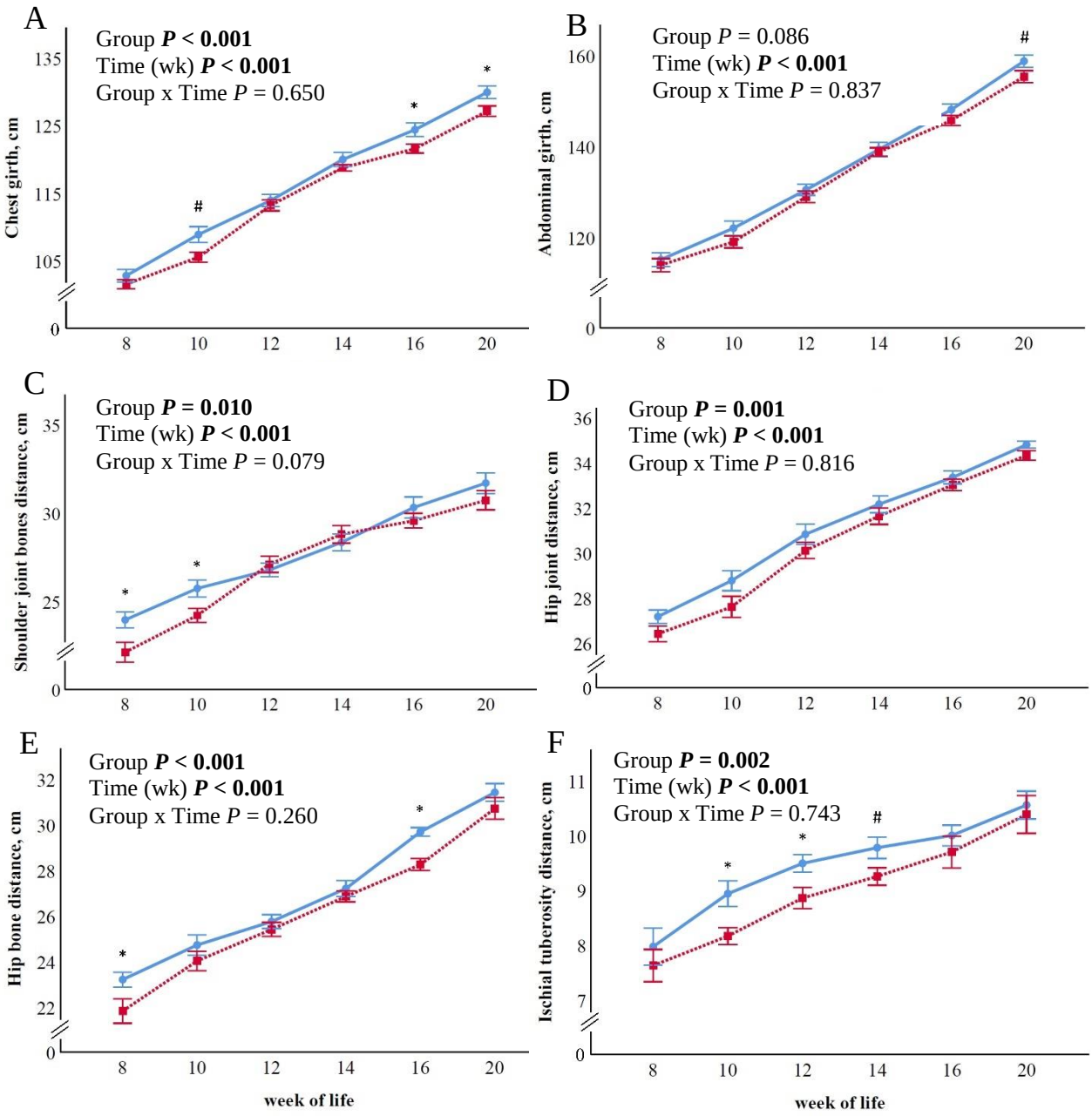


Figure 4. Pre- and postweaning changes (means ± SEM) of calves´ morphometry, including (A) chest girth, (B) abdominal girth at the last rib, (C) shoulder joint bones distance, (D) hip joint distance, (E) hip bone distance, and (F) ischial tuberosity distance of calves fed at a high level of milk replacer (MR; HIGH; 10 L/d; blue solid line; n = 18) or at a restricted level (RES; 5.7 L/d; red intermittent line; n = 19). The MR intake was gradually reduced in wk 13 and 14 to 2 L/d in both groups. Postweaning, calves were moved to a new group pen in another stable where they had free access to a total mixed ration (Seibt et al., 2021). Asterisks indicate differences ($P < 0.05$) and hashtags # a trend for differences ($0.05 \leq P < 0.1$) between groups within individual time points (wk of life).

Performance in the first lactation

From the 23 heifers that were kept on the farm (HIGH n = 11; RES n = 12) performance data from their first 7 wks in the first lactation could be recorded. These heifers did not differ in the age at first calving (HIGH: 765 d ± 50; RES: 745 d ± 37; means ± SD, $P = 0.284$). The average daily milk yield (means ± SD) during the first 7 wk in the first lactation was greater (32.2 kg/d ± 6.0) in the former HIGH group as compared to the RES group (27.6 kg/d ± 9.1; $P = 0.002$). The linear mixed model confirmed the feeding group ($P < 0.001$) and time (wk relative to calving, $P = 0.027$) as significant fixed effects, without the respective interaction ($P = 0.849$). However, 2 heifers in the RES group were detected as outliers (Z-standardization, SD > 2), showing lesser milk yields (12.4 kg/d +- 6.1) than the average in HIGH and RES group. These two outlier cows (RES) had also high somatic cell count numbers > 200,000 cells/mL up to 1,090,000 cells/mL in weekly milk samples in wk 2 – 4 in lactation. When excluding those 2 animals from the data set (n = 21 cows), the daily milk yields increased with time ($P < 0.001$), but were not different between the groups ($P = 0.489$) without interactions during the first 7 wk in the first lactation. In total, within the first 7 wks in lactation, 5.6% of all weekly milk somatic cell measurements, i.e. of all 23 lactating cows including also the low performers in the RES group, indicated 200,000 – 400,000 cells/mL (n = 9 cases) and 3.7% more than 400,000 cells/mL (n = 6 cases): 3 cases occurred in HIGH and 12 cases in RES heifers. Additionally, the average ECM (kg/d; means ± SD) during the first 7 wk in first lactation by excluding the 2 outlier cows and outliers (SD > 2; n = 2) was 33.9 kg/d ± 6.2 in HIGH and 32.2 kg/d ± 5.1 in RES ($P = 0.029$), but the linear mixed model analysis yielded neither an influence of the feeding group ($P = 0.204$) nor of time (wk relative to calving, $P = 0.627$) or interactions thereof ($P = 0.955$). Previous studies showed that an accelerated preweaning BW gain can be positively associated with the milk yield in the first lactation (Soberon and van Amburgh, 2013; Korst et al., 2017; Chester-Jones et al., 2017). Preweaning nutrient intake, from milk or MR, can have profound effects on the development of the calf that enhance first lactation and lifetime productivity (Soberon and van Amburgh, 2013). In contrast, the study of Raeth-Knight et al. (2009) and Kiezebrink et al. (2015) showed no effect of an preweaning intensive milk or MR feeding regimen on the performance in first lactation. Additionally, the preweaning amount of MR intake might have influenced the SCC in heifers in the first lactation, since Svensson et al. (2006) showed that the growth rate from birth to weaning, and feeding e.g., the amount of concentrates intake were associated with clinical mastitis and elevated SCC (≥200,000 cells/mL) at −7 to 30 d after calving in the first lactation. In addition to metabolic clues as possible explanation for the impaired udder health, the calves in the RES group may have had

a higher risk for udder health disturbances triggered by mutual suckling and being suckled by their fellows; however, the calves of both groups were not in separated pens, and we also have no records of behavior other than feeding behavior for substantiating this. Nevertheless, the greater incidence of elevated SCC in heifers originating from the RES group also implies the need for an antibiotic therapy (or culling) for such animals. However, the number of animals in our study that could be pursued into their first lactation is a limitation in our study. In summary, HIGH feeding regimen in early life was advantageous for body growth and potentially also for udder health in later life.

CONCLUSION

Ultrasonic measurements of PAR and SURR structures provide a noninvasive way to monitor MG growth and the development and dynamic of its structures. Even there were no differences in pixel values between feeding groups, the pixel value measurement according to the Ultrasonographic Atlas of the developing bovine mammary gland (USAtlasMG) is a suitable tool to assess this MG development. Concerning the body growth of heifers in the first 5 months of life and their performance in the first lactation, the preweaning feeding regimen increasing MR or milk quantities for 14 wk of life in comparison to the restrictive regimen should be preferred with regard to milk yield and udder health.

ACKNOWLEDGMENTS

The authors declare no conflicts of interest. K. D. S. is a recipient of a scholarship from the H. Wilhelm Schaumann Foundation (Hamburg, Germany). The authors thank the staff of the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle in Münchweiler a. d. Alsenz in Rhineland-Palatinate (Germany), especially A. Brubacher, E. Therre, A. Schoenborn, S. Jobelius, K. Korst, D. Karch, E. Kröger, and the veterinary D. Lang for their support and effort. This project would not have been possible without their daily commitment, workforce and help. The authors thank also C. Sommer and C. Bold of the Association Bezirksverband Pfalz for their support in programming Fiji Image J. For her workforce in digitalization of body dimensions the authors also thank F.-J. Kersting. The data that support the findings and which are presented in this study are available on request from the corresponding author, H. Sauerwein, sauerwein@unibonn.de.

REFERENCES

Akers, R. M., and D. M. Denbow. 2013. Anatomy and physiology of domestic animals. 2. ed. John Wiley & Sons Inc, Ames, Iowa.

Albino, R. L., S. E. F. Guimarães, K. M. Daniels, M. M. S. Fontes, A. F. Machado, G. B. Dos Santos, and M. I. Marcondes. 2017. Technical note: Mammary gland ultrasonography to evaluate mammary parenchymal composition in prepubertal heifers. J. Dairy Sci. 100(2):1588–1591. https://doi.org/10.3168/jds.2016-11668.

Albino, R. L., M. I. Marcondes, R. M. Akers, E. Detmann, B. C. Carvalho, and T. E. Silva. 2015. Mammary gland development of dairy heifers fed diets containing increasing levels of metabolisable protein: Metabolisable energy. J. Dairy Res. 82(1):113–120. https://doi.org/10.1017/S0022029914000697.

Brown, E. G., M. J. VandeHaar, K. M. Daniels, J. S. Liesman, L. T. Chapin, J. W. Forrest, R. M. Akers, R. E. Pearson, and M. W. Nielsen. 2005. Effect of Increasing Energy and Protein Intake on Mammary Development in Heifer Calves. J. Dairy Sci. 88(2):595–603. https://doi.org/10.3168/jds.S0022-0302(05)72723-5.

Chester-Jones, H., B. J. Heins, D. Ziegler, D. Schimek, S. Schuling, B. Ziegler, M. B. de Ondarza, C. J. Sniffen, and N. Broadwater. 2017. Relationships between early-life growth, intake, and birth season with first-lactation performance of Holstein dairy cows. J. Dairy Sci. 100(5):3697–3704. https://doi.org/10.3168/jds.2016-12229.

Cinti, S. 2018. Pink Adipocytes. Trends Endocrinol. Metab. 29(9):651–666. https://doi.org/10.1016/j.tem.2018.05.007.

Delorme, S., and J. Debus. 2005. Sonographie. 2., vollst. überarb. und erw. Aufl. Duale Reihe. Thieme, Stuttgart, Germany.

Deutsche Landwirtschaftsgesellschaft (DLG). 2000. Empfehlungen zum Einsatz von Mischrationen bei Milchkühen. DLG-Information 1/2000. DLG-Verlag, Frankfurt am Main, Germany.

Deutscher Holstein Verband e.V. (DHV e.V.). 2020. Exterieurbeurteilung: Einzelne Merkmale der linearen Beschreibung. Accessed Sep 20, 2020. https://www.holstein-dhv.de/seiteninhalte/exterieur.html.

Esselburn, K. M., T. M. Hill, H. G. Bateman, F. L. Fluharty, S. J. Moeller, K. M. O'Diam, and K. M. Daniels. 2015. Examination of weekly mammary parenchymal area by ultrasound, mammary mass, and composition in Holstein heifers reared on 1 of 3 diets from birth to 2 months of age. J. Dairy Sci. 98(8):5280–5293. https://doi.org/10.3168/jds.2014-9061.

Ferreira, T., and W. Rasband. 2012. ImageJ User Guide: ImageJ/Fiji 1.46. Accessed May 07, 2021. https://imagej.nih.gov/ij/docs/guide.

Franz, S., M. Floek, and M. Hofmann-Parisot. 2009. Ultrasonography of the bovine udder and teat. Vet. Clin. North Am. Food Anim. Pract. 25(3):669-85, Table of Contents. https://doi.org/10.1016/j.cvfa.2009.07.007.

Furini, P. M., R. A. Azevedo, S. R. A. Rufino, F. S. Machado, M. M. Campos, L. G. R. Pereira, T. R. Tomich, B. C. Carvalho, G. B. Santos, and S. G. Coelho. 2018. The effects of increasing amounts of milk replacer powder added to whole milk on mammary gland measurements using ultrasound in dairy heifers. J. Dairy Sci. 101(1):767–773. https://doi.org/10.3168/jds.2017-12798.

Geiger, A. J. 2019. Review: The pre-pubertal bovine mammary gland: unlocking the potential of the future herd. Animal 13(S1):s4-s10. https://doi.org/10.1017/S1751731119001204.

Geiger, A. J., C. L. M. Parsons, R. E. James, and R. M. Akers. 2016. Growth, intake, and health of Holstein heifer calves fed an enhanced preweaning diet with or without postweaning exogenous estrogen. J. Dairy Sci. 99(5):3995–4004. https://doi.org/10.3168/jds.2015-10405.

Hill, T. M., J. D. Quigley, H. G. Bateman, F. X. Suarez-Mena, T. S. Dennis, and R. L. Schlotterbeck. 2016. Effect of milk replacer program on calf performance and digestion of nutrients in dairy calves to 4 months of age. J. Dairy Sci. 99(10):8103–8110. https://doi.org/10.3168/jds.2016-11239.

Hovey, R. C., T. B. McFadden, and R. M. Akers. 1999. Regulation of Mammary Gland Growth and Morphogenesis by the Mammary Fat Pad: A Species Comparison. J. Mammary Gland Biol. Neoplasia, Vol. 4, No. 1, 1999. https://doi.org/10.1023/A:1018704603426.

Kiezebrink, D. J., A. M. Edwards, T. C. Wright, J. P. Cant, and V. R. Osborne. 2015. Effect of enhanced whole-milk feeding in calves on subsequent first-lactation performance. J. Dairy Sci. 98(1):349–356. https://doi.org/10.3168/jds.2014-7959.

Korst, M., C. Koch, J. Kesser, U. Müller, F.-J. Romberg, J. Rehage, K. Eder, and H. Sauerwein. 2017. Different milk feeding intensities during the first 4 weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation. J. Dairy Sci. 100(4):3096–3108. https://doi.org/10.3168/jds.2016-11594.

Lawrence, T. L. J., and V. R. Fowler. 2002. growth of farm animals: 5 Mammary Gland Growth and Product Yield. 2nd ed. CABI Publishing, London, UK.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006a. Developmental and Nutritional Regulation of the Prepubertal Bovine Mammary Gland: II. Epithelial Cell Proliferation, Parenchymal Accretion Rate, and Allometric Growth. J. Dairy Sci. 89(11):4298–4304. https://doi.org/10.3168/jds.S0022-0302(06)72476-6.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006b. Developmental and Nutritional Regulation of the Prepubertal Heifer Mammary Gland: I. Parenchyma and Fat Pad Mass and Composition. J. Dairy Sci. 89(11):4289–4297. https://doi.org/10.3168/jds.S0022-0302(06)72475-4.

Nguyen, L. T., L. F. Zacchi, B. L. Schulz, S. S. Moore, and M. R. S. Fortes. 2018. Adipose tissue proteomic analyses to study puberty in Brahman heifers. J. Anim. Sci. 96(6):2392–2398. https://doi.org/10.1093/jas/sky128.

Nishimura, M., T. Yoshida, S. El-Khodery, M. Miyoshi, H. Furuoka, J. Yasuda, and K. Miyahara. 2011. Ultrasound imaging of mammary glands in dairy heifers at different stages of growth. J. Vet. Med. Sci. 73(1):19–24. https://doi.org/10.1292/jvms.09-0503.

Omidi-Mirzaei, H., M. Khorvash, G. R. Ghorbani, B. Moshiri, M. Mirzaei, A. Pezeshki, and M. H. Ghaffari. 2015. Effects of the step-up/step-down and step-down milk feeding procedures on the performance, structural growth, and blood metabolites of Holstein dairy calves. J. Dairy Sci. 98(11):7975–7981. https://doi.org/10.3168/jds.2014-9260.

Penninck, D., and M.-A. d'Anjou. 2015. Atlas of Small Animal Ultrasonography. 2nd ed. Wiley, Somerset.

Raeth-Knight, M., H. Chester-Jones, S. Hayes, J. Linn, R. Larson, D. Ziegler, B. Ziegler, and N. Broadwater. 2009. Impact of conventional or intensive milk replacer programs on Holstein heifer performance through six months of age and during first lactation. J. Dairy Sci. 92(2):799–809. https://doi.org/10.3168/jds.2008-1470.

Rowson, A. R., K. M. Daniels, S. E. Ellis, and R. C. Hovey. 2012. Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. Sem. Cell Dev. Biol. 23(5):557–566. https://doi.org/10.1016/j.semcdb.2012.03.018.

Salomon, F.-V., H. Geyer, and U. Gille. 2005. Anatomie für die Tiermedizin. Enke, Stuttgart.

Sawa, A., M. Bogucki, S. Krężel-Czopek, and W. Neja. 2013. Relationship between Conformation Traits and Lifetime Production Efficiency of Cows. ISRN Vet. Sci. 2013:124690. https://doi.org/10.1155/2013/124690.

Schwarzkopf, S., A. Kinoshita, J. Kluess, S. Kersten, U. Meyer, K. Huber, S. Dänicke, and J. Frahm. 2019. Weaning Holstein Calves at 17 Weeks of Age Enables Smooth Transition from Liquid to Solid Feed. Animals 9(12). https://doi.org/10.3390/ani9121132.

Seibt, K. D., M. H. Ghaffari, T. Scheu, C. Koch, and H. Sauerwein. 2021. Characteristics of the Oxidative Status in Dairy Calves Fed at Different Milk Replacer Levels and Weaned at 14 Weeks of Age. Antioxidants 10(2):260. https://doi.org/10.3390/antiox10020260.

Shanks, R. D., and S. L. Spahr. 1982. Relationships Among Udder Depth, Hip Height, Hip Width, and Daily Milk Production in Holstein Cows. J. Dairy Sci.(65 (9)):1771–1775. https://doi.org/10.3168/jds.S0022-0302(82)82415-6.

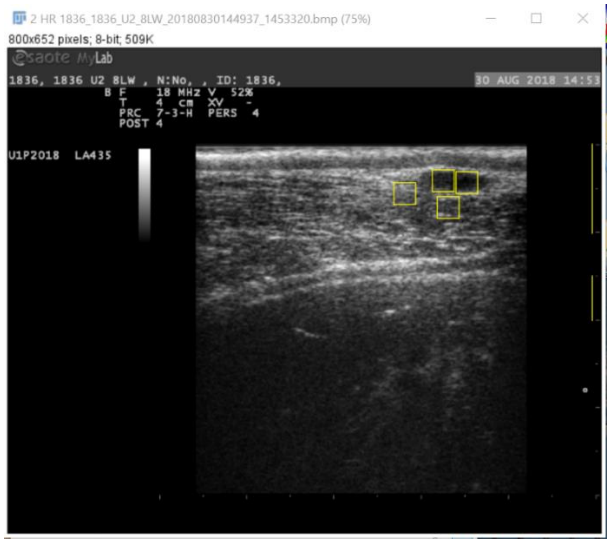
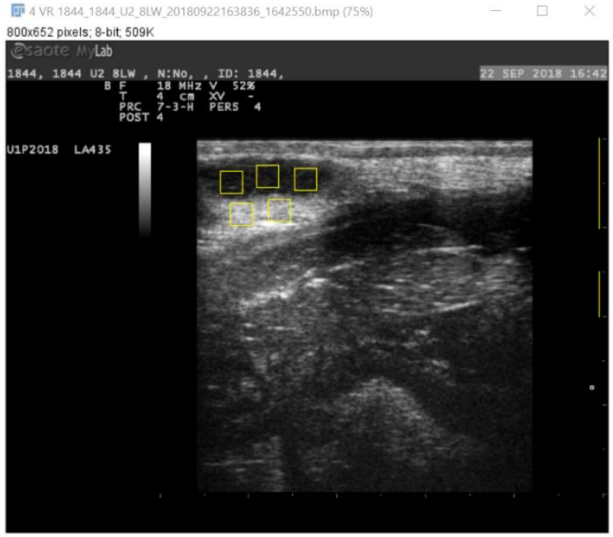
Silva, A. L., E. Detmann, J. Dijkstra, A. M. Pedroso, L. H. P. Silva, A. F. Machado, F. C. Sousa, G. B. Dos Santos, and M. I. Marcondes. 2018. Effects of rumen-undegradable protein on intake, performance, and mammary gland development in prepubertal and pubertal dairy heifers. J. Dairy Sci.. https://doi.org/10.3168/jds.2017-13230.

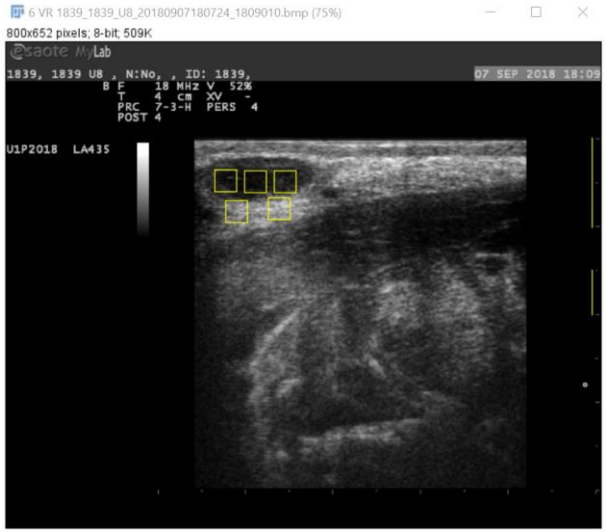
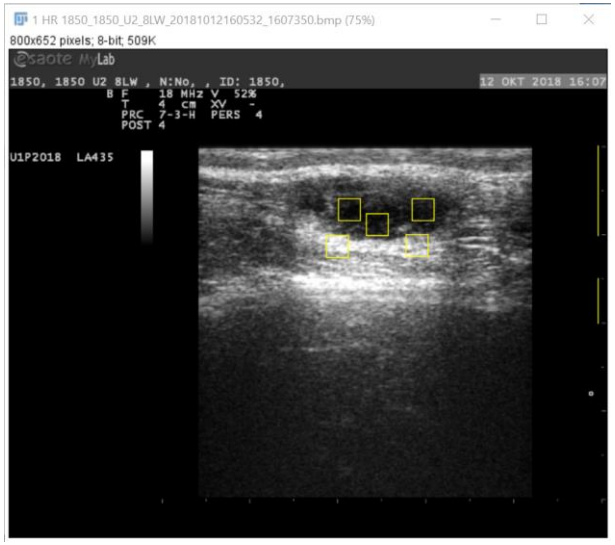
Soberon, F., and M. E. van Amburgh. 2013. Lactation Biology Symposium: The effect of nutrient intake from milk or milk replacer of preweaned dairy calves on lactation milk yield as adults: a meta-analysis of current data. J. Anim. Sci. 91(2):706–712. https://doi.org/10.2527/jas.2012-5834.

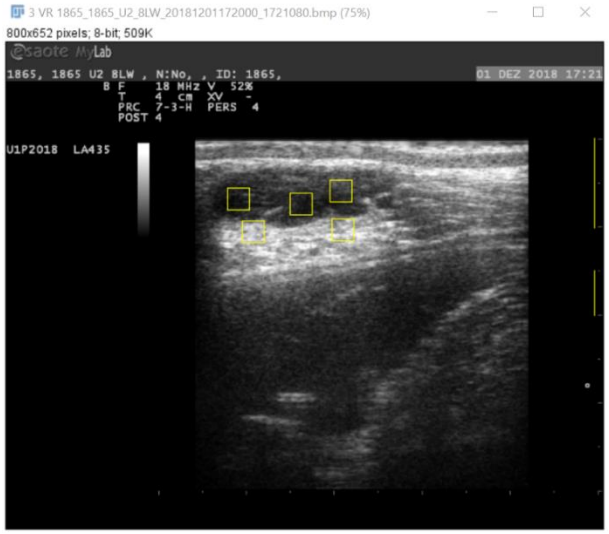
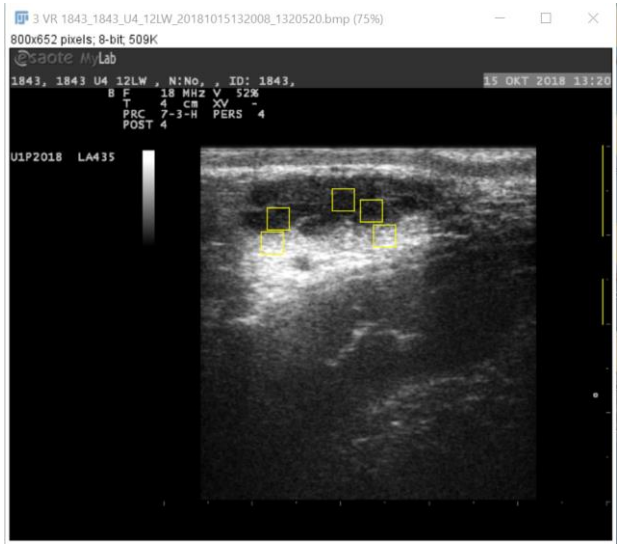
Svensson, C., A.-K. Nyman, K. P. Waller, and U. Emanuelson. 2006. Effects of Housing, Management, and Health of Dairy Heifers on First-Lactation Udder Health in Southwest Sweden. J. Dairy Sci. 89(6):1990–1999. https://doi.org/10.3168/jds.S0022-0302(06)72266-4.

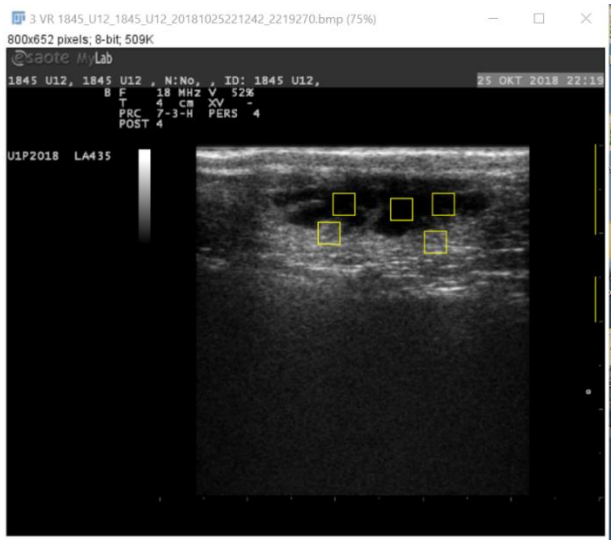
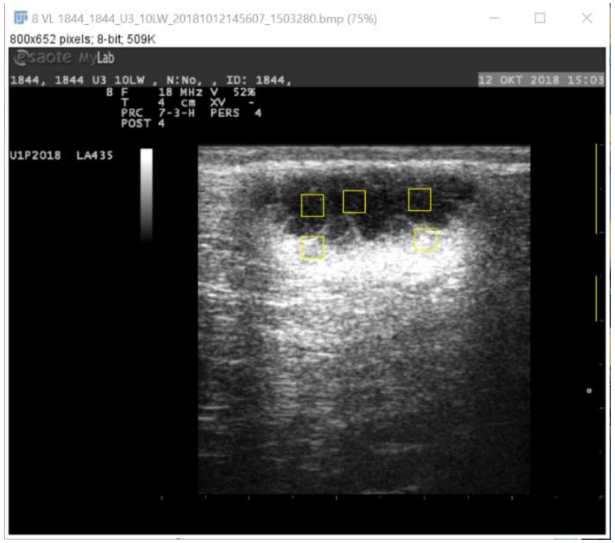
Supplementary Table 1. Ultrasound image evaluation and categories of the bovine mammary gland development (USAtlasMG) by *K. D. Seibt et al.*

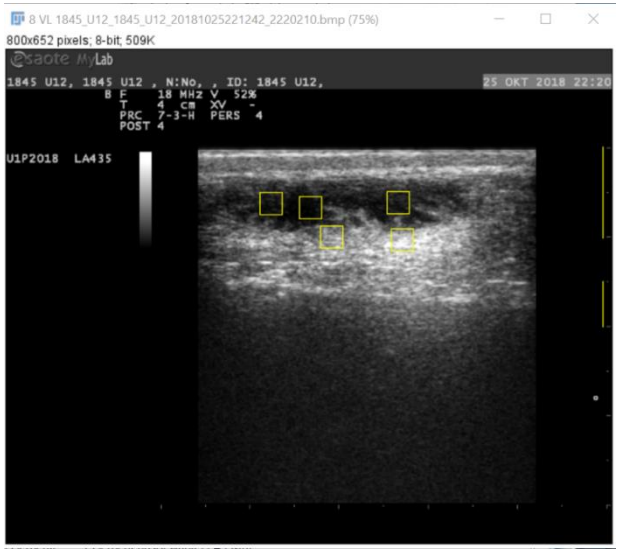
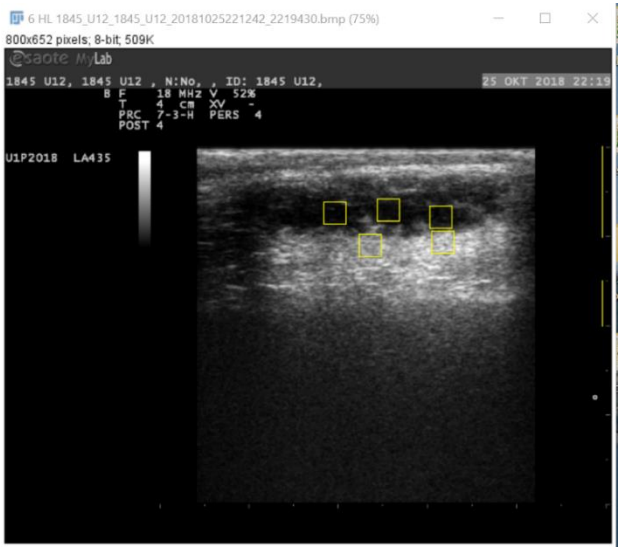
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
1		Visible teat base between the skin layers Just teat base visible, but no round formation of a liquid filled structure (hypoechoic = black; parenchyma (PAR)) Just white grey flecked tissue between skin layers and abdominal layers Tissue layers as borderlines to abdomen visible Example image = 3rd week of life	2 squares, position of the squares close by the visible teat base, under the skin layer, directly at borderline to skin layer if possible => 2 squares / image
1		Visible teat base between the skin layers but also maybe a first very small formation of parenchyma (PAR) or liquid filled structure (hypoechoic = black) close to teat base under the skin layers within white / grey flecked tissue structure Small rice in right square visible under the teat base embedded in grey flecked tissue between skin layers and layers of the abdomen Example image = 3rd week of life	2 squares, position of the squares close by the visible teat base, under the skin layer, directly at borderline to skin layer If a small dark liquid filled formation is visible, then should at least lay one square over this region => 2 squares / image

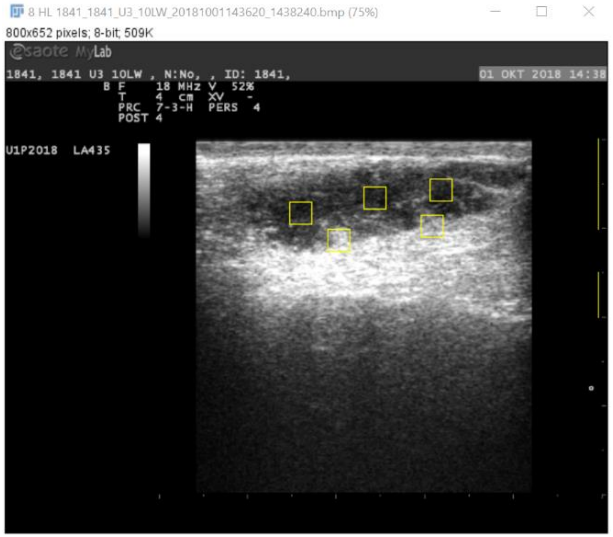
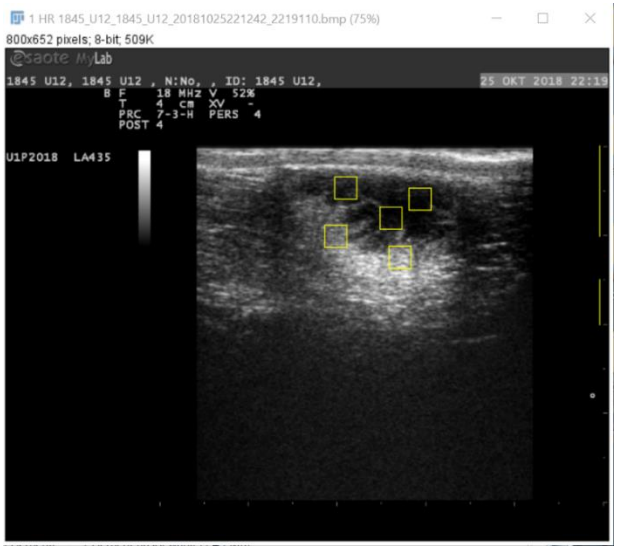
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
2		Teat base is not clearly detectable but the skin layers are clearly separating skin from tissue underneath Small round /oval /circular dark structure – hypoechoic = black – liquid filled – clear borderlines to surrounding - hypoechogenic parenchyma (PAR) according to descriptions of Esselburn et al. (2015) Clearly delimited from grey flecked and white tissue – hyperechoic also layers (white) as borderlines between tissue and skin and abdomen clearly visible Example image = 8th week of life	3 squares, positioned inside of dark round structure (size of round PAR individually different, sometimes it is just possible to lay in just 2 squares) & 2 squares, next to dark round structures borderlines => 4 – 5 squares / image
2		Teat base is not clearly detectable but the skin layers are clearly separating skin from tissue Round /oval /circular dark structure – hypoechoic = black – liquid filled – clear borderlines to surrounding - “hypoechogenic PAR” Esselburn et al. (2015) Clearly delimited from grey flecked and white tissue – hyperechoic – white structure underneath adjoining it - “mammary fat pad hyperechogenic” Esselburn et al. (2015) Also layers (white) as borderlines between tissue and skin and abdomen clearly visible Example image = 8th week of life	3 squares, positioned inside of dark round structure (size of round PAR individually different, sometimes it is just possible to lay in just 2 squares) & 2 squares, next to dark round structures borderlines => 5 squares / image

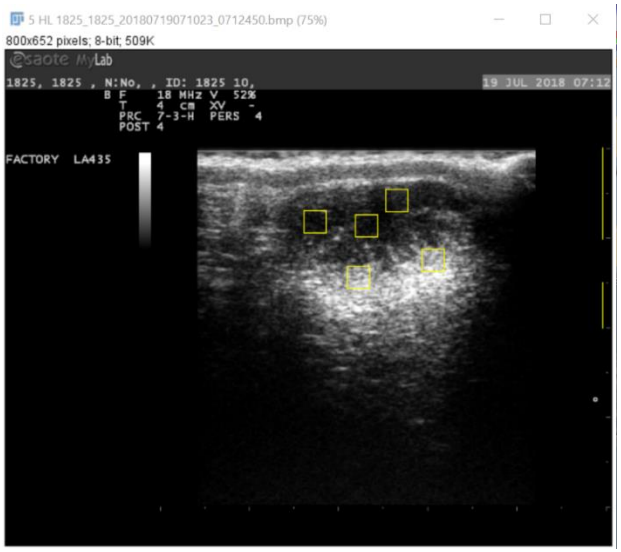
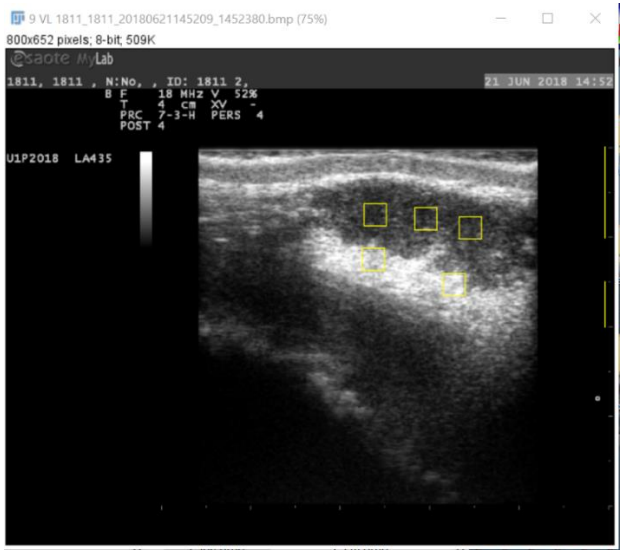
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
2		Teat base is not clearly detectable but the skin layers are clearly separating skin from tissue Round /oval /circular dark structure – hypoechoic = black – liquid filled – clear borderlines to surrounding - "hypoechogenic PAR" Esselburn et al. (2015) Clearly delimited from grey flecked and white tissue – hyperechoic – white structure underneath adjoining it - "mammary fat pad hyperechogenic" Esselburn et al. (2015) Also layers (white) as borderlines between tissue and skin and abdomen slightly visible Example image = 10th week of life	3 squares, positioned inside of dark round structure (size of round PAR individually different, sometimes it is just possible to lay in just 2 squares) & 2 squares, next to dark round structures borderlines => 5 squares / image
3		Round dark structure – hypoechoic = black – liquid filled – but dorsal first formation of cauliflower structures – Dissolving circularity Clear borderlines, and no exact round form of liquid filled structure (hypoechoic) anymore Delimited from grey flecked and white – hyperechoic – tissue White structure underneath adjoining maybe fat pad bordered to abdomen - "mammary fat pad hyperechogenic" Esselburn et al. (2015) Example image = 8th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image

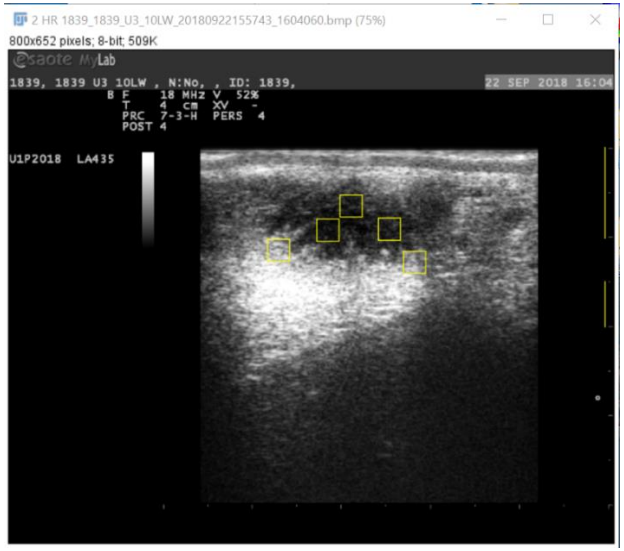
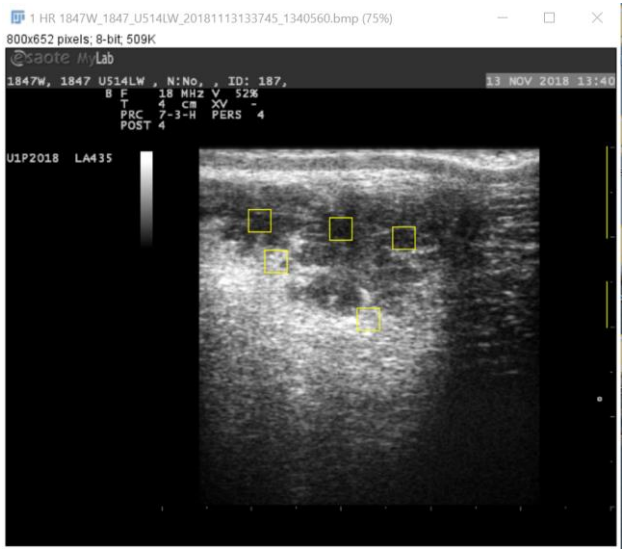
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
3		Round dark structure – hypoechoic = black – liquid filled – but dorsal first formation of cauliflower structures – Dissolving circularity Clear borderlines, and no exact round form of liquid filled structure (hypoechoic) anymore Delimited from grey flecked and white – hyperechoic – tissue White structure underneath adjoining, maybe fat pad bordered to abdomen - “mammary fat pad hyperechogenic” Esselburn et al. (2015) Example image = 8th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image
3		Round dark structure – hypoechoic = black – liquid filled – but dorsal first formation of cauliflower structures – Dissolving circularity Clear borderlines, and no exact round form of liquid filled structure (hypoechoic) anymore Delimited from grey flecked and white – hyperechoic – tissue White structure underneath adjoining, maybe fat pad bordered to abdomen - “mammary fat pad hyperechogenic” Esselburn et al. (2015) Example image = 12th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image

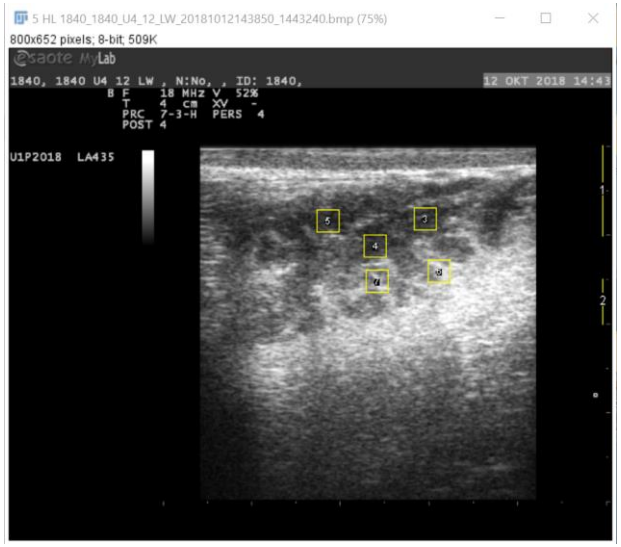
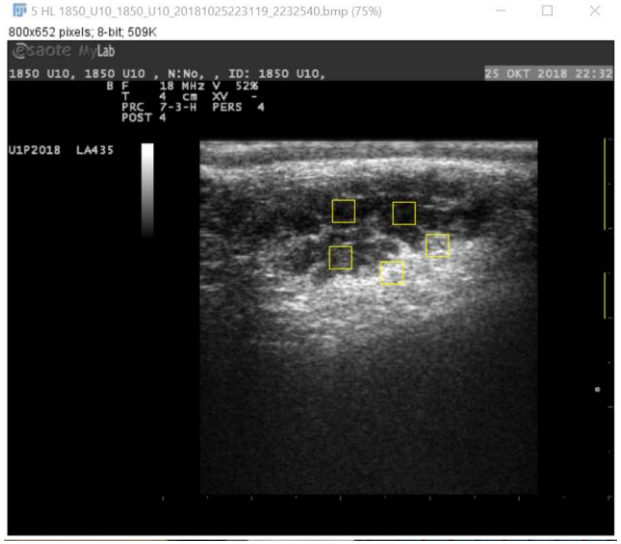
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
3		Round dark structure – hypoechoic = black – liquid filled – but dorsal first formation of cauliflower structures – Dissolving circularity Clear borderlines, and no exact round / oval form of liquid filled structure (hypoechoic) anymore Delimited from grey flecked and white – hyperechoic – tissue White structure underneath adjoining, maybe fat pad bordered to abdomen - "mammary fat pad hyperechogenic" Esselburn et al. (2015) Example image = 12th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image
3		Teat base is not clearly detectable but the skin layers are clearly separating skin from tissue Round dark structure – hypoechoic = black– liquid filled – but dorsal first formation of cauliflower shape/ structures – dissolving circularity Clear borderlines to surrounded tissue clearly delimited from grey flecked and white – hyperechoic – tissue White structure underneath adjoining, maybe "mammary fat pad hyperechogenic" Esselburn et al. (2015) Example image = 10th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image

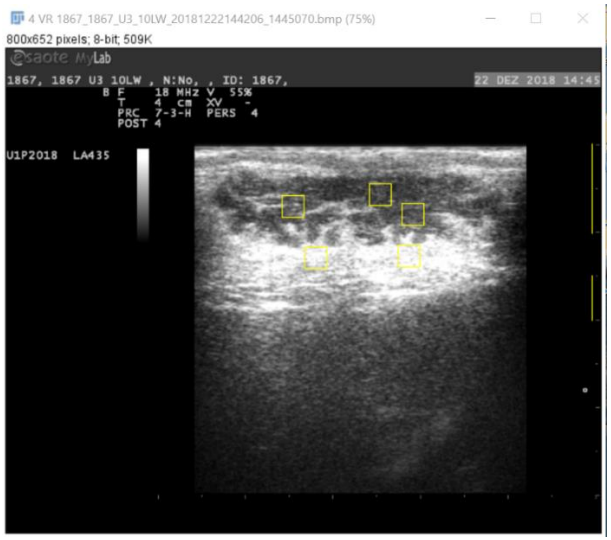
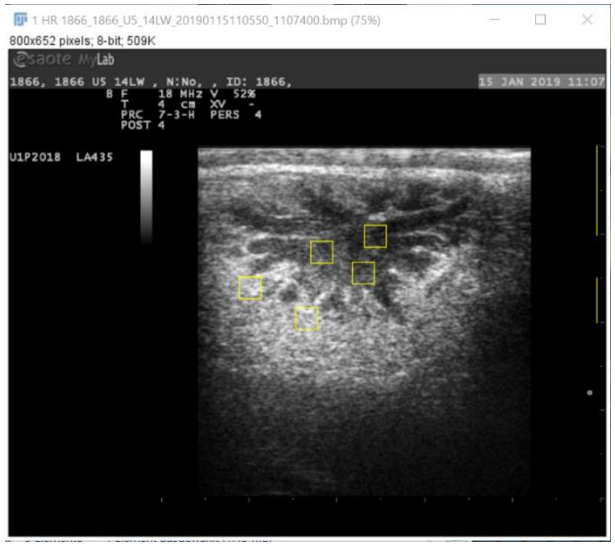
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
4		Flat oval structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) –begins to disappear / dissolve, still clearly identifiable hypoechoic = black – liquid filled area But still no clear cauliflower structures Border lines / contours of oval shape / structure dissolving Example image = 12th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image
4		Flat oval structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) –begins to disappear / dissolve, still clearly identifiable hypoechoic = black – liquid filled area But still no clear cauliflower structures Border lines / contours of oval shape / structure dissolving Example image = 12th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines (of maybe early cauliflower) => 5 squares / image

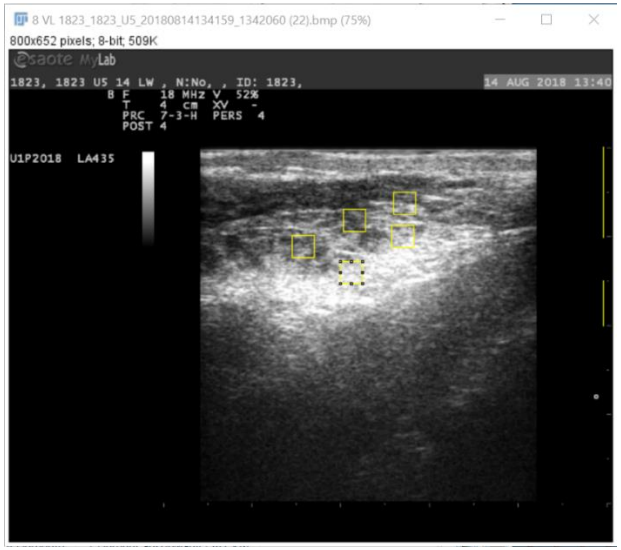
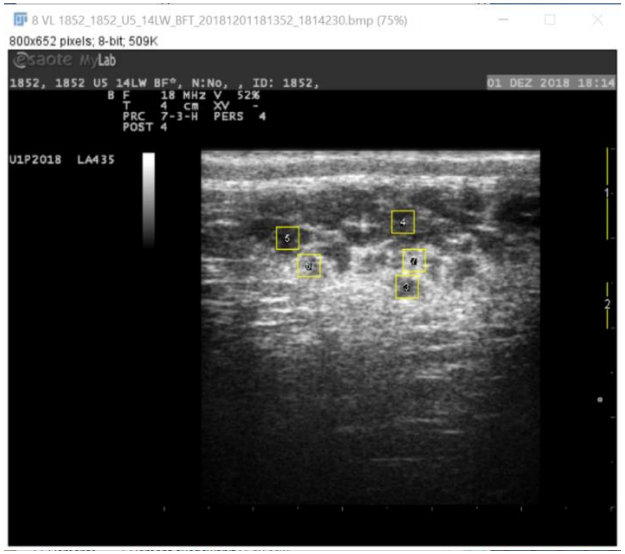
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
4		Flat oval structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) – begins to disappear / dissolve, still clearly identifiable hypoechoic = black – liquid filled area But still no clear cauliflower structures Border lines / contours of oval shape / structure dissolving Example image = 10th week of life	3 squares positioned inside of dark structure & 2 squares next to dark structures borderlines (of maybe early cauliflower) => 5 squares / image
5		Transition to cauliflower structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) – begins to disappear / dissolve Comparable with level 4 There are cauliflower structures already visible in most parts But still no clearly visible cauliflower Example image = 12th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines => 5 squares / image

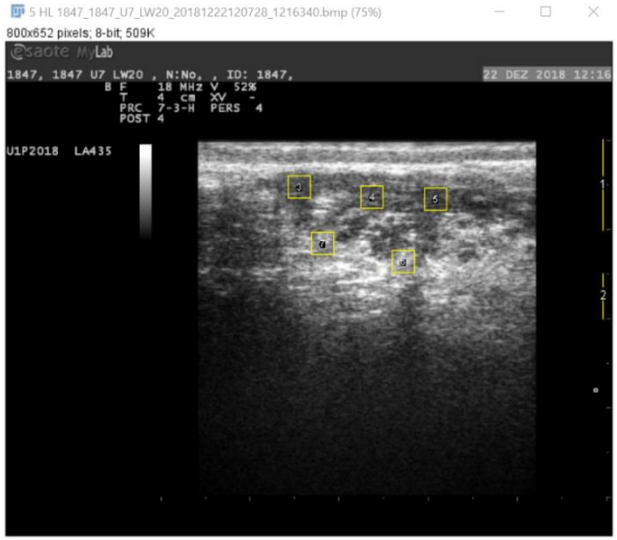
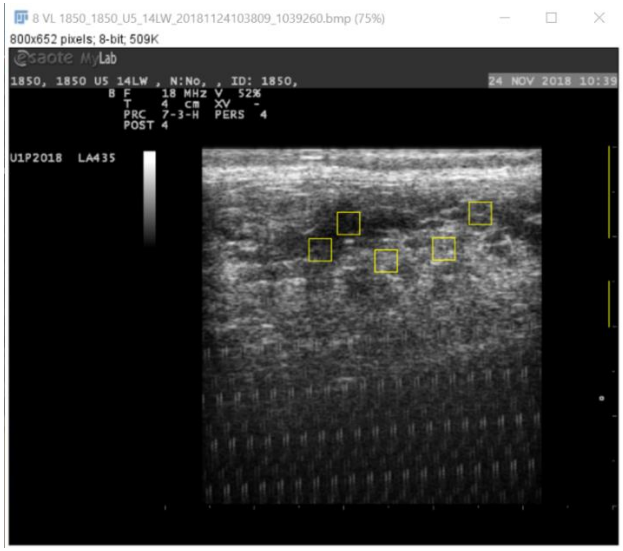
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
5		Transition to cauliflower structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) – begins to disappear / dissolve Still comparable with category 4 There are cauliflower structures already visible in most parts But still no clearly visible cauliflower Example image = 10th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines => 5 squares / image
5		Transition to cauliflower structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) – begins to disappear / dissolve Still comparable with category 4 There are cauliflower structures already visible in most parts But still no clearly visible cauliflower Example image = 16th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines => 5 squares / image

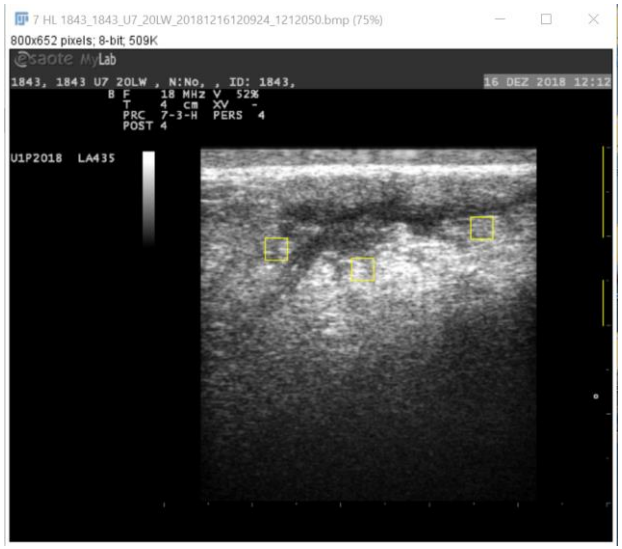
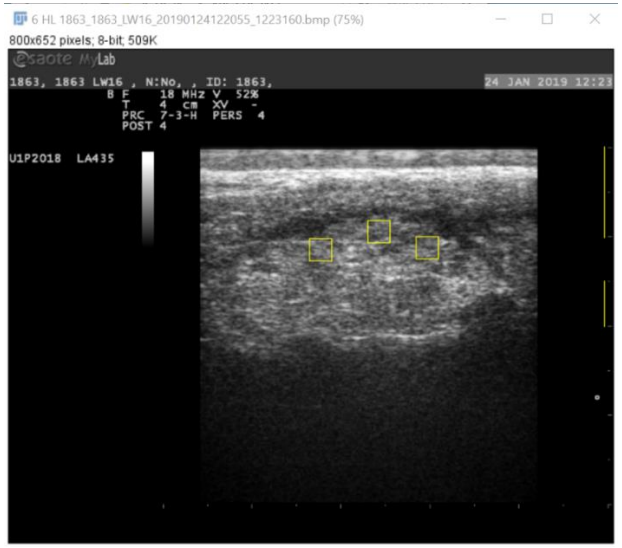
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
5		Transition to cauliflower structure No circular borderline anymore Borderlines and circular shape of dark structure (hypoechoic PAR) – begins to disappear / dissolve Still comparable with category 4 There are cauliflower structures already visible in most parts But still no clearly visible cauliflower Example image = 10th week of life	3 squares, positioned inside of dark round structure & 2 squares, next to dark round structures borderlines => 5 squares / image
6		Large cauliflower, obviously visible cauliflower shape There is no round formation, no circular bordered structure anymore round spongy structures – hypoechoic – liquid filled without grand ducti formation Example image = 14th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches of cauliflower => 5 squares / image

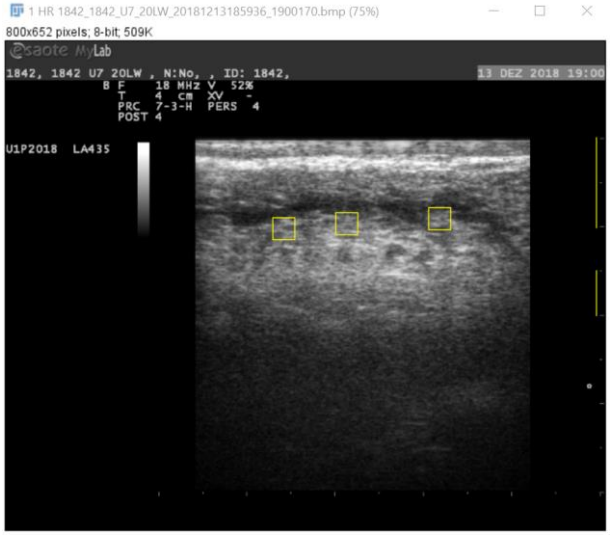
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
6		Large cauliflower, obviously visible cauliflower shape There is no round formation, no circular bordered structure anymore Round spongy structures – hypoechoic – liquid filled Without grand ducti formation Example image = 12th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches of cauliflower => 5 squares / image
6		Large cauliflower, obviously visible cauliflower shape There is no round formation, no circular bordered structure anymore Round spongy structures – hypoechoic – liquid filled Without grand ducti formation Example image = 10th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches of cauliflower => 5 squares / image

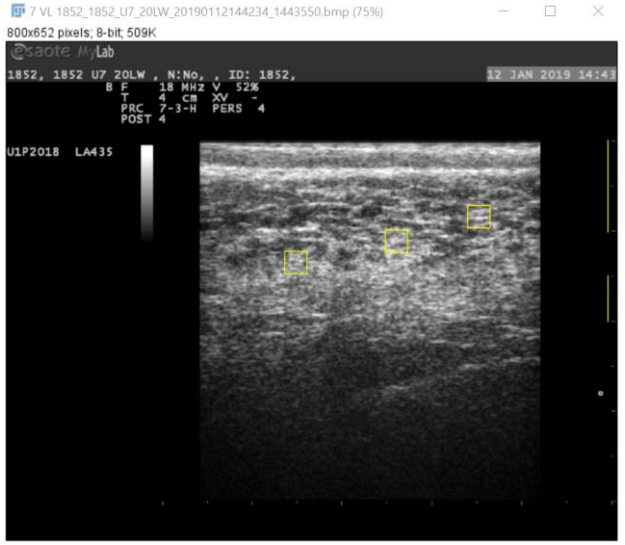
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
6		Special flat cauliflower, obviously visible cauliflower shape There is no round formation, no circular bordered structure anymore Round spongy structures – hypoechoic – liquid filled Without grand ducti formation Example image = 10th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches of cauliflower => 5 squares / image
7		Coral structure, The typical cauliflower structure is dissolving, Formation of first slender branches But there is still clearly identifiable hypoechoic = black – liquid filled area Example image = 14th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches => 5 squares / image

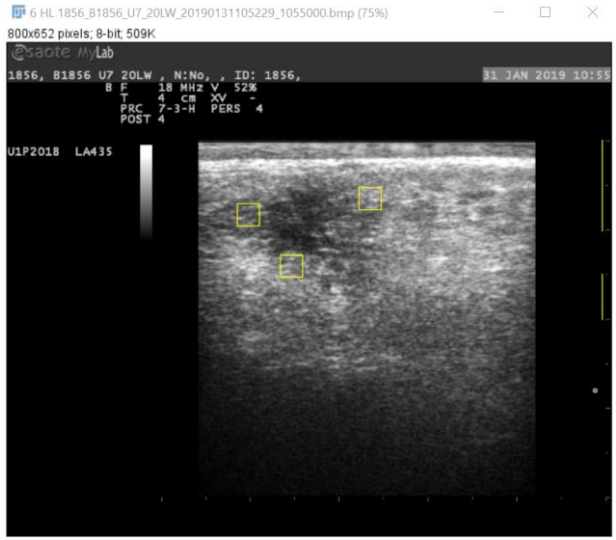
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
7		Coral structure, The typical cauliflower structure is dissolving, Formation of first slender branches But there is still clearly identifiable hypoechoic = black – liquid filled area Example image = 14th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark branches => 5 squares / image
8		No cauliflower structure anymore Increasing of disintegration of cauliflower structure into more separate pieces Spongy flecked grey tissue in surrounding Beginning of branching / formation of main ductus but still no formation of a clear main ductus Example image = 14th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark pieces => 5 squares / image

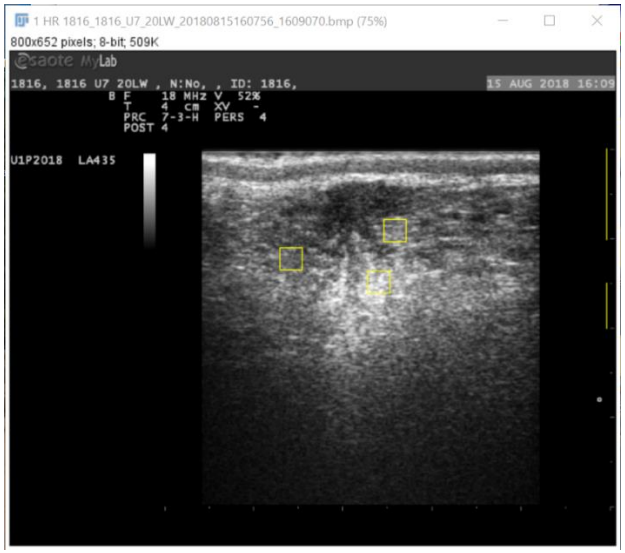
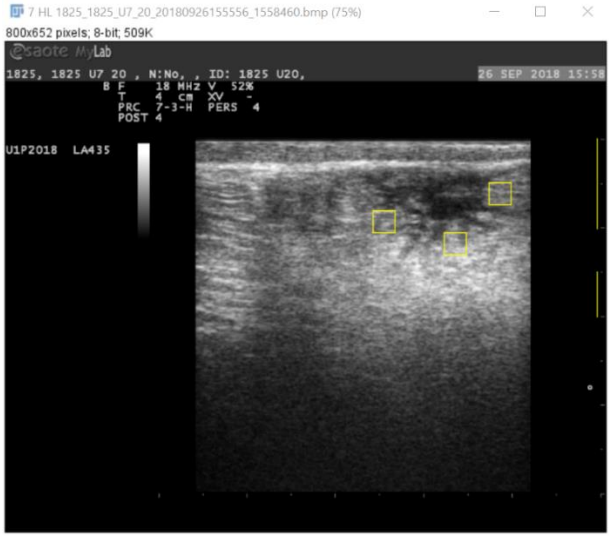
Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
8		No cauliflower structure anymore Increasing of disintegration of cauliflower structure into more separate pieces Spongy flecked grey tissue in surrounding Beginning of branching / formation of main ductus but still no formation of a clear main ductus Example image = 20th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark pieces => 5 squares / image
8		No cauliflower structure anymore Increasing of disintegration of cauliflower structure into more separate pieces Spongy flecked grey tissue in surrounding Beginning of branching / formation of main ductus but still no formation of a clear main ductus Example image = 14th week of life	3 squares, positioned inside of dark structure & 2 squares, adjacent to dark structures, dark pieces => 5 squares / image

Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
9		One main ductus is clearly visible, Clearly identifiable hypoechoic = black – liquid filled but also some smaller ducti Flecked grey, spongy tissue in surrounding Example image = 20th week of life	3 squares, positioned directly next to main ductus within flecked grey surrounding => 3 squares / image
9		One main ductus is clearly visible, Clearly identifiable hypoechoic = black – liquid filled Flecked grey, spongy tissue in surrounding Example image = 16th week of life	3 squares, positioned directly next to main ductus within flecked grey surrounding => 3 squares / image

Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
9		One main ductus is clearly visible, Clearly identifiable hypoechoic = black – liquid filled Flecked grey, spongy tissue in surrounding Example image = 20th week of life	3 squares, positioned directly next to main ductus within flecked grey surrounding => 3 squares / image
10		Totally dissolved structures, No clear visible main ductus anymore Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable A clear demarcation between ducti and surroundings is not possible anymore Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, => 3 squares / image

Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
10		Totally dissolved structures, no clear visible main ductus anymore Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable A clear demarcation between ducti and surroundings is not possible anymore Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, => 3 squares / image
10		Totally dissolved structures, no clear visible main ductus anymore Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable A clear demarcation between ducti and surroundings is not possible anymore Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, => 3 squares / image

Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
11		Cistern Black, hypoechoic – liquid filled area Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, close to area of cistern => 3 squares / image
11		Cistern Black, hypoechoic – liquid filled area Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, close to area of cistern => 3 squares / image

Category	Ultrasound image	Description of the category of mammary gland development	Position of normed Fiji Image J squares (0.25 cm^2)
11		Cistern Black, hypoechoic – liquid filled area Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, close to area of cistern => 3 squares / image
11		Cistern Black, hypoechoic – liquid filled area Just a grey flecked / black and white spotted structure / tissue Some larger black hypoechoic – liquid filled spots identifiable Example image = 20th week of life	3 squares, randomly positioned inside of flecked grey tissue, close to area of cistern => 3 squares / image

6 General Discussion

In the present thesis, the development of German Holstein heifer calves during the postnatal, preweaning and postweaning phases was investigated until wk 20 of life focusing on oxidative status and mammary gland development.

The overall aim of the present project and thesis was to investigate to what extent the amount of milk replacer (MR) per day can influence the development of the mammary gland and the systemic oxidative status in heifer calves, and also how this might influence performance in later life as dairy cows. In addition, the preweaning phase was prolonged to 14 weeks of calves' life in comparison to common preweaning periods of 6 - 8 weeks of life in conventional farming systems (Lohakare et al., 2012).

In this study, it was hypothesized that a high feeding level of 10 L MR/d in comparison to a restrictive and low feeding level of 5.7 L/d to heifer calves would enhance growth and development, especially of the mammary gland, would improve calves' welfare, and may imprint performance in the first lactation. Moreover, within this study, it was possible to follow the early postnatal and prepubertal mammary gland development in dairy heifer calves using ultrasonographic scans. This approach was pursued previously only in a few studies and not in detail.

6.1 Starter intake influenced by milk replacer (MR) intake

The high MR feeding level of 10 L/d did not compromise solid starter feed intake in calves in our study. Hence, the starter intake did not differ between the two feeding groups. Therefore, the higher intake of metabolizable energy (ME) in HIGH fed calves was maintained until weaning by MR intake. The HIGH fed calves could thus gain more energy than RES calves, resulting in higher body weights in HIGH than RES over the 20 weeks of the trial.

For decades, the natural process of calf rearing based on *ad libitum* milk intake has been counteracted in conventional farming systems by restrictive feeding regime and early weaning age. On the one hand, the rearing of calves was considered economically unproductive, on the other hand, it was assumed that restrictive MR feeding stimulates the solid feed intake in calves which should advance the rumen development, growth, and weight gain from starter feed which in turn reduce rearing costs (Davis Rincker et al., 2011; Lohakare et al., 2012; Hulbert and Moisá, 2016; Kertz et al., 2017).

Previous studies showed that rumen development was stimulated by an early and high intake of solid feed, i.e., pelleted, protein-rich calf starter feed and hay with a high fiber content (Kertz et al., 2017; Diao et al., 2019). Furthermore, the results of the study of Heinrichs and Heinrichs (2011) implied that the dry matter intake at weaning is associated with milk production in adult life. It was often observed that the milk or MR intake of calves is negatively correlated with the intake of solid or starter feed (Passillé et al., 2011; Miller-Cushon et al., 2013), while in our study in both feeding groups the starter intake was similar and independent of the MR intake. Also in the study of Silper et al. (2014) comparing two MR feeding levels (4 L/d vs. 6 L/d for 60 days) no difference in starter intake was found, while the higher feeding level resulted in greater weight gains, confirming our findings. Furthermore, the different MR feeding strategies did not affect ruminal development, i.e., papillae length or ruminal epithelium thickness (Silper et al., 2014). In the study of Jasper and Weary (2002) comparing *ad libitum* milk feeding with restrictive feeding (5 L/d, 10% of body weight), the starter intake between feeding groups differed only during the preweaning phase until d 37 of life, but was equal during weaning and postweaning. Also the study of Korst et al. (2017) confirmed no difference in starter intake between *ad libitum* and restrictively fed calves until d 69 of life.

Nevertheless, the forestomach and rumen need time, until around wk 12 – 16 of life, to functionally mature (Huber, 1969; Huber, 2018), while the intake of fiber-rich hay, silage or straw, and starter feed increase (Khan et al., 2016). The developmental stage of the rumen can directly affect the intake of different types of feed, as well as the nutrient digestibility, influencing the amount of energy intake and thus growth (Diao et al., 2019). In our study, the starter intake increased mainly from wk 8 of age onwards, which is the time when calves are normally weaned in conventional farming systems. Therefore, in our trial, calves had twice as much time until weaning in wk 14 than conventionally weaned calves. This timeframe might have covered the phase to establish ruminant digestion fully, but in our study, conventional weaning ages have not been considered and were therefore not compared. Neave et al. (2019) observed a large range in ages between wk 3 to latest wk 11 of age when calves began to consume calf starter. The age at which weaning was completed at 1,300 g/d of solid feed intake also varied over a wide range of 6 and 12 weeks (Neave et al., 2019). They concluded that weaning age is variable when calves are weaned in an automated weaning program based on solid feed intake, but not only due to their developmental stage, but also due to their individual characteristics in the early life, such as fearful and slow learners or exploratory active strong-drinkers (Neave et al., 2019). Hence, finding the right timing for weaning with regard to the

transition from functional monogastric to rumination is still a difficult task in calves‘ rearing management.

In conventional dairy farming, the focus is still on milk production and this excludes the opportunity of natural rearing of a calf with its dam. It is already possible to feed calves individually and on defined levels or *ad libitum* by using automated feeders (Korst et al., 2017). Additionally, there are already organic farm systems that keep calves with their dams and allow for the natural nursing of calves (Johnsen et al., 2016). Individual weaning, by reducing milk or MR allowance according to individual starter intake is also possible when using automated feeders (Roth et al., 2009; Passillé and Rushen, 2012; Neave et al., 2019).

Feeding calves with automated feeders for milk, MR, and starter feed not only saves working time, but also allows a comprehensive collection of data that can be associated with the individual calf and its feed intake behaviour (Paula Vieira et al., 2008; Rosenberger et al., 2017; Korst et al., 2017). In our trial, we used this possibility of automatic data collection by automated feeders. The collected raw data were either sent from the feeders to a PC or to a cloud where a specially adapted software processed the data. This allowed for a good overview of the current and daily MR and starter intake by the individual calves and also showed which calf had no intake recordings yet, i.e., not yet drunk, and therefore needed to be checked for technical or health problems. Power failures during the feeding phase in our trial meant that the feeders could no longer transmit the raw data to the PC or the cloud. Hence, data could not be processed further by the software and data gaps occurred in data sets for the daily recording of MR and starter intake as well as in data concerning the feeding behaviour. Fortunately, the automated feeder ran on an emergency power supply and stored the raw data in the form of a logbook on an inserted SD card. However, the raw data in logbook format were recorded precisely to the minute for each event when the feeder acted and not as daily summed and processed data by the software. Therefore, with a lot of effort, these logbook data from SD card could be converted to Excel tables, summed up and data gaps within software tables could be filled. Hence, in this trial, we gained the experience that automatic monitoring of the calves is possible, but it does not yet replace the daily personal check of the calves in the barn.

6.2 Calves’ feeding behaviour and welfare

Several studies focused on the effect of different milk allowance and rearing calf management on welfare according to the calves’ behaviour (Paula Vieira et al., 2008; Costa et al., 2016; Rosenberger et al., 2017). Especially the feeding behaviour of calves recorded by automated

feeders can provide information about signs of hunger, competitive behaviour, or concurrence in calves (Paula Vieira et al., 2008; Passillé et al., 2011; Rosenberger et al., 2017; Korst et al., 2017). The weaning process itself is one of the points which are important for behavior and welfare. Calves are expected to show restlessness if the weaning process did not meet all aspects of the natural behaviour (Bracke and Hopster, 2006). Restrictively fed calves, fed on low levels (6 L/d), which are more busy checking the automatic feeding station for new admission to milk, showed this restlessness in more visits as compared to calves fed on a high level (12 L/d; Rushen et al., 2012).

In the study described in the current thesis, the restrictively fed calves visited the automated feeder more often and had more unrewarded visits than HIGH fed calves throughout the MR feeding period, indicating more signs of hunger in RES calves than in HIGH. This confirms the findings and observations from several studies indicating a higher frequency of total and unrewarded visits to the automated feeder in restrictively fed calves (4 – 6 L/d) in comparison to *ad libitum* or on a high level (10 – 12 L/d) fed calves (Paula Vieira et al., 2008; Passillé et al., 2011; Korst et al., 2017). On average 11.1 ± 0.73 unrewarded visits/d in calves receiving 6 L/d and 0.4 ± 0.78 unrewarded visits/d by receiving 12 L/d were shown in the study of Rosenberger et al. (2017). In our study, we observed that restrictively fed calves had in average 15.7 ± 10.1 SD unrewarded visits/d during the whole MR feeding period of 14 weeks, which was 3-fold higher than in calves fed with 10 L MR/d (5.28 ± 0.58 SD) and comparable to the results of Rosenberger et al. (2017).

Furthermore, the results of our study showed that even when the liquid feeding period is prolonged to 14 weeks as in our study which is double as long as the conventional periods, restrictively fed calves showed this typical feeding behaviour of more visits during the whole MR feeding period. Passillé et al. (2011) observed that during early weaning in wk 7 of life calves fed on high levels of 12 L milk/d reached a peak and showed the same numbers of total visits/d as the restrictively fed calves (6 L/d) at the same weaning age, whereas late weaned calves (12 weeks of life) were staying constantly on low numbers on visits. This indicates that also the weaning age contributes to the calves' ability to replace milk with starter feed and thus satisfied their hunger.

However, the high numbers of unrewarded visits to the milk feeder by calves indicate persistent hunger (Paula Vieira et al., 2008; Rosenberger et al., 2017; Korst et al., 2017). Hunger influences also calves' social behaviour so that restricted fed calves are spending more time per day standing in front of the suckling teat of the automated feeder, they were more competitive

in behaviour and are more likely to displace other calves from the feeder (Paula Vieira et al., 2008). In our study, HIGH and RES calves were kept in the same group pen and had access to the same automated feeders. Competitive behaviour was observed by daily routine controls but was not documented. For documentation of behaviour, video analyses with exact behaviour protocols have to be carried out, which were not foreseen in our project. In our project behavioural data were derived only from the automated feeder, i.e., total visits, unrewarded and rewarded visits, and drinking speed.

Restricted fed calves were also observed to ingest their available milk allotment more rapidly during a rewarded visit than high fed calves which can be assessed as the so-called drinking or sucking speed (Paula Vieira et al., 2008). With our study, we cannot confirm this observation, since there were no differences in drinking speed, except for wk 13, the beginning of weaning.

Therefore, several studies focusing on feeding behaviour including our study could show that the conventional restricted feeding of 6 L/d or less violates one of the principal features of animal welfare, i.e., the freedom from thirst, hunger, and malnutrition as one of the five freedoms formulated by Webster (1997). This fact alone should be a decisive point to reconsider the outdated views on calf feeding and to change the feeding regime to a higher milk allowance to avoid abnormal behaviour and hunger in calves. Feeding behaviour data derived from automated milk feeders not only provide information about the appearance of hunger but can also be used to identify diseased calves (Svensson and Jensen, 2007; Borderas et al., 2009; Sutherland et al., 2018).

6.3 Health status of dairy heifer calves

Maintaining calves in a good health status is an additional point concerning other principal features of animal welfare, i.e., the freedom from pain, injury, and disease as one of the five freedoms (Webster, 1997), and with regard to the future performance of the animals and thus for better economic efficiency by saving costs for medical treatments or losses in future milk yield.

Dairy calves can suffer from a variety of illnesses and diseases caused by several pathogens (Svensson and Jensen, 2007; Hulbert and Moisá, 2016). After birth, the passive immunity of calves is built up by maternal antibodies provided by colostrum. The calves' active immunity, based on antibodies produced by the calf itself, must first develop after birth. When passive immunity decreases and the calf´s active immunity slowly increases, the so-called immunity

gap occurs around 2 - 3 weeks of life, making calves more susceptible to various diseases and health problems (Hulbert and Moisá, 2016).

In our trial, we observed no differences in the number of diseased calves, in the incidence of health disturbances, or in the concentrations of the acute-phase protein haptoglobin (Hp) between the feeding groups during the first 20 weeks of life. The incidences of health disturbances and diseases were relatively low during these 20 weeks: 62.2% of all calves were healthy and 37.8% were diseased once or more often (7 calves of each feeding group). Hence, with this study, we could neither see an effect of the MR feeding level nor an effect of weaning at 14 weeks of age on the health status of the calves. On the other hand, in our study, we did not take blood samples for Hp analysis during the period of the immunity gap, i.e., between birth and wk 8 of life. However, weekly health data did not show any abnormalities or diseases during this time. Only in wk 6 of life, after dehorning in wk 5, the incidence of cases of fever or elevated body temperature (>39.5 °C) peaked in calves indicating inflammatory processes after dehorning in calves.

Nevertheless, conducting weekly health checks, which included 15 health parameters in our trial, was time-consuming. Health checks were performed only once per wk and therefore the health status was only assessed on one day out of 7 days. Hence, health disturbances outside the day of health check were potentially not seen or not recorded, although daily routine controls of calves were performed, but not in detail as in the weekly health checks. For a complete record of the health status, each calf has to be checked daily. This could be achieved by recording only a few health parameters out of 15, including body temperature. However, this would be far more time-consuming and labour-intensive than weekly health checks. Catching and holding the calf can take up to 5 min and the health check with e.g. 5 parameters takes approximately again 5 min: calculating with 10 min per calf and 37 calves per day, the working time for health checks alone would be 6.17 h/d. Farmers cannot manage this daily workload on their own, which is why several ideas for techniques and the so-called Wisconsin calf health scorer as an App (Food Animal Production Medicine, University of Wisconsin-Madison, Madison, USA) have been developed in recent years to monitor health status with a minimal amount of time as possible: For example, “smart thermometers” with integrated digital recording of the health parameters (Urban Vital Control, Urban, Wüsting, Germany) or connected to smartphone apps recording the health status (CalfApp Vital, Förster-Technik GmbH, Engen, Germany).

However, the immune response in case of inflammation or infection is an energy-consuming process and can impair growth and development. In the study of Ballou (2012), the higher plane

of MR nutrition (11.8 kg MR/d vs. restrictive 4.54 kg MR/d) may improve postweaning resistance to disease or enhance immune responses. Furthermore, calves fed on higher levels of MR had greater concentrations of haptoglobin (Hp) in plasma 24 h after an LPS challenge than restrictively fed calves (Ballou, 2012), which can indicate both a higher and faster immune reaction in case of an infection of caves fed on high levels.

If it is assumed that feeding does not have a direct effect on the health status, but programs the metabolism and thus shows a disposition for increased health problems in the future, then these should be detected during first lactation at the latest (Svensson et al., 2006; Kesser et al., 2017; Korst et al., 2017).

6.4 Development and body growth of dairy heifer calves

Overall, the HIGH feeding resulted in greater preweaning and postweaning body weights in calves in group HIGH than in calves in group RES. The ADG declined in both groups within the first 3 weeks of life, whereas RES calves showed a stronger decline in ADG than HIGH calves, which is in accordance with most of the studies concerning different preweaning feeding levels (Hulbert et al., 2011; Obeidat et al., 2013; Kesser et al., 2017; Korst et al., 2017).

Due to the immunity gap which can occur around 2 - 3 weeks of life, calves are not only more susceptible to diseases, but also show a decrease in ADG (Hulbert and Moisá, 2016), which could explain the decline in ADG within the first 3 weeks of life in our trial. Nevertheless, RES calves had a stronger decline than HIGH fed calves demonstrating that RES calves had less energy to invest in growth and body weight in comparison to high fed calves. Several studies suggested to increase preweaning milk and MR feeding levels or its nutritional content to attain high ADG already in the preweaning phase (Raeth-Knight et al., 2009; Davis Rincker et al., 2011; Soberon et al., 2012).

Also in body growth, HIGH fed calves showed a linear increase with increasing age in all variables of body dimensions and body length, growth of thorax (with the exception of the abdominal girth), and hip were affected by feeding regime and were higher in high fed calves than in RES. Only body height was not influenced by the feeding regime. Overall, the calves fed on the HIGH level showed a growth advantage over the RES calves in all variables except for the body height. Therefore, the calves of group HIGH appeared broader and stronger than the animals of group RES at the same age. Also Raeth-Knight et al. (2009) demonstrated that an intensive feeding with a high content of solids, and a high level of MR feeding resulted in increased body weight in calves as well as in higher hip heights in comparison to calves fed at

a conventional restrictive regime with low fat and protein MR content, and this grow advantage was maintained also in the postweaning period after 6 weeks of life (d 42). Several studies confirmed this positive effect of high levels of MR feeding on the overall body growth in calves (Kiezebrink et al., 2015; Omidi-Mirzaei et al., 2015).

Since calves in our trial showed no differences in their health status during the first 20 weeks of life, which is an important factor influencing for calves' growth (Donovan et al., 1998), the positive effect in body growth can be attributed to the HIGH MR feeding regime.

6.5 Mammary gland development of dairy heifer calves

There is growing evidence that a high preweaning milk or MR intake leads to higher milk production in the first lactation of heifers (Khan et al., 2011; Korst et al., 2017). This might imply that the development of the mammary gland is enhanced in dairy heifers fed on a high allowance of milk or MR.

In previous studies both beneficial and adverse effects of high feeding levels were reported: a high-energy feeding level and an increase in nutrient intake in the early life of heifer calves before 3 months of age can increase body and mammary gland growth and improves (Bar-Peled et al., 1997; Geiger et al., 2016; Korst et al., 2017) or does not affect the future milk yield (Davis Rincker et al., 2011; Kiezebrink et al., 2015). Others found that prepubertal high plane of nutrition in general repressed mammary growth, impaired mammary gland PAR development in heifers, and could lead to decreased milk yields in the first lactation (Sejrsen et al., 1982; Radcliff et al., 2000). Meyer et al. (2006a, 2006b) suggested that chronological age is the main determining factor on development and growth of mammary PAR in prepubertal dairy heifers and not only the level of nutrition. Geiger et al. (2016) showed that calves receiving an enhanced MR feeding had 5.2-fold more trimmed mammary gland mass after dissection in wk 8 of life in comparison to restrictively fed calves. In this thesis and study, the atlas categories of PAR developmental stages expectedly increased with age, but were not affected by the MR feeding level.

A system for recording, tracking, and assessing the growth and development of the mammary gland of calves while aging and growing up is essential to draw conclusions about the effects of preweaning feeding regime. Non-invasive methods are necessary in order to avoid slaughtering the heifer calves, to avoid damage to their tissue of the developing mammary gland and pain by biopsies, and to follow up the performance in the first lactation. Hence, in our study,

tissue samples deriving from biopsies were not taken and the animals were not slaughtered; instead, palpation and ultrasound scans of the mammary gland were carried out.

The main innovation of this current thesis was the creation of the ultrasonographic atlas of the developing bovine mammary gland (USAtlasMG) and its application as a tool for defining and recognizing the developmental stages of the mammary parenchyma (PAR) that are visible in ultrasound images. In addition, the targeted measurement of changes in tissue structures by measuring the brightness as pixel values based on the atlas categories was established. The USAtlasMG provides the opportunity to classify and differentiate between 11 categories of visible developmental stages of the mammary PAR and its surrounding (SURR) within the first 20 weeks of calves' life. Such developmental stages have already been depicted in rodent models (Richert et al., 2000) and human models (Howard and Gusterson, 2000; Macias and Hinck, 2012) in order to follow the development of the mammary gland and PAR, but not yet in dairy heifer calves.

The creation of the atlas required the viewing and comparison of all 2054 ultrasound images from our trial by one person. The subsequent categorization of the visible PAR, i.e., the assignment to the respective categories of the USAtlasMG, also required the viewing of all images by the same person again. Thus, this method is time-consuming. In modern human medicine, there are already image analysis tools and software for ultrasound diagnostic imaging to detect specific tissue structures and structural changes such as cancer tumours in breast or prostate or kidney stones (Suarez-Ibarrola et al., 2020). The basis for these diagnostic tools is an artificial neural network analysis, also referred to as artificial intelligence (AI) and its subfield machine learning (Goldenberg et al., 2019; Suarez-Ibarrola et al., 2020). This AI-based method could be transferable also on mammary gland ultrasound images of calves and could be used for automated detection of structural changes in the mammary gland and categorization of the developmental stages automatically according to the USAltasMG. This would save time and allow larger scale trials with more animals and ultrasound scans.

Additional palpation of the mammary gland and PAR was a good tool to assess the PAR´s development as well, but around wk 14 of life, the mammary gland reached a status in which different structures were not palpable and therefore the stage of development no longer assessable. Esselburn et al. (2015) and Furini et al. (2018) observed a moderate positive correlation between the palpation score of mammary PAR and PAR area (mm^2/gland) in ultrasound images scanned in 2 - 8 wk old heifer calves, but the palpation score was not affected by the preweaning feeding regime, confirming the results of our study. Measuring the PAR area

(mm^2/gland) in ultrasound images to estimate the so-called amount of PAR was suitable in these studies since the PAR´s area until wk 8 of life was not exceeding the scope of the ultrasonic image. In our study, most calves at the age of 8 weeks showed PAR developmental categories between 1 and 6 of the USAtlasMG: in these categories, PAR was mostly smaller than the ultrasonic scanned section of tissues in the image, so that it would have been possible to measure the PAR´s area in those images like Esselburn et al. (2015) and Furini et al. (2018) did. From wk 10 of life onwards, this opportunity to measure and quantify PAR´s area was not given any more in our study, because PAR´s area mostly exceed the border of the scanned section on the ultrasound image and from category 8 onwards the round PAR structure increasingly dissolved into small pieces, as can be seen in the atlas in category 8, 9, 10, and 11. In wk 10 and higher ages, higher categories of the USAtlasMG and PAR developmental stages (categories ≥ 7) were reached by the majority of calves. In those categories ≥ 7, the area of PAR was larger than the scanned section on the ultrasound image, i.e., PAR´s area was mostly beyond the scope of the ultrasonic image and could be not measured. Therefore, measuring the PAR´s area was not a suitable method in our study to compare its development between the different feeding groups. Furthermore, the measurement of PAR´s area cannot clearly reflect the stage of development according to our observations and the atlas derived therefrom.

Another possibility and established method of evaluating ultrasound images was the measurement of the brightness of different tissue structures of the PAR and its surrounding, the so-called pixel values (Albino et al., 2015; Albino et al., 2017; Silva et al., 2018). Feeding MR at a high or a restrictive level for 14 weeks in the study of this thesis did not alter the brightness of mammary tissue structures: the PAR and SURR pixel values as well as delta pixel values changed with time, but were not affected by feeding. With progressing PAR development, its pixel brightness increased from wk 10 to 20 of life, i.e., PAR became more hyperechogenic since it spread into its SURR, showing the increasing growth of PAR, independently of MR feeding regime.

Geiger et al. (2016) reported that the degree of expansion of the epithelial tissue into the adjacent stromal tissue and the complexity of ductal development were minimal in 8-wk old calves fed restrictively (0.45 kg MR/d) and increased in calves fed on an elevated MR feeding level of 1.13 kg MR/d. In our study, there was neither a difference between the visible developmental stages (USAtlasMG) nor in the pixel brightness of PAR or SURR at the age of 8 weeks between both feeding groups. Albino et al. (2015, 2017) and Silva 2018 measured the pixel brightness in mammary glands of calves postweaning and observed that different ratios of dietary metabolizable protein (MP) and metabolizable energy (ME) may influence body

weight gain as well as the growth of the mammary fat pad (MFP) and the pixel brightness in SURR.

Further studies with more frequent measuring intervals are needed for elucidating the influence of different feeding levels or *ad libitum* feeding on mammary gland growth and especially on the pixel brightness and to precisely determine the age at which developmental stages firstly appear. The period between wk 3 and wk 8 of life should be explicitly considered.

6.6 Milk yield in first lactation and performance

The rearing of heifer calves is essential as it can have an impact on growth, development, and milk production performance in adult life (Diao et al., 2019). Previous studies have already shown that an accelerated preweaning body weight gain can be positively associated with the milk yield in the first lactation (Soberon and van Amburgh, 2013; Korst et al., 2017; Chester-Jones et al., 2017). Preweaning nutrient intake, from milk or MR, can have profound effects on the development of the calf that enhance first lactation and lifetime productivity (Soberon and van Amburgh, 2013).

The records of lactation performance were limited to 23 heifers from our study (HIGH n = 11; RES n = 12) and their first 7 weeks of lactation. Cows from the former HIGH group produced on average 4.6 kg/d more milk than those from the RES group. Previously, it was already shown that increasing age at first calving can have negative effects on first lactation performance and milk production of Holstein cows (Heinrichs and Heinrichs, 2011), but this effect can be ruled out since the age at first calving was not different between cows of the two preweaning MR feeding groups in our study. In the study of Kiezebrink et al. (2015), the age at first calving as well as milk production parameters were not affected by the preweaning whole-milk feeding level of 4 L/d or 8 L/d for 8 weeks in calves, although preweaning starter intake of calves fed on the lower level was higher. Their results suggest that enhanced whole milk feeding improved growth performance until 3 months of age, while results of the first-lactation indicated no benefits in lactation performance in dairy calves fed on increased feeding levels and thus improved growth performance during the milk-fed period. In another study of Davis Rincker et al. (2011) comparing preweaning high and low energy diets resulted in younger ages at first calving (2 wk less) in high-energy fed calves than in restrictively fed ones, but the 305-d milk yield was not different between the different preweaning feeding groups. Raeth-Knight et al. (2009) also detected no difference in first-lactation performance between conventional low intensive and high and intensive MR feeding. Korst et al. (2017) detected numerically but

insignificantly higher milk yields (305 d) in *ad libitum* MR fed calves in comparison to restrictively fed ones (6.78 kg MR/d) in the first lactation. In the study of Davis Rincker et al. (2011) milk yield tended to be higher in those animals fed the intensive diet of a high protein MR in comparison to a standard MR.

Heinrich and Heinrichs (2011) assumed that the dry matter intake at weaning was associated with an improved 305-d ME milk, fat and protein production in Holstein dairy cows and that a further increase in dry matter intake correspondingly increases the milk performance and production in the first lactation. In our study, on which this thesis is based, the starter feed intake in both feeding groups was not different, so that differences in energy intake came only from MR intake and could influence mammary gland development and subsequent lactation performance.

The difference in milk yield between feeding groups in our study was mainly attributable to two low-performing animals in the RES group that also had elevated somatic cell counts (> 200,000 cells/mL up to 1,090,000 cells/mL) in weekly milk samples in wk 2–4 in lactation. These two heifers in the RES group were detected as outliers showing lesser milk yields (12.4 kg/d +- 6.1) than the average in the HIGH and the RES group. After excluding these two cows, there was no difference in average daily milk yield and somatic cell counts (SCC) anymore. Also Davis Rincker et al. (2011) did not see any effect of the preweaning MR feeding intensity on SCC in the first lactation. Svensson et al. (2006) showed with calculations in their study that a higher growth rate from birth to weaning and feeding of more roughage to calves at weaning was associated with a reduced risk of clinical mastitis 7 d before to 30 d after first calving and of elevated SCC (≥200,000 cells/mL) on d 21 after calving in the first lactation. In our study, calves receiving MR on the HIGH level showed higher ADG and body growth than calves fed on restricted level in group RES. Therefore, HIGH calves could also benefit in terms of udder health.

Overall, feeding costs of intensive feeding regime are higher in comparison to restrictive ones (Davis Rincker et al., 2011; Korst et al., 2017), but the advantage for calves in development might overweigh this, although there is often just a numerical but not significant higher milk yield in HIGH fed calves (Korst et al., 2017). In addition, it can be confirmed that an intensive preweaning feeding regime of calves had no negative effects on milk yield in the first lactation. Keeping cows healthy during the first lactation could additionally save costs of veterinary treatments and costs due to losses from discarded milk, which should be calculated in future studies as well. More data and studies are needed to confirm this.

6.7 Oxidative status and antioxidative potential in calves

The oxidative status describes the amounts of oxidants and antioxidants (Costantini, 2019) and depends on the production of reactive oxygen species (ROS), which could lead to cell damages, and their antagonists, the antioxidants (Sies, 1985). If oxidants cannot be equilibrated by antioxidants, oxidative stress arises, and this, in turn, may lead to various diseases (Sies, 1985; Aruoma, 1998; Kaushal et al., 2019).

In the present thesis, different variables defining the oxidative status and its changes were assessed in blood plasma of the calves until wk 20 of life. The values of dROM, FRAP, and total protein content in plasma increased with age, whereas TBARS, AOPP, and the GSH-Px activity decreased. Feeding group effects were limited to AOPP (oxidative damage to proteins) and the antioxidative capacity FRAP. Both variables had greater concentrations in RES than in HIGH from birth until wk 12 of life, whereas at the end of weaning in wk 14 the levels were equal in both feeding groups.

In newborns, oxygen species (ROS) are generated due to the abrupt environmental change at birth, from hypoxic to hyperoxic environment (Ranade et al., 2014) and therefore the antioxidative defense mechanism should react to the same extent as oxidants appear. Previously, it was assumed that the cellular antioxidative defence mechanisms in human neonates are not completely developed and could not adequately compensate for the increasing ROS (Saugstad, 1990, 1996), whereas Gaál et al. (2006) concluded that newborns are prepared to deal with oxidative stress.

Results of the study of Ranade et al. (2014) showed that antioxidative defence in plasma of new-born calves increased from birth onwards and was related to protein oxidation processes (AOPP). After calves' birth, the biological antioxidative potential determined in fresh blood was higher than in wk 6 and wk 12 of life, while the concentration of reactive oxygen metabolites (dROM test) did not change from birth until weaning in wk 18 (Ranade et al., 2014). In our study, dROM showed the lowest values after birth (36 – 42 h p.n.) and an increase with age, while the antioxidative activity of GSH-Px was highest after birth compared to the following time points until wk 20 of life. The higher GSH-Px activity after birth could be a reaction due to the greater production of oxidative H_2O_2. Therefore, it can be assumed that also new-born calves are able to react to increasing ROS.

Although ROS are considered as deleterious by-products of the cell metabolism, mostly generated during the mitochondrial oxidative phosphorylation, they also play an essential role in maintaining the redox homeostasis within the cell, as long as they do not exceed a certain

physiological level (Kaushal et al., 2019). There are no reference values for any of the variables used to characterize the oxidative status for differentiating oxidative stress from basal levels in the blood of calves. Hence, a high level of ROS or dROM in plasma can indicate both, a higher activity of the mitochondria or a reduced antioxidative capacity. Furthermore, it should be noted that no conclusions can be drawn from the measured values in plasma with regard to the origin of the investigated variables. Therefore, it is unclear exactly which organ, tissue, or cells are affected by oxidative stress (Briviba et al., 2008).

Oxidative stress is supposed to be an important factor that can contribute to dysfunctional inflammatory responses in metabolically stressed cows (Sordillo, 2016). Moreover, tissue injury itself can cause ROS generation, e.g., by causing the activation of phagocytes or releasing transition metal ions from damaged cells into the surrounding tissue or blood (Aruoma, 1998). Macrophages and neutrophilic granulocytes showing phagocytic activity during inflammatory processes. During phagocytosis, bacteria are internalized within phagosomes that contain hydrolytic enzymes and generate bactericidal ROS, a reaction which is also known as neutrophilic oxidative burst (Sordillo, 2016). In his study Ballou (2012) could show that higher preweaning planes of MR nutrition (11.8 kg MR/d vs. restrictive 4.54 kg MR/d) increased the postweaning rate of neutrophil oxidative burst intensity in calves' blood and the whole-blood killing responses when cultured with *E. coli* for 10 min at d 77 of life. In our study, damages caused by oxidative stress on lipids were measured in our study in plasma as TBARS (Gutteridge and Halliwell, 1988) and on proteins by assessing AOPP (Witko-Sarsat et al., 1996). Pro-oxidants can hardly be directly analyzed due to their instability and short half-life blood (Leeuwenburgh and Heinecke, 2001). Thus we used the dROM test according to Alberti et al. (2000) in which reactive oxygen metabolites (ROM) are indirectly assessed as derivatives (d) of ROM by considering the Fenton reaction, i.e., the reaction between Fe(II) and H_2O_2 yielding hydroxyl radicals, and coupled processes. "The alkoxy and peroxy radicals formed in the degradation of hydroperoxides brought about by transition metal ions in acidic media can convert substrates with suitably low oxidation potentials, such as *N*,*N*-diethyl-*para*-phenylendiamine" (DEPPD), i.e., the substrate used in the dROM test, "to the corresponding radical cations"($DEPPD^{\bullet+}$; Alberti et al., 2000). The latter ones are proportional to the concentration of ROS in the sample. Hence, in our study the amount of ROS was not measured directly, only their consequences.

Greater AOPP concentrations in RES calves may indicate a greater portion of oxidized proteins in RES, but the relevance of this finding is yet unknown. In general, the tests used herein to characterize the oxidative status can only provide a rough estimate and have to be interpreted

with caution. Hence we can neither conclude on inflammatory processes in calves showing higher AOPP, TBARS or dROM values nor on the health status of the animals in general.

However, diet-derived antioxidants might be particularly important in protecting against oxidative stress. Levels of vitamins or other diet-derived antioxidative factors were not measured in blood from calves in this trial. Nevertheless, the preweaning intake of MR which contains supplemental amounts of vitamins and micronutrients was higher in calves fed on HIGH level than those fed RES. Therefore, also the intake of vitamins and micronutrients via MR, which are important for the antioxidative defence and also for the involved enzymes such as GSH-Px, was higher in HIGH than in RES. However, in our study, no difference was found in GSH-Px activity between the calves in the different feeding groups, whereas the antioxidative capacity (measured as FRAP) was influenced by the preweaning MR feeding level and was higher in RES than in HIGH fed calves. In the case of FRAP, the higher values in RES calves than in HIGH were already apparent before the differential feeding of MR and feed intake (36–48 h p.n.). Also, potential differences in their respective dams or a correlation between FRAP values of calves and dams were excluded. Hence, this effect on FRAP could not be clearly attributed to the MR feeding regime. Possibly, the composition of the dams' colostrum or its redox balance might have been different. However, the colostrum samples were not specifically analyzed in this regard.

The weaning process is another stress inducing factor and can cause transient neutrophilia, suppressed neutrophil phagocytic, and oxidative burst responses in calves (Hulbert et al., 2011). In the study of Ranade et al. (2014), dROM in calves' plasma and the respective oxidative stress index were not affected by weaning in wk 18 of life. In our study, dROM values were higher in HIGH than in RES calves in wk 14 at the end of weaning, but reached the same level in wk 16. Weaning is potentially stressful for calves because of the sudden change in their feeding routine and nutrition. Stress is known to suppress many leukocyte responses and increases the relative risk of developing an infectious disease (Hulbert et al., 2011; Obeidat et al., 2013). Hulbert et al. (2011) could show that neutrophil responses and the neutrophil oxidative burst were suppressed at weaning in calves fed on a restrictive or low MR feeding level. Obeidat et al. (2013) showed that the neutrophil responses including the oxidative burst in calves fed on a restrictive low preweaning MR level (4.18 kg MR/d of DM) were more active in the preweaning phase than in calves fed on higher MR levels (7.47 kg MR/d and 10.10 kg MR/d of DM), but not in the postweaning phase. In the study of Ballou (2012), the higher plane of MR nutrition (11.8 kg MR/d vs. restrictive 4.54 kg MR/d) increased the postweaning rate of neutrophil oxidative burst intensity in calves blood and whole-blood killing responses when cultured with

E. coli for 10 min at d 77 of life, irrespective of the calves' breed. However, indicators for neutrophilic response or oxidative burst can be released as ROS such as H_2O_2 into blood which was not specifically measured, but included in dROM analysis of free radicals in plasma in our study. At the end of weaning, in wk 14 of life, dROM values were significantly higher in HIGH fed calves than in RES fed calves, but an overall influence of the feeding regime on dROM was not found. The values of the other variables of oxidative status assessed in our trial were not different between feeding groups. Therefore, the results of our study cannot confirm a negative influence of the weaning procedure on the oxidative status of calves.

The oxidative status, with its many different interfaces, is a complex phenomenon and its assessment depends on the type of target variables which are assessed to describe the current status in blood or within cells. Therefore, it is not directly possible to conclude from the oxidative status to a better health status or an advantage of the animals. Further studies are needed to gain deeper insights into these dynamics of oxidative status in developing calves and heifers. Moreover, further research should emphasize the assessment of the oxidative status in new-born calves directly after birth to clarify whether the antioxidative defence is already active at birth or increases in the first hours of life. However, it still needs to be elucidated to what extent variables of oxidative status in blood circulation influence the development of metabolism of dairy calves.

7 Conclusions and future perspectives

The results of the calf trial presented herein showed that an increased MR feeding level of 10 L MR/d enhanced the body growth and body weight gain in comparison to restrictive feeding of 5.7 L MR/d as it is usually done in conventional farming. The high MR feeding level reduced signs of hunger, while starter feed intake was not compromised. The prolonged MR feeding period of 14 weeks probably also contributed to these effects even though in this study shorter preweaning periods as in conventional farming could not be considered. Hence, an investment of milk or MR is worthwhile with regard to beneficial effects on growth, development, and an increase in calf welfare, which confirms our hypothesis.

Even though there was no effect of MR feeding level on mammary gland growth and developmental categories, the herein developed ultrasonographic atlas of bovine mammary gland development (USAtlasMG) and its categories provide an overview of developmental stages and the structural development in the prepubertal bovine mammary gland and can serve as a basis for further studies. Further studies should find out the age at which PAR formation in category 2 appears for the first time in ultrasound images. The period between the wk 3 and wk 8 of life should be explicitly considered.

Furthermore, the categorization of mammary gland developmental stages in all ultrasound images based on the Atlas (USAtlasMG) was conducted out by one single person and was time-consuming. In future studies, software analysis tools from human medicine for ultrasound diagnostic imaging based on artificial neural network analysis, also referred to as artificial intelligence (AI) could be adapted for mammary gland ultrasound images of calves. An automated detection of structural changes in the mammary gland and automated categorization of the developmental stages according to the USAltasMG would be possible. This would save time, allow for conducting out larger scale trials with more animals and ultrasound scans, allow for the objective categorizations of the developmental stages, and could be used by anyone without the need for persons to be specifically trained in image analysis. Currently, the USAtlasMG and its categories are used in such a new research project that uses artificial intelligence for the automatic categorisation of the calves' mammary gland development in ultrasound images.

8 Summary

The growth and the development of heifer calves in the early life are considered as the most influential factors concerning future health and profitability of dairy cows. Both, growth and development not only depend on the genetic potential of a calf, but are mainly affected by feed intake, i.e., the energy intake. Therefore, the farmer has the opportunity to force growth and development via feeding. Due to the fact that calves are born as functional monogastrics, their nutrient and energy intake is based on liquid feed, i.e., colostrum, milk, or milk replacer (MR), until the rumen is fully matured and they become ruminants. A paradigm shift in rearing of heifer calves from restricted milk or MR feeding and early weaning towards greater feed allowances and later weaning ages is ongoing. There is growing evidence that increasing the daily allowance for milk or MR positively affects growth, development, and animal welfare by reducing hunger and avoiding abnormal behaviour. The development of the mammary gland contributes to the final performance of dairy cows. Gaining a deeper insight into mammary gland development in the early life is important not only for basic research, but also for testing different feeding and rearing management strategies in terms of mammary gland development. It is becoming evident that the period between wk 8 and wk 10 of life, when calves are commonly weaned, is critical for the imprinting of the mammary gland capacity for milk production. The preweaning feeding regime can also influence mammary gland growth and development.

The effects of different milk or MR feeding regimes in a prolonged preweaning period of 14 weeks are less explored concerning the mammary gland development and the systemic oxidative status in calves.

Therefore, the aim of the herein presented study and thesis was to investigate to what extent the MR feeding level before weaning at week 14 of life can influence the oxidative status, growth and development of dairy heifer calves, and in particular the development of the mammary gland.

In the first manuscript (I), presented in chapter 3, which is already published in Antioxidants, the hypothesis was that calves fed on high levels of daily MR in comparison to calves fed on a restricted and low level would show higher rates of development without extreme declines after weaning, fewer signs of hunger, and good health condition. Furthermore, it was hypothesised that feeding high planes of MR would reduce stress during weaning and also alter the blood profiles variables describing the oxidative status, as well as of haptoglobin (Hp), leptin, and adiponectin.

In this study, 37 Holstein heifer calves fed with MR (14% solids, 140 g MR/L) either at a high (HIGH; 10 L MR/d, n = 18) level or a restrictive (RES; 5.7 L/d, n = 19) level from day 5 until linear weaning in wk 13 and wk 14 of life. All calves were examined from birth until wk 20 of life.

The new-born calves were initially kept in individual hutches and were transferred to group pens at day 10 of life. Pelleted calf starter was fed *ad libitum*. The intake of both MR and pelleted calf starter, as well as the number of rewarded and unrewarded visits and the drinking speed, were recorded individually by the automated feeder until the end of weaning at the end of wk 14 of life. After weaning, the calves were moved to a new group pen where they had free access to a total mixed ration. Water and hay offered ad libitum to the calves throughout the study.

The body weight (BW) and health status of calves were assessed weekly. Blood samples were taken after birth (36 - 48 h p.n.), then fortnightly from wk 8 - 16 of life and in wk 20 of life. The concentrations of leptin, adiponectin and haptoglobin (Hp) were measured in serum. The oxidative status was assessed in plasma by measuring the antioxidant capacity as the ferric reducing ability of plasma (FRAP), the antioxidative activity of the glutathione peroxidase (GSH-Px), the oxidative damages to lipids and proteins measured as thiobarbituric acid reactive substances (TBARS) and as advanced oxidation products of proteins (AOPP), and the level of derivatives of reactive oxygen metabolites (dROM).

Feeding of MR at different levels before weaning resulted in greater preweaning and postweaning BW in HIGH fed calves than in RES calves throughout the study period until wk 20 of life. There was no difference in health status between feeding groups. The average daily gains (ADG) increased from wk 3 onwards and decreased postweaning in both groups, whereby the RES calves had a more pronounced growth depression than HIGH in wk 16. The intake of starter feed did not differ between the two feeding groups and the higher intake of metabolizable energy (ME) in HIGH until weaning could thus be attributed to the higher MR intake in HIGH. The RES calves visited the feeder more often and had more unrewarded visits than HIGH calves throughout the milk feeding period. This indicated more signs of hunger in RES than in HIGH calves. However, the feeding groups did not differ in their drinking speed. Adiponectin, an insulin-sensitising hormone, was influenced by calves´ age (time) and MR feeding level. Adiponectin concentrations were higher in HIGH-fed calves than in RES-fed calves. Leptin was only influenced by the calves´ age, but not by the feeding group. Neither the health status nor the Hp concentrations in serum differed between the feeding groups. The time course of

variables describing the oxidative status during the first 20 weeks of life showed characteristic patterns: values of reactive oxygen metabolites (dROM) and the antioxidative capacity (FRAP) increased with age, whereas the oxidative damage to lipids (TBARS) and to proteins (AOPP) decreased. Differences between feeding groups were limited to adiponectin, AOPP, and FRAP, while in FRAP this difference was already apparent 36 – 48 h after birth.

In summary, the HIGH feeding level improved animal welfare by reducing signs of hunger, while starter intake was not compromised. The differences in FRAP which are already seen in newborns were thus rather not related to the feeding level. Greater AOPP concentrations in RES may indicate a greater portion of oxidized proteins in RES, but the relevance of this finding is yet unknown. The results of oxidative status in calves could not show clear benefit or disadvantage for either feeding group in terms of oxidative loads.

In the second and third manuscripts (II and III), presented in chapter 4 and 5 of this thesis, the focus was on the development of the mammary gland and differentiation of defined mammary gland structures in heifer calves. Moreover, in this study it was possible to follow and record the early postnatal and prepubertal mammary gland development of the 37 dairy heifer calves via ultrasonographic scans until wk 20 of life. It was hypothesized that increasing the daily allowance for MR for calves could enhance the mammary gland growth and development in the preweaning phase which in turn could imprint also the first lactation performance as dairy cows.

In manuscript II, the development of the mammary gland was captured in ultrasonic images from the 37 Holstein dairy heifer calves from wk 3 to 20 of life. Ultrasound scans of the mammary gland were performed first in wk 3 of life, fortnightly from wk 8–16, and in wk 20 of life, from each udder quarter of each calf, in standing position, using a B-mode ultrasound device equipped with a linear probe, at a frequency of 18 MHz, and in a standardized position of 45° inclination to the teat. Before the ultrasonic scanning, the mammary gland quarters were palpated for assessing the size of the mammary parenchyma (PAR) and the teat length was measured. For assigning the structures of PAR visible in the ultrasound images to defined stages of PAR development, schematic exemplary drawings of mammary gland structures per stage were elaborated forming an ultrasonographic atlas of the developing bovine mammary gland (USAtlasMG). This atlas provides a tool for the qualitative categorisation of the tissue types, i.e., PAR and its surrounding tissue (SURR) visible in ultrasound images, and thus also for classifying the developmental stages. The USAtlasMG comprises 11 categories of PAR development. In addition, the mean visible category number (MVC) was calculated as the

average of visible mammary gland category numbers from ultrasound images of 4 quarters of the udder, as well as for the mean palpation score. With increasing age of the calves, higher atlas categories of PAR developmental stages were shown in ultrasonic images. The preweaning daily MR allowance had neither an effect on the MVC of mammary PAR development nor on mean palpation score or teat length, but all variables increased with increasing age. The USAtlasMG can serve as a template for the categorisation and qualitative assessment of mammary gland structures and furthermore for the quantitative measurement of PAR´s development by measuring the pixel brightness. Hence, quantitative measurements by measuring the pixel brightness can be standardized based on this atlas, because well-defined tissue structures of interest can be specifically selected and subjected to assess the brightness as pixel values.

In manuscript III, the pixel brightness of distinct mammary gland structures was measured according to the previously developed ultrasonographic atlas of the developing bovine mammary gland (USAtlasMG). In addition, ultrasonic evaluations of the back-fat thickness (BFT) and the rib-fat thickness (RFT) were performed in 30 calves (HIGH n = 16; RES n = 14) of pluriparous cows only. Furthermore, the body growth of the calves was determined using morphometric traits for body height, body length, thorax, and hip. Feeding MR at a high or a restrictive level for 14 weeks did not alter the brightness of mammary tissue structures in mammary glands. However, with progressing PAR development, its pixel brightness increased from wk 10 to 20 of life, i.e., PAR became more hyperechogenic since it spread into its surrounding tissue (SURR), which in turn showed the increasing growth of PAR, independently of MR feeding regime. Both, BFT and RFT increased with age, whereby HIGH calves had greater RFT and tended to have greater BFT values than RES calves from wk 8 until the end of weaning. In the 30 calves of pluriparous cows, all variables of the body dimensions showed a linear increase in body growth with age, but except for the height growth. HIGH calves showed an overall growth advantage over RES calves within the observed 20 weeks of life.

Records of lactational performance were limited to 23 heifers (HIGH n = 11; RES n = 12) and the first 7 weeks of their first lactation. The age at first calving was not different between the former feeding groups, but cows from the former HIGH group produced on average 4.6 kg/d more milk than those from the former RES group. This difference was mainly attributed to two low-performing animals in the RES group, which also had increased somatic cell counts (> 200.000 cells/mL).

In summary, this study and thesis characterise the changes in oxidative status of dairy heifer calves with increasing age and confirm the benefits of a high MR feeding level in terms of development and animal welfare. The feeding of MR at a high or a restrictive level for 14 weeks did not alter the brightness of mammary gland tissue structures in ultrasound images, but a high MR feeding level had advantages in body growth in length and width, and potentially also for udder health in the first lactation. Thus, the welfare of dairy heifer calves can be improved by simple and more natural rearing strategies. Further studies should find out the age at which PAR formation appears for the first time in ultrasound images, focusing on the timeframe between wk 3 and 8 of life. Furthermore, in future, software analysis tools for ultrasound image diagnosis, based on artificial neural network analysis also referred to as artificial intelligence (AI), could be adapted for mammary gland ultrasound images of calves according to the herein created USAtlasMG.

9 Zusammenfassung

Das Wachstum und die Entwicklung von Färsenkälbern in der frühen Lebensphase werden als die einflussreichsten Faktoren für die zukünftige Gesundheit und Rentabilität von Milchkühen angesehen. Beides, Wachstum und Entwicklung hängen nicht nur vom genetischen Potenzial eines Kalbes ab, sondern werden hauptsächlich durch die Futteraufnahme, d.h. die Energieaufnahme, beeinflusst. Daher hat der Landwirt die Möglichkeit, Wachstum und Entwicklung durch die Fütterung zu forcieren. Da Kälber als funktionale Monogastrier geboren werden, basiert ihre Nährstoff- und Energieaufnahme bis zur vollständigen Pansenentwicklung eines Wiederkäuer auf flüssigem Futter, d.h. auf Kolostrum, Milch oder Milchaustauscher (MR). Derzeit findet ein Paradigmenwechsel in der Aufzucht von Färsenkälbern von einer restriktiven Milch- oder MR-Fütterung und frühem Absetzen hin zu größeren Futtermengen und einem späterem Absetzalter statt. Es gibt immer mehr Belege dafür, dass eine Erhöhung der täglichen Milch- oder MR-Fütterungsmenge, das Wachstum, die Entwicklung und das Wohlbefinden der Tiere positiv beeinflusst, indem Hunger reduziert und dadurch abnormes Verhalten vermieden wird. Die Entwicklung der Milchdrüse ist essentiell für die spätere Leistung von Milchkühen. Ein tieferer Einblick in die Milchdrüsenentwicklung zu Lebensbeginn und in der frühen Lebensphase ist nicht nur für die Grundlagenforschung wichtig, sondern auch um verschiedene Fütterungs- und Aufzuchtmanagementstrategien im Hinblick auf die Entwicklung der Milchdrüse zu prüfen. Es wird immer deutlicher, dass der Zeitraum zwischen der 8. und 10. Lebenswoche, in dem Kälber üblicherweise abgesetzt werden, entscheidend für die Prägung der Milchdrüsen und damit deren künftige Milchproduktion ist. Die Fütterung vor dem Absetzen kann das Wachstum und die Entwicklung der Milchdrüse beeinflussen.

Die Auswirkungen verschiedener Fütterungsregime in einer verlängerten Milchtränkephase von 14 Wochen sind hinsichtlich der Milchdrüsenentwicklung und des systemischen oxidativen Status bei Kälbern weniger erforscht.

Daher war das Ziel der hier vorgestellten Studie und Dissertation, zu untersuchen, inwieweit die gefütterte MR-Menge vor dem Absetzen in der 14. Lebenswoche den oxidativen Status, das Wachstum und die Entwicklung der Färsenkälber, insbesondere der Entwicklung der Milchdrüse, beeinflussen kann.

Im ersten Manuskript, vorgestellt in Kapitel 3, welches bereits in „Antioxidants“ veröffentlicht wurde, war die Hypothese, dass mit hohen täglichen MR-Mengen gefütterte Kälber im Vergleich zu restriktiv gefütterten Kälbern sowohl höhere Entwicklungsraten ohne extreme

Rückgänge nach dem Absetzen, als auch weniger Anzeichen von Hunger zeigen und einen guten Gesundheitszustand aufweisen würden. Des Weiteren wurde angenommen, dass die Fütterung von höheren MR-Mengen Stress während des Absetzens von der Milchtränke reduzieren und auch die Profile ausgewählter Parameter des oxidativen Status im Blut sowie von Haptoglobin (Hp), Leptin und Adiponectin verändern würde.

In dieser Studie wurden 37 Holstein-Färsen-Kälbern vom 5. Lebenstag bis zum linearen Absetzen in der 13. und 14. Lebenswoche entweder mit hohen Mengen (HIGH; 10 L/d, n = 18) an Milchaustauscher (MR, 14% Trockenmasse, 140 g MR/L) oder restriktiv (RES; 5,7 L/d, n = 19) gefüttert und bis zur 20. Lebenswoche untersucht.. Die neugeborenen Kälber wurden zunächst in Einzelbuchten gehalten und nach dem 10. Lebenstag in Gruppenbuchten umgestellt. Dort wurde zusätzlich Kälberstarter *ad libitum* gefüttert. Die tägliche Aufnahme von MR und pelletiertem Kälberstarter, sowie die Anzahl der belohnten und unbelohnten Besuche und die Trinkgeschwindigkeit wurden bis zum Ende des Absetzens am Ende der 14. Lebenswoche tierindividuell vom Tränkeautomaten erfasst und aufgezeichnet. Nach dem Absetzen wurden die Kälber in einen größeren Stall umgestallt, wo sie freien Zugang zu einer totalen Mischration hatten. Wasser und Heu, standen den Kälbern während des gesamten Versuchszeitraums *ad libitum* zur Verfügung.

Das Körpergewicht (BW) und der Gesundheitszustand der Kälber wurden wöchentlich erfasst und untersucht. Blutproben wurden nach der Geburt (36 - 48 h p.n.) und anschließend vierzehntägig von Lebenswoche 8 - 16 und in Woche 20 entnommen. Die Konzentrationen von Leptin, Adiponektin und Haptoglobin (Hp) wurden im Serum gemessen. Der oxidative Status wurde im Plasma durch Messungen der antioxidativen Kapazität als Eisen(III)-Reduktionsvermögen des Plasmas (FRAP), der oxidativen Schädigungen von Lipiden und Proteinen (TBARS und AOPP), des Gehalts an reaktiven Sauerstoffmetaboliten (dROM) und der antioxidativen Aktivität der Glutathion-peroxidase (GSH-Px) erfasst.

Die Fütterung unterschiedlicher MR-Mengen vor dem Absetzen führte dazu, dass die Kälber, die auf einem hohen Niveau (HIGH) gefütterten wurden vor und nach dem Absetzen bis zur 20. Lebenswoche ein höheres BW aufwiesen als restriktiv gefütterte Kälber (RES). Beim Gesundheitsstatus zeigte sich kein Unterschied zwischen den Fütterungsgruppen. Die durchschnittlichen Tageszunahmen (ADG) stiegen ab Woche 3 an und nahmen nach dem Absetzen in beiden Gruppen ab, wobei die Kälber der Gruppe RES in Woche 16 einen stärkeren Wachstumsrückgang aufwiesen als die Kälbern der Gruppe HIGH. Die Starterfutteraufnahme unterschied sich nicht zwischen den beiden Fütterungsgruppen, sodass die höhere Aufnahme

an umsetzbarer Energie (ME) bei Kälbern der Gruppe HIGH bis zum Absetzen auf die höhere MR-Aufnahme zurückgeführt werden konnte. Die restriktiv gefütterten Kälber (RES) besuchten die Tränke häufiger und zeigten während der gesamten Milchfütterungsphase mehr unbelohnte Besuche als Kälber der Gruppe HIGH. Dies deutete auf mehr Anzeichen von Hunger bei den restriktiv gefütterten Kälbern RES hin als bei den Kälbern der Gruppe HIGH. Die Fütterungsgruppen unterschieden sich jedoch nicht in ihrer Trinkgeschwindigkeit. Adiponectin, ein Insulin-sensibilisierendes Hormon, wurde durch das Alter der Kälber (Zeit) und durch die Fütterungsmenge an MR beeinflusst. Die Adiponectin-Konzentrationen waren bei Kälbern der Gruppe HIGH höher als bei den Kälbern der Gruppe RES. Leptin wurde nur durch das Alter der Kälber, aber nicht durch die Fütterungsgruppe beeinflusst. Weder der Gesundheitsstatus noch die Hp-Konzentrationen im Serum unterschieden sich zwischen den Fütterungsgruppen. Die Verlaufskurven der hier gemessenen Parameter, die den oxidativen Status beschreiben, zeigten charakteristische Muster während der ersten 20 Lebenswochen: Die Werte der reaktiven Sauerstoffmetabolite dROM und der antioxidativen Kapazität FRAP stiegen mit zunehmendem Alter an, während die oxidative Schädigungen von Lipiden (TBARS) und von Proteinen (AOPP) abnahmen. Unterschiede zwischen den Fütterungsgruppen beschränkten sich auf Adiponectin, AOPP und FRAP, wobei bei FRAP ein Unterschied zwischen den Gruppen bereits 36 - 48 h nach der Geburt sichtbar war.

Zusammenfassend lässt sich sagen, dass die hohe MA-Fütterungsmege (HIGH) das Wohlbefinden der Tiere verbessert hat, indem Anzeichen von Hunger reduziert wurden, während die Starteraufnahme durch das höhere Fütterungsniveau nicht beeinträchtigt wurde. Die Unterschiede zwischen den Gruppen in FRAP, die bereits kurz nach der Geburt und vor Beginn der unterschiedlichen MR-Fütterung auftraten, standen somit nicht im Zusammenhang mit dem MR-Fütterungsniveau. Höhere AOPP-Konzentrationen in Kälbern der Gruppe RES im Vergleich zu HIGH könnten auf größere Mengen an oxidierten Proteinen hinweisen, jedoch ist die Relevanz dieses Ergebnisses noch unbekannt. Die Ergebnisse des oxidativen Status bei den Kälbern konnten keine eindeutigen Vor- oder Nachteile für eine der beiden Fütterungsgruppen in Bezug auf die oxidativen Belastungen zeigen.

Im zweiten und dritten Manuskript (II und III), vorgestellt in Kapitel 4 und 5, lag der Fokus auf der Entwicklung und der strukturellen Differenzierung der Milchdrüse bei Färsenkälbern. Im Rahmen dieser Studie war es möglich, die frühe postnatale und präpubertäre Milchdrüsenentwicklung der 37 Färsenkälber mittels Ultraschallaufnahmen bis zur 20. Lebenswoche zu erfassen. Es wurde die Hypothese aufgestellt, dass eine Erhöhung der täglichen Fütterungsmenge an MR für Kälber das Wachstum und die Entwicklung der

Milchdrüsen in der Phase vor dem Absetzen und darüber hinaus fördern könnte, was sich wiederum auf die Leistung in der ersten Laktation als Milchkuh auswirken könnte.

In Manuskript II wurde die Entwicklung der Milchdrüse und deren Erfassung in Ultraschallbildern von 37 Holstein-Färsen-Kälbern von der 3. bis zur 20. Lebenswoche beschrieben. Ultraschalluntersuchungen der Milchdrüse wurden zuerst in der 3. Lebenswoche, dann vierzehntägig von der 8. bis zur 16. Lebenswoche und in der 20. Lebenswoche von jedem Euterviertel eines jeden Kalbes in stehender Position mit einem Ultraschallgerät im B-Modus, das mit einer Linearsonde ausgestattet war, mit einer Frequenz von 18 MHz, in einer standardisierten Position von 45°-Winkel zur Zitze, durchgeführt. Vor Beginn der Ultraschalluntersuchung wurden die Milchdrüsenviertel zur Beurteilung der Größe des Milchdrüsenparenchyms (PAR) abgetastet und die Länge der Zitzen gemessen. Um die in Ultraschallbildern sichtbaren Strukturen des PAR definierten Stadien der PAR-Entwicklung zuordnen zu können, wurden schematische Beispielzeichnungen der Milchdrüsen-Gewebestrukturen pro Stadium erstellt, die einen „ultrasonographischen Atlas der sich entwickelnden bovinen Milchdrüse“ (USAtlasMG) umfassen. Dieser Atlas bietet die Möglichkeit zur qualitativen Kategorisierung der Gewebetypen in Ultraschallbildern, d.h. des PAR und des umgebenden Gewebes (SURR), und somit auch zur Klassifizierung des Entwicklungsstadiums. Der USAtlasMG umfasst 11 Kategorien der PAR-Entwicklung. Zusätzlich wurde die durchschnittliche sichtbare Kategorienzahl (MVC) als Mittelwert der sichtbaren Kategorienzahl aus den Ultraschallbildern von 4 Vierteln des gesamten Euters berechnet, ebenso wie der Mittelwert des Palpationswertes auf Grundlage einer zugehörigen Skala (Palpationsscore).

Mit zunehmendem Alter der Kälber zeigten sich in den Ultraschallbildern höhere Atlas-Kategorien der PAR-Entwicklungsstadien. Die täglich gefütterte MR-Menge vor dem Absetzen hatte weder einen Einfluss auf die MVC der mammaren PAR-Entwicklung noch auf den Mittelwert des Palpationsscores oder die Zitzenlänge, dennoch zeigte sich ein Anstieg aller Werte mit zunehmendem Alter der Kälber. Der USAtlasMG kann als Vorlage für die Kategorisierung und qualitative Bewertung von Milchdrüsenstrukturen und darüber hinaus auch für die quantitative Messung der PAR-Entwicklung durch Messung der Pixelhelligkeit dienen. Daher können quantitative Messungen durch die Messung der Pixelhelligkeit auf der Grundlage dieses Atlas standardisiert werden, da gezielt bestimmte und definierte Gewebestrukturen ausgewählt und einer Helligkeitsmessung (Pixelwerte) unterzogen werden können.

In Manuskript III wurde die Messung der Pixelhelligkeit verschiedener Milchdrüsenstrukturen gemäß dem zuvor entwickelten ultrasonographischen Atlas der sich entwickelnden bovinen Milchdrüse (USAtlasMG) beschrieben. Zusätzlich wurden Ultraschallauswertungen der Rückenfettdicke (BFT) und der Rippenfettdicke (RFT) bei 30 Kälbern (HIGH n = 16; RES n = 14) von ausschließlich pluriparen Kühen durchgeführt. Des Weiteren wurde das Körperwachstum der Kälber anhand morphometrischer Merkmale für Körpergröße, Körperlänge, Thorax und Hüfte bestimmt.

Die Fütterung von MR auf einem hohem (HIGH) oder restriktivem Niveau (RES) für 14 Wochen hatte keine Einfluss auf die Helligkeit der Gewebestrukturen in der Milchdrüse und veränderte diese auch nicht. Jedoch nahm mit fortschreitender Entwicklung von PAR, dessen Pixelhelligkeit von der 10. bis zur 20. Lebenswoche zu, d.h. PAR wurde hyperechogener, da es sich in das umgebende Gewebe (SURR) ausbreitete, was wiederum das zunehmende Wachstum von PAR unabhängig vom MR-Fütterungsregime zeigte. Sowohl BFT als auch RFT nahmen mit zunehmendem Alter zu, wobei Kälber der Gruppe HIGH ab der 8. Lebenswoche bis zum Ende des Absetzens eine größere RFT und tendenziell höhere BFT-Werte als Kälber der RES-Gruppe aufwiesen. Bei den 30 Kälbern pluriparer Kühe zeigte sich bei allen Körpermaßen, jedoch mit Ausnahme des Höhenwachstums, ein zunehmendes lineares Größenwachstum mit zunehmendem Alter. Die Kälber der Gruppe HIGH zeigten innerhalb der beobachteten 20 Lebenswochen insgesamt einen Wachstumsvorteil gegenüber den restriktiv gefütterten Kälbern der Gruppe RES.

Die Erfassung der Laktationsleistung beschränkten sich auf 23 Färsen (HIGH n = 11; RES n = 12) und die ersten 7 Wochen in ihrer ersten Laktation. Das Erstkalbealter unterschied sich nicht zwischen den beiden ehemaligen Fütterungsgruppen, aber die Kühe aus der ehemaligen Gruppe HIGH produzierten im Durchschnitt 4,6 kg/d mehr Milch als die Kühe aus der ehemaligen Gruppe RES. Dieser Unterschied konnte hauptsächlich auf zwei leistungsschwache Tiere in der RES-Gruppe zurückgeführt werden, die auch erhöhte somatische Zellzahlen (> 200.000 Zellen/mL) aufwiesen.

Zusammenfassend charakterisieren diese Studie und Dissertation die Veränderungen im oxidativen Status von Milchkälbern mit zunehmendem Alter und bestätigen die Vorteile einer hohen MR-Fütterung in Bezug auf die Entwicklung der Kälber und auf das Tierwohl. Die Fütterung von MR auf einem hohen oder einem restriktiven Niveau für 14 Wochen veränderte zwar nicht die Helligkeit der Gewebestrukturen der Milchdrüse in Ultraschallbildern, jedoch zeigte ein hohes MR-Fütterungsniveau Vorteile für das Körperwachstum in Länge und Breite

und möglicherweise auch für die Eutergesundheit in der ersten Laktation. Somit kann das Wohlbefinden von Milchfärsenkälbern durch einfache und natürlichere Aufzuchtstrategien verbessert werden. Weitere Studien sollten herausfinden, in welchem Alter die Struktur des PAR zum ersten Mal in Ultraschallbildern erscheint, mit Fokus auf dem Zeitraum zwischen der 3. und 8. Lebenswoche. Darüber hinaus könnten in Zukunft bereits bestehende Software-Analysewerkzeuge für die Ultraschallbilddiagnose, die auf der Analyse künstlicher neuronaler Netze, auch als künstliche Intelligenz (KI) bezeichnet, auf der Basis des in dieser Arbeit erstellten USAtlasMG, für Milchdrüsen-Ultraschallbilder von Kälbern adaptiert werden.

10 References

Akers, R. M. 2017. A 100-Year Review: Mammary development and lactation. J. Dairy Sci. 100(12):10332–10352. https://doi.org/10.3168/jds.2017-12983.

Albera, E., and M. Kankofer. 2011. The comparison of antioxidative/oxidative profile in blood, colostrum and milk of early post-partum cows and their newborns. Reprod. Domest. Anim. 46(5). https://doi.org/10.1111/j.1439-0531.2010.01737.x.

Alberti, A., L. Bolognini, D. Macciantelli, and M. Caratelli. 2000. The radical cation of N,N-diethyl-para-phenylendiamine: A possible indicator of oxidative stress in biological samples. Res. Chem. (26):253–267.

Albino, R. L., S. E. F. Guimarães, K. M. Daniels, M. M. S. Fontes, A. F. Machado, G. B. Dos Santos, and M. I. Marcondes. 2017. Technical note: Mammary gland ultrasonography to evaluate mammary parenchymal composition in prepubertal heifers. J. Dairy Sci. 100(2):1588–1591. https://doi.org/10.3168/jds.2016-11668.

Albino, R. L., M. I. Marcondes, R. M. Akers, E. Detmann, B. C. Carvalho, and T. E. Silva. 2015. Mammary gland development of dairy heifers fed diets containing increasing levels of metabolisable protein: metabolisable energy. J. Dairy Res. 82(1):113–120. https://doi.org/10.1017/S0022029914000697.

Aruoma, O. I. 1998. Free radicals, oxidative stress, and antioxidants in human health and disease. J. Am. Oil Chem.' Soc. 75(2):199–212. https://doi.org/10.1007/s11746-998-0032-9.

Bach, A. 2012. Ruminant Nutrition Symposium: Optimizing Performance of the Offspring: nourishing and managing the dam and postnatal calf for optimal lactation, reproduction, and immunity. J. Anim. Sci. 90(6):1835–1845. https://doi.org/10.2527/jas.2011-4516.

Baldwin, R. L., K. R. McLeod, J. L. Klotz, and R. N. Heitmann. 2004. Rumen Development, Intestinal Growth and Hepatic Metabolism In The Pre- and Postweaning Ruminant. J. Dairy Sci. 87:E55-E65. https://doi.org/10.3168/jds.S0022-0302(04)70061-2.

Ballou, M. A. 2012. Immune responses of Holstein and Jersey calves during the preweaning and immediate postweaned periods when fed varying planes of milk replacer. J. Dairy Sci. 95(12):7319–7330. https://doi.org/10.3168/jds.2012-5970.

Bar-Peled, U., B. Robinzon, E. Maltz, H. Tagari, Y. Folman, I. Bruckental, H. Voet, H. Gacitua, and A. R. Lehrer. 1997. Increased Weight Gain and Effects on Production Parameters of Holstein Heifer Calves That Were Allowed to Suckle from Birth to Six Weeks of Age. J. Dairy Sci. 80(10):2523–2528. https://doi.org/10.3168/jds.S0022-0302(97)76205-2.

Benzie, I. F., and J. J. Strain. 1996. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal. Biochem. 239(1):70–76. https://doi.org/10.1006/abio.1996.0292.

Berryhill, G. E., J. F. Trott, A. L. Derpinghaus, and R. C. Hovey. 2017. Triennial lactation symposium/Bolfa: Dietary regulation of allometric ductal growth in the mammary glands. J. Anim. Sci. 95(12):5664–5674. https://doi.org/10.2527/jas2017.1901.

Borderas, T. F., A. M. B. de Passillé, and J. Rushen. 2009. Feeding behavior of calves fed small or large amounts of milk. J. Dairy Sci. 92(6):2843–2852. https://doi.org/10.3168/jds.2008-1886.

Bordignon, R., A. Volpato, P. Glombowsky, C. F. Souza, M. D. Baldissera, R. Secco, W. A. B. Pereira, M. L. R. Leal, M. Vedovatto, and A. S. Da Silva. 2019. Nutraceutical effect of vitamins and minerals on performance and immune and antioxidant systems in dairy calves during the nutritional transition period in summer. J. Therm. Biol. 84:451–459. https://doi.org/10.1016/j.jtherbio.2019.07.034.

Bracke, M. B. M., and H. Hopster. 2006. Assessing the Importance of Natural Behavior for Animal Welfare. J. Agric. Environ. Ethics 19(1):77–89. https://doi.org/10.1007/s10806-005-4493-7.

Briviba, K., B. Heinzow, and M. Schwenk. 2008. Oxidativer Stress und Möglichkeiten seiner Messung aus umweltmedizinischer Sicht: Mitteilung der Kommission "Methoden und Qualitätssicherung in der Umweltmedizin". Bundesgesundheitsblatt, Gesundheitsforschung, Gesundheitsschutz 51(12):1464–1482. https://doi.org/10.1007/s00103-008-0720-5.

Brown, E. G., M. J. VandeHaar, K. M. Daniels, J. S. Liesman, L. T. Chapin, J. W. Forrest, R. M. Akers, R. E. Pearson, and M. W. Nielsen. 2005. Effect of Increasing Energy and Protein Intake on Mammary Development in Heifer Calves. J. Dairy Sci. 88(2):595–603. https://doi.org/10.3168/jds.S0022-0302(05)72723-5.

Bruckmaier, R. M., and J. W. Blum. 1992. B-mode ultrasonography of mammary glands of cows, goats and sheep during alpha- and beta-adrenergic agonist and oxytocin administration. J. Dairy Res. 59(2):151–159. https://doi.org/10.1017/s0022029900030399.

Buddecke, E. 1994. Grundriß der Biochemie: F R Studierende Der Medizin, Zahnmedizin Und Naturwissenschaften. De Gruyter, Berlin.

Capuco, A. V., and R. M. Akers. 2010. Management and Environmental Influences on Mammary Gland Development and Milk Production. p. 259–292. *In* P. L. Greenwood, A. W. Bell, P. E. Vercoe, and G. J. Viljoen (eds.). Managing the Prenatal Environment to Enhance Livestock Productivity. Springer Netherlands, s.l.

Cartee, R. E., A. K. Ibrahim, and D. McLeary. 1986. B-mode ultrasonography of the bovine udder and teat. J. Am. Vet. Med. Assoc. 188(11):1284–1287.

Celi, P. 2011. Biomarkers of oxidative stress in ruminant medicine. Immunopharmacol. Immunotoxicol. 33(2):233–240. https://doi.org/10.3109/08923973.2010.514917.

Chester-Jones, H., B. J. Heins, D. Ziegler, D. Schimek, S. Schuling, B. Ziegler, M. B. de Ondarza, C. J. Sniffen, and N. Broadwater. 2017. Relationships between early-life growth, intake, and birth season with first-lactation performance of Holstein dairy cows. J. Dairy Sci. 100(5):3697–3704. https://doi.org/10.3168/jds.2016-12229.

Ciampi, F., L. M. Sordillo, J. C. Gandy, M. Caroprese, A. Sevi, M. Albenzio, and A. Santillo. 2020. Evaluation of natural plant extracts as antioxidants in a bovine in vitro model of oxidative stress. J. Dairy Sci. 103(10):8938–8947. https://doi.org/10.3168/jds.2020-18182.

Costa, J. H. C., M. A. G. von Keyserlingk, and D. M. Weary. 2016. Invited review: Effects of group housing of dairy calves on behavior, cognition, performance, and health. J. Dairy Sci. 99(4):2453–2467. https://doi.org/10.3168/jds.2015-10144.

Costantini, D. 2019. Understanding diversity in oxidative status and oxidative stress: the opportunities and challenges ahead. J. Exp. Biol. 222(Pt 13). https://doi.org/10.1242/jeb.194688.

Davis Rincker, L. E., M. J. VandeHaar, C. A. Wolf, J. S. Liesman, L. T. Chapin, and M. S. Weber Nielsen. 2011. Effect of intensified feeding of heifer calves on growth, pubertal age, calving age, milk yield, and economics. J. Dairy Sci. 94(7):3554–3567. https://doi.org/10.3168/jds.2010-3923.

Delorme, S., and J. Debus. 2005. Sonographie. 2., vollst. überarb. und erw. Aufl. Duale Reihe. Thieme, Stuttgart.

Diao, Q., R. Zhang, and T. Fu. 2019. Review of Strategies to Promote Rumen Development in Calves. Animals 9(8). https://doi.org/10.3390/ani9080490.

Donovan, G., I. R. Dohoo, D. M. Montgomery, and F. L. Bennett. 1998. Calf and disease factors affecting growth in female holstein calves in Florida, USA. Prev. Vet. Med. 33(1-4):1–10. https://doi.org/10.1016/S0167-5877(97)00059-7.

Drackley, J. K. 2008. Calf nutrition from birth to breeding. Vet. Clin. North Am. Food Anim. Pract. 24(1):55–86. https://doi.org/10.1016/j.cvfa.2008.01.001.

Eckert, E., H. E. Brown, K. E. Leslie, T. J. DeVries, and M. A. Steele. 2015. Weaning age affects growth, feed intake, gastrointestinal development, and behavior in Holstein calves fed an elevated plane of nutrition during the preweaning stage. J. Dairy Sci. 98(9):6315–6326. https://doi.org/10.3168/jds.2014-9062.

Esselburn, K. M., T. M. Hill, H. G. Bateman, F. L. Fluharty, S. J. Moeller, K. M. O'Diam, and K. M. Daniels. 2015. Examination of weekly mammary parenchymal area by ultrasound, mammary mass, and composition in Holstein heifers reared on 1 of 3 diets from birth to 2 months of age. J. Dairy Sci. 98(8):5280–5293. https://doi.org/10.3168/jds.2014-9061.

Franz, S., M. Floek, and M. Hofmann-Parisot. 2009. Ultrasonography of the Bovine Udder and Teat. Vet. Clin. North Am. Food Anim. Pract 25(3):669–685. https://doi.org/10.1016/j.cvfa.2009.07.007.

Frieten, D., C. Gerbert, C. Koch, G. Dusel, K. Eder, E. Kanitz, J. M. Weitzel, and H. M. Hammon. 2017. Ad libitum milk replacer feeding, but not butyrate supplementation, affects growth performance as well as metabolic and endocrine traits in Holstein calves. J. Dairy Sci. 100(8):6648–6661. https://doi.org/10.3168/jds.2017-12722.

Furini, P. M., R. A. Azevedo, S. R. A. Rufino, F. S. Machado, M. M. Campos, L. G. R. Pereira, T. R. Tomich, B. C. Carvalho, G. B. Santos, and S. G. Coelho. 2018. The effects of increasing amounts of milk replacer powder added to whole milk on mammary gland measurements using ultrasound in dairy heifers. J. Dairy Sci. 101(1):767–773. https://doi.org/10.3168/jds.2017-12798.

Gaál, T., P. Ribiczeyné-Szabó, K. Stadler, J. Jakus, J. Reiczigel, P. Kövér, M. Mézes, and L. Sümeghy. 2006. Free radicals, lipid peroxidation and the antioxidant system in the blood of cows and newborn calves around calving. Comparative biochemistry and physiology. Part B, Biochem. Mol. Biol. 143(4):391–396. https://doi.org/10.1016/j.cbpb.2005.12.014.

Geiger, A. J. 2019. Review: The pre-pubertal bovine mammary gland: unlocking the potential of the future herd. Animal 13(S1):s4-s10. https://doi.org/10.1017/S1751731119001204.

Geiger, A. J., C. L. M. Parsons, R. E. James, and R. M. Akers. 2016. Growth, intake, and health of Holstein heifer calves fed an enhanced preweaning diet with or without postweaning exogenous estrogen. J. Dairy Sci. 99(5):3995–4004. https://doi.org/10.3168/jds.2015-10405.

Gerbert, C., D. Frieten, C. Koch, G. Dusel, K. Eder, T. Stefaniak, J. Bajzert, P. Jawor, A. Tuchscherer, and H. M. Hammon. 2018. Effects of ad libitum milk replacer feeding and butyrate supplementation on behavior, immune status, and health of Holstein calves in the postnatal period. J. Dairy Sci. 101(8):7348–7360. https://doi.org/10.3168/jds.2018-14542.

Goldenberg, S. L., G. Nir, and S. E. Salcudean. 2019. A new era: artificial intelligence and machine learning in prostate cancer. Nature Reviews. Urology 16(7):391–403. https://doi.org/10.1038/s41585-019-0193-3.

Gutteridge, J. M., and B. Halliwell. 1988. The deoxyribose assay: an assay both for 'free' hydroxyl radical and for site-specific hydroxyl radical production. Biochem. J. 253(3):932–933. https://doi.org/10.1042/bj2530932.

Heinrichs, A. J., and B. S. Heinrichs. 2011. A prospective study of calf factors affecting first-lactation and lifetime milk production and age of cows when removed from the herd. J. Dairy Sci. 94(1):336–341. https://doi.org/10.3168/jds.2010-3170.

Hornick, J., C. van Eenaeme, O. Gérard, I. Dufrasne, and L. Istasse. 2000. Mechanisms of reduced and compensatory growth. Domest. Anim. Endocrinol. 19(2):121–132. https://doi.org/10.1016/S0739-7240(00)00072-2.

Hovey, R. C., and L. Aimo. 2010. Diverse and active roles for adipocytes during mammary gland growth and function. J. Mammary Gland Biol. Neoplasia 15(3):279–290. https://doi.org/10.1007/s10911-010-9187-8.

Hovey, R. C., T. B. McFadden, and R. M. Akers. 1999. Regulation of mammary gland growth and morphogenesis by the mammary fat pad: a species comparison. J. Mammary Gland Biol. Neoplasia 4(1):53–68. https://doi.org/10.1023/a:1018704603426.

Howard, B. A., and B. A. Gusterson. 2000. Human breast development. J. Mammary Gland Biol. Neoplasia 5(2):119–137. https://doi.org/10.1023/a:1026487120779.

Huber, J. T. 1969. Development of the Digestive and Metabolic Apparatus of the Calf. J. Dairy Sci. 52(8):1303–1315. https://doi.org/10.3168/jds.S0022-0302(69)86744-5.

Huber, K. 2018. Invited review: resource allocation mismatch as pathway to disproportionate growth in farm animals - prerequisite for a disturbed health. Animal 12(3):528–536. https://doi.org/10.1017/S1751731117002051.

Hulbert, L. E., C. J. Cobb, J. A. Carroll, and M. A. Ballou. 2011. The effects of early weaning on innate immune responses of Holstein calves. J. Dairy Sci. 94(5):2545–2556. https://doi.org/10.3168/jds.2010-3983.

Hulbert, L. E., and S. J. Moisá. 2016. Stress, immunity, and the management of calves. J. Dairy Sci. 99(4):3199–3216. https://doi.org/10.3168/jds.2015-10198.

Jasper, J., and D. M. Weary. 2002. Effects of Ad Libitum Milk Intake on Dairy Calves. J. Dairy Sci. 85(11):3054–3058. https://doi.org/10.3168/jds.S0022-0302(02)74391-9.

Johnsen, J. F., K. A. Zipp, T. Kälber, A. M. de Passillé, U. Knierim, K. Barth, and C. M. Mejdell. 2016. Is rearing calves with the dam a feasible option for dairy farms?—Current and future research. Appl. Anim. Behav. Sci. 181:1–11. https://doi.org/10.1016/j.applanim.2015.11.011.

Kaushal, G. P., K. Chandrashekar, and L. A. Juncos. 2019. Molecular Interactions Between Reactive Oxygen Species and Autophagy in Kidney Disease. Int. J. Mol. Sci. 20(15). https://doi.org/10.3390/ijms20153791.

Kenéz, Á., C. Koch, M. Korst, J. Kesser, K. Eder, H. Sauerwein, and K. Huber. 2018. Different milk feeding intensities during the first 4 weeks of rearing dairy calves: Part 3: Plasma metabolomics analysis reveals long-term metabolic imprinting in Holstein heifers. J. Dairy Sci. 101(9):8446–8460. https://doi.org/10.3168/jds.2018-14559.

Kertz, A. F., T. M. Hill, J. D. Quigley, A. J. Heinrichs, J. G. Linn, and J. K. Drackley. 2017. A 100-Year Review: Calf nutrition and management. J. Dairy Sci. 100(12):10151–10172. https://doi.org/10.3168/jds.2017-13062.

Kesser, J., M. Korst, C. Koch, F.-J. Romberg, J. Rehage, U. Müller, M. Schmicke, K. Eder, H. M. Hammon, H. Sadri, and H. Sauerwein. 2017. Different milk feeding intensities during the first 4 weeks of rearing dairy calves: Part 2: Effects on the metabolic and endocrine status during calfhood and around the first lactation. J. Dairy Sci. 100(4):3109–3125. https://doi.org/10.3168/jds.2016-11595.

Khan, M. A., A. Bach, D. M. Weary, and M. A. G. von Keyserlingk. 2016. Invited review: Transitioning from milk to solid feed in dairy heifers. J. Dairy Sci. 99(2):885–902. https://doi.org/10.3168/jds.2015-9975.

Khan, M. A., D. M. Weary, and M. A. G. von Keyserlingk. 2011. Invited review: effects of milk ration on solid feed intake, weaning, and performance in dairy heifers. J. Dairy Sci. 94(3):1071–1081. https://doi.org/10.3168/jds.2010-3733.

Kiezebrink, D. J., A. M. Edwards, T. C. Wright, J. P. Cant, and V. R. Osborne. 2015. Effect of enhanced whole-milk feeding in calves on subsequent first-lactation performance. J. Dairy Sci. 98(1):349–356. https://doi.org/10.3168/jds.2014-7959.

Kirchgeßner, M., G. I. Stangl, F. J. Schwarz, F. X. Roth, K.-H. Südekum, and K. Eder. 2014. Tierernährung: Leitfaden für Studium, Beratung und Praxis. 14., aktualisierte Aufl. DLG-Verl., Frankfurt am Main.

Klein, R. D., R. L. Kincaid, A. S. Hodgson, J. H. Harrison, J. K. Hillers, and J. D. Cronrath. 1987. Dietary Fiber and Early Weaning on Growth and Rumen Development of Calves. J. Dairy Sci. 70(10):2095–2104. https://doi.org/10.3168/jds.S0022-0302(87)80259-X.

Korst, M., C. Koch, J. Kesser, U. Müller, F.-J. Romberg, J. Rehage, K. Eder, and H. Sauerwein. 2017. Different milk feeding intensities during the first 4 weeks of rearing in dairy calves: Part 1: Effects on performance and production from birth over the first lactation. J. Dairy Sci. 100(4):3096–3108. https://doi.org/10.3168/jds.2016-11594.

Kressin, M., R. Brehm, and B. Schnorr. 2019. Embryologie der Haustiere. 7., völlig überarbeitete Auflage. Georg Thieme Verlag, Stuttgart.

Lawrence, T. L., V. R. Fowler, and J. E. Novakofski. 2012. Growth of farm animals. 3. ed. CABI Publ, Wallingford.

Leeuwenburgh, C., and J. W. Heinecke. 2001. Oxidative stress and antioxidants in exercise. Curr. Med. Chem. 8(7):829–838. https://doi.org/10.2174/0929867013372896.

Lohakare, J. D., K.-H. Südekum, and A. K. Pattanaik. 2012. Nutrition-induced Changes of Growth from Birth to First Calving and Its Impact on Mammary Development and First-lactation Milk Yield in Dairy Heifers: A Review. Asian Australas. J. Anim. Sci. 25(9):1338–1350. https://doi.org/10.5713/ajas.2012.12282.

Macias, H., and L. Hinck. 2012. Mammary gland development. Wiley interdisciplinary reviews. Dev. Biol. 1(4):533–557. https://doi.org/10.1002/wdev.35.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006a. Developmental and Nutritional Regulation of the Prepubertal Bovine Mammary Gland: II. Epithelial Cell Proliferation, Parenchymal Accretion Rate, and Allometric Growth. J. Dairy Sci. 89(11):4298–4304. https://doi.org/10.3168/jds.S0022-0302(06)72476-6.

Meyer, M. J., A. V. Capuco, D. A. Ross, L. M. Lintault, and M. E. van Amburgh. 2006b. Developmental and Nutritional Regulation of the Prepubertal Heifer Mammary Gland: I. Parenchyma and Fat Pad Mass and Composition. J. Dairy Sci. 89(11):4289–4297. https://doi.org/10.3168/jds.S0022-0302(06)72475-4.

Miller, J. K., E. Brzezinska-Slebodzinska, and F. C. Madsen. 1993. Oxidative Stress, Antioxidants, and Animal Function. J. Dairy Sci. 76(9):2812–2823. https://doi.org/10.3168/jds.S0022-0302(93)77620-1.

Miller-Cushon, E. K., R. Bergeron, K. E. Leslie, and T. J. DeVries. 2013. Effect of milk feeding level on development of feeding behavior in dairy calves. J. Dairy Sci. 96(1):551–564. https://doi.org/10.3168/jds.2012-5937.

Mutinati, M., M. Pantaleo, M. Roncetti, M. Piccinno, A. Rizzo, and R. L. Sciorsci. 2014. Oxidative stress in neonatology: a review. Reprod. Domest. Anim. 49(1):7–16. https://doi.org/10.1111/rda.12230.

Neave, H. W., J. H. C. Costa, J. B. Benetton, D. M. Weary, and M. A. G. von Keyserlingk. 2019. Individual characteristics in early life relate to variability in weaning age, feeding behavior, and weight gain of dairy calves automatically weaned based on solid feed intake. J. Dairy Sci. 102(11):10250–10265. https://doi.org/10.3168/jds.2019-16438.

Nishimura, M., T. Yoshida, S. El-Khodery, M. Miyoshi, H. Furuoka, J. Yasuda, and K. Miyahara. 2011. Ultrasound imaging of mammary glands in dairy heifers at different stages of growth. J. Vet. Med. Sci. 73(1):19–24. https://doi.org/10.1292/jvms.09-0503.

Noya, A., I. Casasús, J. Ferrer, and A. Sanz. 2019. Effects of Developmental Programming Caused by Maternal Nutrient Intake on Postnatal Performance of Beef Heifers and Their Calves. Animals 9(12). https://doi.org/10.3390/ani9121072.

Obeidat, B. S., C. J. Cobb, M. D. Sellers, A. R. Pepper-Yowell, T. J. Earleywine, and M. A. Ballou. 2013. Plane of nutrition during the preweaning period but not the grower phase influences the neutrophil activity of Holstein calves. J. Dairy Sci. 96(11):7155–7166. https://doi.org/10.3168/jds.2013-6699.

Omidi-Mirzaei, H., M. Khorvash, G. R. Ghorbani, B. Moshiri, M. Mirzaei, A. Pezeshki, and M. H. Ghaffari. 2015. Effects of the step-up/step-down and step-down milk feeding procedures on the performance, structural growth, and blood metabolites of Holstein dairy calves. J. Dairy Sci. 98(11):7975–7981. https://doi.org/10.3168/jds.2014-9260.

Paglia, D. E., and W. N. Valentine. 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. J. Lab. Clin. Med. 70(1):158–169.

Passillé, A. M. de, F. Borderas, and J. Rushen. 2011. Cross-sucking by dairy calves may become a habit or reflect characteristics of individual calves more than milk allowance or weaning. Appl. Anim. Behav. Sci. 133(3-4):137–143. https://doi.org/10.1016/j.applanim. 2011.04.020.

Passillé, A. M. de, and J. Rushen. 2012. Adjusting the weaning age of calves fed by automated feeders according to individual intakes of solid feed. J. Dairy Sci. 95(9):5292–5298. https://doi.org/10.3168/jds.2012-5521.

Paula Vieira, A. de, V. Guesdon, A. M. de Passillé, M. A. G. von Keyserlingk, and D. M. Weary. 2008. Behavioural indicators of hunger in dairy calves. Appl. Anim. Behav. Sci. 109(2-4):180–189. https://doi.org/10.1016/j.applanim.2007.03.006.

Penninck, D., and M.-A. d'Anjou. 2015. Atlas of Small Animal Ultrasonography. 2nd ed. Wiley, Somerset.

Przybylska, J., E. Albera, and M. Kankofer. 2007. Antioxidants in bovine colostrum. Reprod. Domest. Anim. 42(4):402–409. https://doi.org/10.1111/j.1439-0531.2006.00799.x.

Radcliff, R. P., M. J. VandeHaar, L. T. Chapin, T. E. Pilbeam, D. K. Beede, E. P. Stanisiewski, and H. A. Tucker. 2000. Effects of Diet and Injection of Bovine Somatotropin on Prepubertal Growth and First-Lactation Milk Yields of Holstein Cows. J. Dairy Sci. 83(1):23–29. https://doi.org/10.3168/jds.S0022-0302(00)74850-8.

Raeth-Knight, M., H. Chester-Jones, S. Hayes, J. Linn, R. Larson, D. Ziegler, B. Ziegler, and N. Broadwater. 2009. Impact of conventional or intensive milk replacer programs on Holstein heifer performance through six months of age and during first lactation. J. Dairy Sci. 92(2):799–809. https://doi.org/10.3168/jds.2008-1470.

Ranade, R., S. Talukder, G. Muscatello, and P. Celi. 2014. Assessment of oxidative stress biomarkers in exhaled breath condensate and blood of dairy heifer calves from birth to weaning. Vet. J. 202(3):583–587. https://doi.org/10.1016/j.tvjl.2014.10.025.

Rauw, W. M. 2008. Resource allocation theory applied to farm animal production. CABI, Wallingford, UK, Cambridge, MA.

Reinhardt, V., and A. Reinhardt. 1981. Natural sucking performance and age of weaning in zebu cattle (Bos indicus). J. Agric. Sci. 96(2):309–312. https://doi.org/10.1017/S0021859600066089.

Richert, M. M., K. L. Schwertfeger, J. W. Ryder, and S. M. Anderson. 2000. An atlas of mouse mammary gland development. J. Mammary Gland Biol. Neoplasia 5(2):227–241. https://doi.org/10.1023/A:1026499523505.

Rosenberger, K., J. H. C. Costa, H. W. Neave, M. A. G. von Keyserlingk, and D. M. Weary. 2017. The effect of milk allowance on behavior and weight gains in dairy calves. J. Dairy Sci. 100(1):504–512. https://doi.org/10.3168/jds.2016-11195.

Roth, B. A., N. M. Keil, L. Gygax, and E. Hillmann. 2009. Influence of weaning method on health status and rumen development in dairy calves. J. Dairy Sci. 92(2):645–656. https://doi.org/10.3168/jds.2008-1153.

Rowson, A. R., K. M. Daniels, S. E. Ellis, and R. C. Hovey. 2012. Growth and development of the mammary glands of livestock: a veritable barnyard of opportunities. Semin. Cell Dev. Biol. 23(5). https://doi.org/10.1016/j.semcdb.2012.03.018.

Rushen, J., N. Chapinal, and am de Passillé. 2012. Automated monitoring of behavioural-based animal welfare indicators. Anim. Welf. 21(3):339–350. https://doi.org/10.7120/09627286.21.3.339.

Saugstad, O. D. 1990. Oxygen toxicity in the neonatal period. Acta Paediatr. Scandin. 79(10):881–892. https://doi.org/10.1111/j.1651-2227.1990.tb11348.x.

Saugstad, O. D. 1996. Mechanisms of tissue injury by oxygen radicals: implications for neonatal disease. Acta Paediatr. Scandin. 85(1):1–4. https://doi.org/10.1111/j.1651-2227.1996.tb13880.x.

Sejrsen, K., J. T. Huber, H. A. Tucker, and R. M. Akers. 1982. Influence of Nutrition on Mammary Development in Pre- and Postpubertal Heifers. J. Dairy Sci. 65(5):793–800. https://doi.org/10.3168/jds.S0022-0302(82)82268-6.

Sies, H. 1985. Oxidative Stress. Elsevier Science, Burlington.

Silper, B. F., A. M. Q. Lana, A. U. Carvalho, C. S. Ferreira, A. P. S. Franzoni, J. A. M. Lima, H. M. Saturnino, R. B. Reis, and S. G. Coelho. 2014. Effects of milk replacer feeding strategies on performance, ruminal development, and metabolism of dairy calves. J. Dairy Sci. 97(2):1016–1025. https://doi.org/10.3168/jds.2013-7201.

Silva, A. L., E. Detmann, J. Dijkstra, A. M. Pedroso, L. H. P. Silva, A. F. Machado, F. C. Sousa, G. B. Dos Santos, and M. I. Marcondes. 2018. Effects of rumen-undegradable protein on intake, performance, and mammary gland development in prepubertal and pubertal dairy heifers. J. Dairy Sci. 101(7):5991–6001. https://doi.org/10.3168/jds.2017-13230.

Soberon, F., E. Raffrenato, R. W. Everett, and M. E. van Amburgh. 2012. Preweaning milk replacer intake and effects on long-term productivity of dairy calves. J. Dairy Sci. 95(2):783–793. https://doi.org/10.3168/jds.2011-4391.

Soberon, F., and M. E. van Amburgh. 2013. Lactation Biology Symposium: The effect of nutrient intake from milk or milk replacer of preweaned dairy calves on lactation milk yield as adults: a meta-analysis of current data. J. Anim. Sci. 91(2):706–712. https://doi.org/10.2527/jas.2012-5834.

Soberon, F., and M. E. van Amburgh. 2017. Effects of preweaning nutrient intake in the developing mammary parenchymal tissue. J. Dairy Sci. 100(6):4996–5004. https://doi.org/10.3168/jds.2016-11826.

Sordillo, L. M. 2016. Nutritional strategies to optimize dairy cattle immunity1. J. Dairy Sci. 99(6):4967–4982. https://doi.org/10.3168/jds.2015-10354.

Sordillo, L. M., and S. L. Aitken. 2009. Impact of oxidative stress on the health and immune function of dairy cattle. Vet. Immunol. Immunopathol. 128(1-3):104–109. https://doi.org/10.1016/j.vetimm.2008.10.305.

Steele, M. A., G. B. Penner, F. Chaucheyras-Durand, and L. Le Guan. 2016. Development and physiology of the rumen and the lower gut: Targets for improving gut health. J. Dairy Sci. 99(6):4955–4966. https://doi.org/10.3168/jds.2015-10351.

Suarez-Ibarrola, R., S. Hein, G. Reis, C. Gratzke, and A. Miernik. 2020. Current and future applications of machine and deep learning in urology: a review of the literature on urolithiasis, renal cell carcinoma, and bladder and prostate cancer. World J. Urol. 38(10):2329–2347. https://doi.org/10.1007/s00345-019-03000-5.

Sutherland, M. A., G. L. Lowe, F. J. Huddart, J. R. Waas, and M. Stewart. 2018. Measurement of dairy calf behavior prior to onset of clinical disease and in response to disbudding using automated calf feeders and accelerometers. J. Dairy Sci. 101(9):8208–8216. https://doi.org/10.3168/jds.2017-14207.

Svensson, C., and M. B. Jensen. 2007. Short Communication: Identification of Diseased Calves by Use of Data from Automatic Milk Feeders. J. Dairy Sci. 90(2):994–997. https://doi.org/10.3168/jds.S0022-0302(07)71584-9.

Svensson, C., A.-K. Nyman, K. P. Waller, and U. Emanuelson. 2006. Effects of Housing, Management, and Health of Dairy Heifers on First-Lactation Udder Health in Southwest Sweden. J. Dairy Sci. 89(6):1990–1999. https://doi.org/10.3168/jds.s0022-0302(06)72266-4.

Swanson, E. W., and J. I. Poffenbarger. 1979. Mammary Gland Development of Dairy Heifers during Their First Gestation. J. Dairy Sci. 62(5):702–714. https://doi.org/10.3168/jds.S0022-0302(79)83313-5.

Sweeney, B. C., J. Rushen, D. M. Weary, and A. M. de Passillé. 2010. Duration of weaning, starter intake, and weight gain of dairy calves fed large amounts of milk. J. Dairy Sci. 93(1):148–152. https://doi.org/10.3168/jds.2009-2427.

Tucker, H. A. 1987. Quantitative Estimates of Mammary Growth During Various Physiological States: A Review. J. Dairy Sci. 70(9):1958–1966. https://doi.org/10.3168/jds.S0022-0302(87)80238-2.

Vannucchi, C. I., L. G. Silva, C. F. Lúcio, and G. A. L. Veiga. 2019. Oxidative stress and acid-base balance during the transition period of neonatal Holstein calves submitted to different calving times and obstetric assistance. J. Dairy Sci. 102(2):1542–1550. https://doi.org/10.3168/jds.2018-14754.

Webster, J. 1997. Animal welfare: A cool eye towards Eden. Blackwell Science, Oxford.

Witko-Sarsat, V., M. Friedlander, C. Capeillère-Blandin, T. Nguyen-Khoa, A. T. Nguyen, J. Zingraff, P. Jungers, and B. Descamps-Latscha. 1996. Advanced oxidation protein products as a novel marker of oxidative stress in uremia. Kidney Int. 49(5):1304–1313. https://doi.org/10.1038/ki.1996.186.

Zanton, G. I., and A. J. Heinrichs. 2005. Meta-Analysis to Assess Effect of Prepubertal Average Daily Gain of Holstein Heifers on First-Lactation Production. J. Dairy Sci. 88(11):3860–3867. https://doi.org/10.3168/jds.S0022-0302(05)73071-X.

11 Acknowledgments/Danksagungen

An dieser Stelle möchte ich mich herzlich bei all denjenigen bedanken, die mich während meiner Promotion unterstützt und begleitet haben.

Ein ganz besonderer Dank gebührt Frau Prof. Dr. Dr. Helga Sauerwein für Ihre freundliche und passionierte Betreuung, Unterstützung und die Möglichkeit in der Tier-Physiologie zu promovieren. Vielen Dank, dass Sie mir dieses außergewöhnliche Promotions-Projekt mit „Kälbern und Co." anvertraut haben. Grazie mille!

Bei Herrn Prof. Dr. Karl-Heinz Südekum möchte ich mich herzlich für die Übernahme des Korreferats bedanken. Den überaus interessanten Austausch während meiner Statusgespräche mit Ihnen und Frau Prof. Sauerwein habe ich sehr geschätzt.

Herrn Dr. Morteza Hosseini-Ghaffari danke ich sehr für den wissenschaftlichen Austausch und die vielen fachlichen Gespräche.

Vielen Dank an alle Kollegen der Abteilung Physiologie des Instituts für Tierwissenschaften, insbesondere Inga Hofs und Isabella Israel, die mich seit meiner Masterarbeit unterstützt, motiviert und mir mit großer Freundlichkeit so viel im Labor gezeigt und beigebracht haben.

Herrn Prof. Dr. Fabrizio Ceciliani danke ich herzlich für die Unterstützung und die Möglichkeit während meines ERASMUS-Aufenthalts in Mailand die Grundlagen der Laborarbeit zu erlernen, welche auch für die Arbeit während meiner Promotion essentiell waren. Grazie mille!

Ein großes Dankeschön gilt Herrn Dr. Christian Koch und Frau Dr. Theresa Scheu von der Lehr- und Versuchsanstalt Hofgut Neumühle, die mit mir bei Wind und Wetter und mit großem Einsatz den Versuch durchgeführt haben.

Vielen herzlichen Dank an das großartige Hofgut Neumühle-Team, meine großartigen Kollegen und Freunde für eure Unterstützung, eure Hilfe und die unvergesslich schöne und arbeitsreiche Zeit „im Herzen der Pfalz". Insbesondere danke ich Anke Brubacher, Elisabeth Therre, Anna Schönborn, Sophie Jobelius nun Rock, Ricarda Günter, Klaus Korst, Daniel Karch, Ewald Kröger und den Doktoranden Dr. Dorotheé Lang, Veronika Drexl, Sonja Bässler und Michael Berger. Ihr seid großartige Kollegen, Freunde und wart eine klasse WG.

Ich bedanke mich von ganzem Herzen bei all meinen Freunden, die an mich glauben, mir Mut geben und Freude teilen, die für mich da sind und mir soviel geben: Dankeschön, Dr. Harald Mayer und Dr. Ulrike Mayer, meine Mailand, ERASMUS und große Italien-Freundin Nina Häberle, sowie mein Kommilitone Matthias Klemm.

Meine lieben Eltern, ich danke Euch von ganzem Herzen für Eure großzügige Liebe, Unterstützung und Geduld. Danke, dass Ihr mir ermöglicht habt meinen Weg zu gehen. Meinen Großeltern habe ich ebenfalls so viel zu verdanken: Opa Hans Widdenhöfer hat mir als Kind Liebe, Geduld und Respekt gegenüber den Tieren vermittelt.

Caro Dennis, Grazie mille! Dankeschön, dass du mich unterstützt und begleitest. Wer wenn nicht du versteht die genauen Mechanismen der Balance sowohl zwischen oxidativen und antioxidativen Prozessen als auch zwischen Promotion und italienischem „dolce vita". Dennis Familie, euch allen, danke ich ebenfalls für die schöne ausgleichende Zeit währenddessen.

Nicht zu vergessen ist ein großer Dank an alle Kälber und deren Mütter, welche im Versuch involviert waren und mit uns kooperiert haben. Auch meinen tierischen Freunden, die mich begleiten, gilt ein großes Dankeschön.

Außerdem bedanke ich mich bei der H. W. Schaumann Stiftung für die finanzielle Unterstützung meiner Promotion.

12 Publications derived from this doctorate thesis

1. **Seibt, K. D.**, T. Scheu, C. Koch, M. H. Ghaffari, and H. Sauerwein. **2021**. Effects of different levels of milk replacer for 14 weeks on mammary gland development in heifers. Proceedings of the 72th Annual Meeting of the European Federation of Animal Science (EAAP). Book of abstracts, p. 555.
2. **Seibt, K. D.**, M. H. Ghaffari, T. Scheu, C. Koch, and H. Sauerwein. **2021**. Characteristics of the oxidative status in dairy calves fed at different milk replacer levels and weaned at 14 weeks of age. Antioxidants, 10, 260. https://doi.org/10.3390/antiox 10020260.
3. **Seibt, K. D.**, M. H. Ghaffari, T. Scheu, C. Koch, and H. Sauerwein. **2021**. Effects of milk replacer allowance on growth performance and oxidative status of dairy heifer calves during an extended preweaning period of 14 weeks. Proceedings of the 75th Annual Meeting of the German Society of Nutrition Physiology (Gesellschaft für Ernährungsphysiologie, GfE). Volume 30, p. 84.
4. **Seibt, K.D.** and the MitoCow consortium[1]. **2020**. MitoCow - Effects of systemic inflammation on telomere length and mitochondrial DNA copy numbers. Proceedings of the 71th Annual Meeting of the EAAP. Book of abstracts, p. 267.
5. **Seibt, K.D.** and the MitoCow consortium[1]. **2020**. MitoCow - Effects of systemic inflammation on the ratio of leukocytes & mitochondrial DNA copy number. Proceedings of the 71th Annual Meeting of the EAAP. Book of abstracts, p. 275.
6. **Seibt, K.D.** and the MitoCow consortium[1]. **2020**. Effects of systemic inflammation on mitochondrial DNA copy number in leukocytes of dairy cows. Proceedings of the 74th Annual Meeting of the GfE. Volume 29, p. 126.

[1] MitoCow is a DFG-funded cooperative project (project number 202989534) on mitochondrial functionality in dairy cows. The MitoCow consortium is formed by Profes. S. Dänicke, K. Huber, H. Sauerwein, and J. Seifert as the principal investigators (https://gepris.dfg.de/gepris/projekt/202989534) and Jennifer Meyer, Susanne Ursula Daniels, Sandra Grindler, Johanna Tröscher-Mußotter, and Mohamadtaher Alaedin as doctorate students.