SERUM CONCENTRATIONS OF GLOBULINS, ALBUMIN AND SERUM AMYLOID A OF NEONATAL LAMBS AND ASSOCIATIONS WITH WEIGHT GAIN DURING SUMMER REARING PERIOD

TALLEDE SEERUMI GLOBULIINIDE, ALBUMIINI JA SEERUMI AMÜLOID A SISALDUS NEONATAALPERIOODIL JA SEOS TALLEDE KAALUIIBEGA SUVISEL KASVUPERIOODIL

Graduation Thesis in Veterinary Medicine
Curriculum of Veterinary Medicine

Supervisors: Professor Toomas Orro, PhD
Kristel Peetsalu, DVM

Tartu 2015
SHORT SUMMARY

The neonatal period is the most vulnerable time in the life of a lamb. The physiological adaptation period to extra uterine life plays a significant role in the survival and growth of the lamb. The present study was undertaken to investigate certain biochemical aspects and their relations with growth rate over the first months of life in neonatal lambs.

In the present study serum samples were collected from total of 322 lambs during the summer rearing period 2011 and 2012 at a sheep farm in southern Estonia. Total of 524 serum samples were analysed by measuring the concentration of albumin, globulins and serum amyloid A (SAA) and gamma-glutamyl transferase (GGT) enzyme activity. Automated colorimetric analysing methods were used in biochemical analysis of albumin, total protein and GGT. The concentration of globulins was calculated by subtracting albumin concentration from total protein concentration. Concentration of SAA was measured by ELISA-method.

According to the outcome of present study there is positive correlation between serum concentrations of albumin and globulins from one-week-old lambs and weight gain during summer rearing period. Negative correlation was found between serum SAA concentration in one-week-old lambs and weight gain during summer rearing period. Enzyme GGT activity in serum did not correlate with weight gain.

Keywords: albumin, globulins, GGT, SAA, lambs, weight gain
TABLE OF CONTENTS

1. INTRODUCTION ........................................................................................................... 5

2. LITERATURE REVIEW ............................................................................................... 6
   2.1. Neonatal period in lambs .................................................................................... 6
       2.1.1. Hypothermia, body weight and energy reserves ................................. 6
       2.1.2. Suckling ............................................................................................... 7
       2.1.3. Colostrum composition........................................................................ 8
       2.1.4. Pathogens ............................................................................................. 8
   2.2. Role of globulins during the neonatal period ..................................................... 9
       2.2.1. Colostrum Immunoglobulins ............................................................. 10
   2.3. Albumin during neonatal period ....................................................................... 11
   2.4. Gamma-glutamyl transeferase (GGT) .............................................................. 12
   2.5. Acute phase reaction ......................................................................................... 12
       2.5.1. Acute phase proteins .......................................................................... 13
       2.5.1.1. Acute phase proteins during neonatal period ..................... 13
   2.6. Serum amyloid-A ............................................................................................. 14

3. AIM OF THE STUDY .................................................................................................. 16

4. MATERIALS AND METHODS .................................................................................. 17
   4.1. Animals and sampling ...................................................................................... 17
   4.2. Laboratory analysis........................................................................................... 18
   4.3. Statistical analysis ............................................................................................ 19

5. RESULTS ....................................................................................................................... 21

6. DISCUSSION ................................................................................................................. 25

7. CONCLUSIONS ............................................................................................................ 28

8. SUMMARY .................................................................................................................... 29

9. REFERENCES .............................................................................................................. 30

10. KOKKUVÕTE .......................................................................................................... 366

11. ACKNOWLEDGEMENTS ...................................................................................... 377
### ABBREVIATIONS

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>APP</td>
<td>acute phase protein</td>
</tr>
<tr>
<td>APR</td>
<td>acute phase response</td>
</tr>
<tr>
<td>BAT</td>
<td>brown adipose tissue</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>FPIT</td>
<td>failure of passive immune transfer</td>
</tr>
<tr>
<td>GGT</td>
<td>gamma-glutamyl transferase</td>
</tr>
<tr>
<td>HDL</td>
<td>high-density lipoproteins</td>
</tr>
<tr>
<td>HRP</td>
<td>horseradish peroxidase</td>
</tr>
<tr>
<td>Hp</td>
<td>haptoglobin</td>
</tr>
<tr>
<td>IgA</td>
<td>immunoglobulin A</td>
</tr>
<tr>
<td>IgG</td>
<td>immunoglobulin G</td>
</tr>
<tr>
<td>IgM</td>
<td>immunoglobulin M</td>
</tr>
<tr>
<td>IL-1β</td>
<td>interleukin-1β</td>
</tr>
<tr>
<td>IL-6</td>
<td>interleukin-6</td>
</tr>
<tr>
<td>PIT</td>
<td>passive immune transfer</td>
</tr>
<tr>
<td>SAA</td>
<td>serum amyloid A</td>
</tr>
<tr>
<td>TMB</td>
<td>tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
</tr>
</tbody>
</table>
1. INTRODUCTION

The neonatal period is one of the most demanding adaptation periods during the life of any animal, including lambs. The adaptation period to the extrauterine life begins immediately after parturition. First challenge lambs face, is fighting hypothermia. Suckling colostrum within the first few hours of life is also crucial for survival (Nowak & Poindron 2006).

Lambs are born without circulating immunoglobulins in the blood, which makes them bound to absorb maternal immunoglobulins from the colostrum during the first day of life (Zarilli et al. 2003). According to previous studies (Esser et al. 1989; Herndon et al. 2011) the ingestion of colostrum must be accomplished during the 24 hours post partum, before intestinal closure. Colostrum is the only main source of proteins, necessary for the adaptation into extrauterine life. The total protein in both colostrum and serum contribute to neonate immunity and growth (Piccione et al. 2007).

Zarilli et al. (2003) have demonstrated a high correlation between the enzyme GGT activity and IgG concentrations in sheep colostrum. Pauli (1983) showed significant positive correlation between GGT serum activity level and serum gamma globulin level, suggesting efficient passive immune transfer.

Acute phase reaction (APR) is the organism’s non-specific systematic reaction to trauma, injury, inflammation and infection. During APR acute phase proteins (APPs) are released into the blood as reviewed by Orro (2008). One of the main acute phase proteins in ruminants is serum amyloid A (SAA), which is considered to be a significant biomarker during inflammatory processes (Ceciliani et al. 2012). Serum amyloid A is mainly produced in hepatocytes in the liver.

It is a complex set of factors and circumstances that influence the neonatal lambs success of development and growth. This study aims to cast a light on the importance of early health and welfare and its effect on future performance.
2. LITERATURE REVIEW

2.1. Neonatal period in lambs

The neonatal period begins after parturition and it can be characterized as being an intensive period in time, when the newborn adapts to the extrauterine life (Mellor 1988). As reviewed by Piccione et al. (2011) the neonatal period is the interval between birth and 28 days of age. Profound metabolic and morphological mechanisms, such as thermoregulatory system, cardiovascular system, respiratory system and metabolic homeostasis, complete maturation during the neonatal period. This period of time is also called as adaptive period (Piccione et al. 2007). It is one of the most vulnerable period in animal’s life and it is connected to the high mortality and morbidity rate, especially during the first few days of life (Piccione et al. 2008; Piccione et al. 2009).

Mortality in the neonate lambs appears in both intensive and extensive systems and remains approximately 15-25% worldwide (Nowak & Poindron 2006). Nowak et al. (2000) found that neonatal deaths are highest, 21%, during the three first days of life. Dwyer (2008) estimated that half of all pre-weaning lamb deaths occur at the parturition day. This is a major economical loss and welfare issue in the sheep farming, and points out the importance of the post-partum periods role in lambs survival.

2.1.1 Hypothermia, body weight and energy reserves

The first couple of hours after birth are highly important for the survival. Hypothermia is one of the first battles the newborn must counteract. During the first 15 minutes after birth the body temperature decreases 1 to 2°C from the intrauterine temperature of 39°C (Nowak & Poindron 2006). Normal body temperature for lambs is considered to be 39-40°C (Eales et al. 2004). Alexander (1962) concluded that depending on the lambs size and prenatal nutrition, the body reserves are sufficient to maintain the lambs body temperature for 5 to 12 hours in cold weather and up to 3 days in good weather.
In order to counteract the heat loss that the lamb faces post partum, the neonate must generate a 15-fold heat production compared to the prenatal period (Alexander 1962). The thermoregulatory capacity is limited for lambs after birth, and depends on glycogen reserves and the thickness of the brown adipose tissue (Bureau & Begin 1982). The heat production of the newborns is produced by two different mechanisms: shivering and non-shivering thermogenesis. Brown adipose tissue (BAT) is the main site for non-shivering thermogenesis. Alexander & Williams (1966) concluded that BAT accounts for 1.5-2.0% of the neonates total body weight. However, Nowak & Poindron (2006) suggested it to cover 2-4.5% of the total body weight.

The one greatest factor affecting to the lamb mortality is birth weight outside of optimum range, which varies between breeds (Fogarty et al. 2000). Lambs with low birth weight might die because of exposure and starvation. Especially single lambs with higher birth weight are more predisposed to death caused by dystocia due to bigger size (Dwyer 2008).

2.1.2. Suckling

Risks that are involved to the lamb survival include as well the reluctance to stand up and suckle colostrum. The longer it takes for the lamb to get up and start suckling, the less the lamb makes an effort for suckling. The maintenance of effective suckling behaviour is considerably reduced if the lamb has not ingested colostrum during the first six hours of life (Alexander & Williams 1966; Nowak & Poindron 2006). As reviewed by Gudex (2001) the number of lambs in the litter have an impact on the amount of colostrum ingested; it was determined that single lambs are able to consume on average 35% more colostrum than twin lambs, and up to 60% more than triplet lambs.

Temperature has an impact on suckling as well; it has been established that when rectal temperature of the lamb falls beneath 37°C the drive for suckling disappears. Hence hypothermia compromises the newborns chances for gaining the important colostrum (Alexander & Williams 1966).
2.1.3. Colostrum composition

Colostrum is a unique food source of neonates and the lack or insufficient intake of colostrum is considered to be the second largest factor to affect neonatal survival, after body reserve (Nowak & Poindron 2006). It is produced immediately before the parturition and it is a compilation of dense nutrients and high levels of immunoglobulins, growth factors, enzymes and hormones. Colostrum contains approximately 7% fat, 4% casein, 5% lactose and 82% water. It provides 2 kcal of energy for each ml (Nowak & Poindron 2006). Mellor (1988) has determined that 180-290 ml of colostrum per kilogram of bodyweight is required for the lamb during the first 18 hours after birth to ensure adequate passive immune transfer.

2.1.4. Pathogens

Neonatal lambs are extremely vulnerable to infectious diseases as they are born immunologically naïve. It has been estimated in Europe, that the prevalence for deaths caused by infectious diseases vary between on average 10 to 35% (Dwyer 2008).

The total amount of immunoglobulins in the colostrum is not the only component to ensure the adequate passive immune transfer for the neonatal lamb; it is also a matter of antibody activity in the different immunoglobulin classes. Previous studies about piglets and calves show that they are more susceptible to infections if they are born into other environments than their mothers have been reared in. This is due to the specific antibodies against infections related to the new environment are missing (Kruse 1983).

As reviewed by Dwyer (2008) a major factor affecting neonatal sensibility to pathogens is the permeable immature gut. This permeability of the gut allows the initial immunoglobulin passage, but increases also the risk for pathogens to enter. However, colostrum ingestion itself accelerates the process of intestinal closure, thereby it is also preventing the route of neonatal infection.
2.2. Role of globulins during the neonatal period

Globulin proteins are serum proteins that are classified into three groups in ruminants; α-, β-, and γ- globulins (Tizard 1987). The α-fraction includes two major acute phase proteins, serum amyloid A (SAA) and haptoglobin (Hp), which both increase during inflammatory process and stress. The most important component in β-fraction is C-reactive protein (CRP), which is also involved in the stress response (Kaneko 1997). The γ-globulin fraction contains mainly immunoglobulins, which are proteins with antibody activity (Kaneko 1997; Thrall 2004; Tizard 1987). Tizard (1987) stated, that sheep have four different isotypes of immunoglobulins; IgG, IgA, IgM and IgE. Part of the immunoglobulin isotypes includes several sub-isotypes. Of these isoforms, IgG is the immunoglobulin found in highest concentration in serum.

Nowak & Poindron (2006) found that, as sheep have an epitheliochorial placenta, the immunoglobulins do not cross the placental barrier and the lamb is born without any circulating antibodies. The passive immune transfer from the ewes’ colostrum to the lamb is of utmost importance for the survival of the offspring, providing it with some resistance against infectious diseases (Nowak & Poindron 2006). But according to Hernandez-Castellano et al. (2014) early colostrum intake has increased also non-immunoglobulin proteins in lamb blood plasma, such as serum amyloid A, plasminogen and fibrinogen. The study suggests that these non-immunoglobulin proteins might promote gastrointestinal growth and the development of the newborn.

Previous studies that measured blood serum parameters in foals, calves (Bauer et al. 1985; Piccione et al. 2009) and lambs (Nowak & Poindron 2006) state a trend for total protein and serum immunoglobulin levels, having a peak on the first day of life (Piccione et al. 2013). Immunoglobulin-synthesis is initiated at approximately 3 weeks of age in neonatal lambs (Klobasa et al. 1985)
2.2.1. Colostrum Immunoglobulins

In ruminants, colostrum is the sole source of acquired immunity (Stelwagen et al. 2009). According to Tizard (1987) sheep colostrum contains IgA 100-700 mg/ml, IgM 400-1200 mg/ml and IgG 4000-6000 mg/ml. IgG represents the major immunoglobulin class to provide the initial protection early on in life (Alves et al. 2015).

The intestines of the ruminants are non-selective in their permeability and can therefore absorb all immunoglobulin isoforms from the colostrum (Yilmaz & Kaşıkçı 2013). However as Nowak & Poindron (2006) states intestinal closure happens approximately 24 hours after birth, meaning that the passive absorption of immunoglobulins in the intestine seize. Yilmaz & Kaşıkçı (2013) found that the time of the intestinal closure for each immunoglobulin type in sheep was for IgG 26.4 hours, for IgM 25 hours and for IgA 26 hours postpartum.

Nowak & Poindron (2006) found that when suckling begins; the level of immunoglobulins in the blood starts to rise rapidly during the first hour and reaches a peak around 24 hours after parturition. Shubber et al. (1979) concluded that larger volumes of colostrum correlate with larger amounts of immunoglobulins. The immunoglobulins are resistant to enzymatic digestion in the intestine. There is also a trypsin inhibitor in the colostrum itself, which additionally adds to this resistance (Yilmaz & Kaşıkçı 2013).

Adequate passive immune transfer (PIT) has been determined in some studies to be reached when the lambs IgG intake was above 30 g during the first 24 h of life (Alves et al. 2015). Consequently some studies suggest that the failure of passive immune transfer (FPIT) for the neonatal lamb has a significant effect on neonatal mortality and losses because of infectious causes correlate positively with low concentrations of serum immunoglobulins (Ahmad et al. 2000).
2.3. Albumin during neonatal period

Albumin is produced in the liver and it represents the main protein in the mammalian blood serum. As a small globular protein found in abundance in serum, it makes a large contribution to plasma colloid osmotic pressure (Piccione et al. 2013). As reviewed by Tothova et al. (2014) serum albumin also acts as a carrier protein for many insoluble organic substances, and plays a role as reserve energy source whenever there is inadequate intake for some reason.

In regards to neonates and serum albumin, a study by Piccione et al. (2013) concluded that the concentration peaked on first day of life after which it decreases during the first two weeks of life, and from week three it starts to increase. Other previous studies have also shown the same trend in albumin levels, suggesting a medium half-life of 14 to 16 days for ruminants. After this period the liver is responsible for synthesizing albumin in the neonate (Kaneko 1997, Thrall 2004). These results suggest that the initial albumin concentrations in neonates originate from the colostrum ingested during the first hours of life.

Other factors affecting to the serum albumin levels have been suggested to be protein intake of the ewe (Thomas et al. 1987). Also gender of the neonate seems to play a role, according to Ahmad et al. (2000) the male neonatal lambs were reported to have higher serum albumin than female lambs.

Colostrum is the only main source of proteins, necessary for the adaptation into extra uterine life for the neonate. Therefore the total serum protein in both colostrum and serum contribute to neonate immunity and growth (Piccione et al. 2007). According to Ahmad et al. (2000) the serum TP of the lambs that survived the neonatal period was significantly higher than in those who died.
2.4. Gamma-glutamyl transeferase (GGT)

Gamma-glutamyl transferase (GGT) is an enzyme that is present in biliary epithelium, hepatocytes, pancreas, kidneys and udder (Foreman 2014). It is widely used as a diagnostic marker for liver disease in medicine. For neonatal lambs GGT has low diagnostic value in diagnosing liver diseases, as it is present in colostrum as well as in milk of the ewe (Foreman 2014).

Zarilli et al. (2003) have demonstrated a high correlation between the enzyme GGT and IgG concentrations in sheep colostrum. It has been suggested that GGT is involved in the process of colostrum synthesis. Pauli (1983) measured that ewes’ colostrum GGT levels were 470 times higher than normal serum levels in adult sheep. Those lambs that had suckled colostrum showed 140-fold increase in serum GGT levels compared to the normal adult sheep GGT serum activity levels. Zarilli et al. (2003) concluded that GGT determination could be used as a marker of colostrum quality in ewes. Pauli (1983) showed significant positive correlation between GGT serum activity levels and serum gamma globulin levels, suggesting efficient passive immune transfer.

Serum GGT seem to have a rapid clearance in lambs. Because of this the correlation between serum GGT levels and serum gamma globulin levels, is more likely to be evident in serum samples that have been taken within 24 hours postpartum (Pauli 1983).

2.5. Acute phase reaction

The acute phase reaction (APR) is an important, non-specific and innate reaction to injury or stress to the body. The injury might have traumatic, infective, immunological or neoplastic origin as reviewed by Tothova et al. (2014). It is a defence response of the whole organism to minimize further damage, regain homeostasis in the organism and promote the healing process (Uhlar & Whitehead 1999). Initiation of the acute phase reaction typically starts within the inflammatory sites, where cells that are involved in the innate immune response, such as macrophages and monocytes release inflammatory mediators, such as cytokines including interleukin-1β, interleukin-6 and tumour necrosis factor-α. These
mediators induce a cascade of local and systemic inflammatory responses (Koj 1996; Tothovan et al. 2014). One of the most important metabolic changes is the increased synthesis of plasma proteins in the liver, also called as acute phase proteins (APPs) (Orro 2008; Tothovan et al. 2014).

2.5.1. Acute phase proteins

Acute phase proteins (APPs) are a large and diverse group of plasma proteins, released into the blood stream in response to APR (Baumann & Gauldie 1994). By definition the APPs are proteins that change >25% in serum concentrations in response to pro-inflammatory cytokine stimulation (Eckersall & Bell 2010). Acute phase proteins are produced mainly in the liver, but also extrahepatic production has also been detected, such as in equine joints (Jacobsen et al. 2005; Marhaug et al. 1997).

APPs can be positive or negative according to the increase or decrease in serum concentration during acute phase reaction (Eckersall & Bell 2010). Positive APP’s are further classified as major, moderate or minor according to their accumulation to the organism. Major proteins often increased markedly within 24 to 48 hours after the triggering event and they are usually rapidly declining due to a short half-life. Moderate and minor APPs may increase more slowly and persist for longer as it was reviewed by Tothovan et al. 2014. Major APPs for sheep are haptoglobin, serum amyloid A; moderate APP is α1-acid glycoprotein; minor APPs are fibrinogen and ceruloplasmin; albumin is a negative APP (Tothova et al. 2014).

2.5.1.1. Acute phase proteins during neonatal period

There have been several studies implying that APPs play an important role during the neonatal period. However the functional mechanisms are still not completely clear (Murata et al. 2004; Orro et al. 2006; 2008). A general view is that the main function of the cellular components in colostrum is to play a role in the development of local immunity and to stimulate the active immunization of the new-borns’ intestinal tract (McDonald et al. 2001).
As reviewed by Orro (2008) the colostrum contains high quantities of inflammatory mediators. Several of the most important cytokines have been found in colostrum and milk, but the effect of these on the neonate is not yet fully determined. According to Yamanaka et al. (2003) in calves these cytokines are transferred from colostrum into the blood. Orro et al. (2008) suggested that these colostrum inflammatory mediators might induce APR in the neonate, and that inflammatory mediators can cross from colostrum to neonate, pass their intestine and stimulate the acute phase protein production in the liver.

The acute phase reaction (APR) is a very important mechanism for the neonate to adapt into the extra uterine life and regain homeostasis. Therefore it might be speculated that the inflammatory response is to be expected in the newborn, this being reflected in the APP concentrations as reviewed by Orro (2008). Several different factors play a role in the APPs concentrations after parturition. These include the foetal synthesis of APPs, the stimulation of APP production by birth trauma, APP containing colostrum intake by the neonate (McDonald et al. 2001) and the neonate’s synthesis capacity of APPs by its liver (Brunn et al. 1998).

2.6. Serum amyloid-A

Serum amyloid-A (SAA) is a multifunctional apolipoprotein that takes part in the acute phase of inflammation. Like other apolipoproteins it has been suggested to inhibit the oxidative tissue damage during inflammation (Uhlar & Whitehead 1999). It is one of the most conserved proteins among mammals and is therefore considered to play a basic and essential role in the innate immune system (Hernandez-Castellano et al. 2014).

As reviewed by Cerón (2005) SAA is an APP in most species of animals, ruminants as well as humans. Serum amyloid A is significant marker of systemic inflammation and it has also been suggested to rise during infection, stress and endotoxemia. For most of the species SAA is a major APP according to the ability to increase up to 1000-fold. Measured from milk SAA has been suggested to have diagnostic value in detecting mastitis (Pyörälä et al. 2011).
Serum amyloid A has been found in the colostrum of several healthy mammals. It has been suggested that it is part of a normal non-pathological process, and plays an important role in the health and wellbeing of newborn animals (McDonald et al. 2001). The mammary-associated serum amyloid A 3 (M-SAA3) has been found to be produced extrahepatically by the mammary glands epithelial cells. It has been found in high concentrations in the colostrum (McDonald et al. 2001).

The mechanism of action is not fully understood, however M-SAA3 expression appears to be part of a natural physiological process, and it seems to not be limited to disease-associated responses. M-SAA3 may promote tissue repair, and by this contribute in protecting the neonate’s gastrointestinal tract from pathogens encountered early on in life (McDonald et al. 2001).
3. AIM OF THE STUDY

The aim of this study was to examine the variation in blood serum concentration of globulins, albumin, SAA and enzyme GGT activity in neonatal lambs up to the age of three weeks and their association with weight gain during summer rearing period (aprox. 3 months).
4. MATERIALS AND METHODS

4.1. Animals and sampling

The material was collected during April and May of 2011 and 2012, at one lamb farm in Southern Estonia. The population size for this study was 322 lambs in total. These were lambs of mixed breed.

The animals are housed as a heard in a free-range barn with access to outside grazing lands. Postpartum the lambs receive immediately colostrum from their mothers. In addition they have free access to water and hay.

The blood samples were collected, both years, from lambs approximately once a week for 1-3 consecutive weeks during the first weeks of their life. Out of the in total 322 lambs, blood was sampled once from 176 lambs, twice from 90 lambs and three times from 56 lambs. Total sample size was 524, in 2011 there was 248 samples and in 2012 a total of 276 samples taken. Sample size distribution by age is given in table 1.

<table>
<thead>
<tr>
<th>Age group of lambs</th>
<th>Sample size 2011</th>
<th>Sample size 2012</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 day</td>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>1-7 days</td>
<td>199</td>
<td>82</td>
</tr>
<tr>
<td>8-14 days</td>
<td>72</td>
<td>104</td>
</tr>
<tr>
<td>15-21 days</td>
<td>0</td>
<td>46</td>
</tr>
<tr>
<td>22-25 days</td>
<td>0</td>
<td>16</td>
</tr>
</tbody>
</table>

Blood was collected jugular venipuncture. Samples were collected into a 4 ml serum clot tube. The collected samples were transported to the laboratory of the Estonian University of Life Sciences. Blood samples were centrifuged at 4000 rpm for 5 minutes thereby separating the serum. The serum samples were stored at -20°C before analysis.
The measuring of weight was at the end of the summer rearing period, at the farm, when the lambs were 2.5-4.5 months of age (mean ± SD age at weighting in 2011 86.3 ± 5.0 days and at 2012 129.9 ± 6.9 days). Weight gain was calculated by subtracting the estimated average weight at birth (2 kg) from the weight measured, and then dividing the result with the lambs age in days, at the time of the weighting.

4.2. Laboratory analysis

Total protein concentration was determined by using a commercial photometric colorimetric test for total proteins liquicolor, developed for human use (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany). The method is based on a biuret test that determines the presence of peptide bonds in proteins. The test measures quantitatively the concentration of total protein using colorimetric method.

Albumin concentration was determined by using a commercial photometric colorimetric test for albumin liquicolor, developed for human use (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany). The method is based on Brom cresol green (BCG) dye-binding method. The test measures quantitatively the presence of serum albumin by colorimetric methods.

Globulins concentration was calculated by subtracting albumin concentration from total protein concentration in the same sample.

Gamma glutamyl transferase concentration was determined by using a commercial colorimetric test L-γ-glutamyl transferase γ-GT liquicolor The test is developed for human diagnostics (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany). The principle of the test method is based on kinetic colorimetric determination of γ-GT activity as according to Persijn & van der Silk (1976).

These analysed were carried out with automated biochemistry analyser Mindray BS-200 (Mindray Medical International Limited, Shenzhen, China).
Serum amyloid A concentration was determined by using a commercially available Spectrophotometric ELISA SAA Assay kit (Tridelta development Ltd, Maynooth, Co. Kildare, Ireland). Analysing was performed manually, according to instructions provided by the manufacturer. The principle of the test is a solid phase sandwich ELISA were SAA specific monoclonal, immobilized antibodies cover the base of the micro titre plate provided in the kit. Serum samples were diluted first into 1:500. When concentration was above the range of a standard curve (150 mg/l) the samples were further diluted into 1:2500. Samples and calibrates with known SAA concentrations were applicated to the micro wells in the plate together with biotinylated monoclonal antibody. This procedure captured and marked any SAA antibodies present in the well. Wells were washed to remove the unbound material after which the plates were incubated. After the first incubation the wells were washed again and Streptavidin-Horse Radish Peroxidase (Streptavidin HRP) was added to the micro wells and the plates were incubated a second time. After washing the tetramethylbenzidine (TMB) substrate was added to the wells. A third incubation a stop solution was added to finish the reaction. The results were read from each well by spectrophotometer with a wavelength of 450 using 630 nm as a reference.

4.3. Statistical analysis

To investigate time changing patterns of the serum concentration in lambs’ proteins (globulins, albumin and SAA) and GGT activity during first 3-4 weeks of life, linear mixed models were used. In these models lambs were included as random intercepts and polynomials of time (days), with interactions with sampling year as fixed effects in increasing order from the beginning of the experiment, were used. Statistical software R 3.1.2 nlme package (R Core Team 2013) was used for those models.

The associations between the protein serum concentrations and GGT serum activity from first 3 weeks of life, and lambs weight gain during 2.5-4.5 months summer rearing period were studied using an linear mixed models. Weight gain in g/day were used as response variable and proteins concentrations, GGT activity as continuous explanatory variables, lambs gender, siblings (one, twin or triplet), ewe body condition, ewes age and study year as grouping explanatory variables in models separately build on 3 datasets containing
samples from lambs with different age groups (1-7, 8-14 and 15-21 respectively). Backward elimination process was carried out for the final models. Ewe was included as random factor in all models. Stata/IC 13.0 statistical software (StataCorp LP, Texas, USA) was used for statistical analyses.
5. RESULTS

Mean serum globulins concentrations of neonatal lambs can be seen in figure 1. It shows that the globulins concentration is lowest on day 0 reaching a peak on day 1 thereafter it shows a decline until day 14. There was no statistical difference in globulins concentration changes between study years.

![Figure 1](image_url)

**Figure 1.** Mean (±SEM) serum concentrations of globulins in lambs blood during the first 25 days of life by study year (samples n = 524; 2011 year n = 248; 2012 year n = 276).

Mean serum albumin concentration of lambs can be seen in figure 2. It shows that albumin has a lowest concentration on day 1 where after it starts to rise steadily until day 14. There was no statistical difference in albumin concentration changes between study years.
Figure 2. Mean (±SEM) serum concentrations of albumin in lambs blood during the first 25 days of life by study year (samples n = 524; 2011 year n = 248; 2012 year n = 276).

Mean Serum amyloid A (SAA) concentrations of neonatal lambs can be seen in figure 3. It shows a low concentration on day 0 where after it reaches a peak on day 1. On days 7 to 8 the concentration shows a decline to a generally low level. There was no statistical difference in SAA concentration changes between study years.

Mean serum GGT activity in lambs can be seen in figure 4. It shows that GGT serum activity started to rise shortly after birth, picked at age of 3 days and then decline until day 14. There was no statistical difference in serum GGT activity changes between study years.
Figure 3. Mean (±SEM) serum concentrations of serum amyloid A (SAA) in lambs blood during the first 25 days of life by study year (samples n = 524; 2011 year n = 248; 2012 year n = 276).

Figure 4. Mean (±SEM) serum activity of gamma-glutamyl transferase (GGT) in lambs blood during the first 25 days of life by study year (samples n = 524; 2011 year n = 248; 2012 year n = 276).

The association between mean weight gain (g/day) and serum albumin, globulins and SAA concentrations at the lambs age of 1-7 days are shown in table 2. There was positive
association of albumin and globulins at the age of 1-7 days with weight gain and negative association of SAA concentrations with weight gain at the same time. There were no significant associations of GGT with weight gain at any time studied (1-7, 8-14 and 15-21 days of age) and no associations between serum proteins and weight gain at lambs second and third week of life.

**Table 2.** Association of albumin, globulins and serum amyloid A (SAA) concentration of 1-7 days old lambs (n = 224) with weight gain (g/day) during 2.5-4.5 months period

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate</th>
<th>95% CI of estimate</th>
<th>p-value</th>
<th>Wald test p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Albumin (g/l)</td>
<td>2.714</td>
<td>0.507; 4.922</td>
<td>0.016</td>
<td></td>
</tr>
<tr>
<td>Globulins (log g/l)</td>
<td>33.56</td>
<td>17.9; 49.14</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>SAA (mg/l)</td>
<td>-0.156</td>
<td>-0.275; -0.037</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>Age of ewe</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 year (n = 48)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 years (n = 73)</td>
<td>41.18</td>
<td>25.60; 56.77</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>3-4 years (n = 41)</td>
<td>70.82</td>
<td>50.51; 91.13</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>5-6 years (n = 34)</td>
<td>47.61</td>
<td>27.02; 68.20</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>7-8 years (n = 28)</td>
<td>31.85</td>
<td>8.87; 54.84</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Sample year</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2011 (n = 78)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2012 (n = 146)</td>
<td>-35.09</td>
<td>-47.07; -23.11</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>No. of siblings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Single (n = 109)</td>
<td>0</td>
<td></td>
<td></td>
<td>0.000</td>
</tr>
<tr>
<td>Twins (n = 105)</td>
<td>-54.76</td>
<td>-67.82; 41.69</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Triplets (n = 10)</td>
<td>-82.61</td>
<td>-113.87; -51.35</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 121)</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female (n = 103)</td>
<td>-27.29</td>
<td>-38.03; -16.56</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Intercept</td>
<td>48.59</td>
<td>-50.43; 147.62</td>
<td>0.336</td>
<td></td>
</tr>
</tbody>
</table>
6. DISCUSSION

It is widely recognised that the level of circulating immunoglobulins reflect the success of antibody absorption from the colostrum. Serum globulins concentration can be considered to represent the main fraction, immunoglobulin G, concentration. Thus it can be assumed that high serum globulins concentration can be used as an indication of sufficient passive immunity received from the colostrum.

Serum proteins (globulins and albumin) also provide an energy resource for the newborn lamb, this gain the neonate in early development and growth. As reviewed by Alves et al. (2015), measuring serum proteins concentration can also be used in assessing sufficiency of passive immune transfer (PIT) as it reflects both the concentration of serum albumin and globulins. With efficient passive immune transfer (PIT) the neonate has a stronger immunological defence against infections from the start, and we might expect it to perform better in growth and weight gain later in life, than would be the case if there was failure of passive immune transfer (FPIT).

Serum albumin is a negative acute phase protein in sheep as reviewed by Tothova et al. (2014). Serum albumin concentrations correlate inversely with serum SAA concentrations. We could speculate that high serum albumin concentration is an indicator that there is no present acute phase reaction, infection or stressor, affecting the neonate. This could suggest a positive impact on development and growth of the lamb.

Results in this study show that high blood SAA concentrations during days 1 to 7 of life seem to predict negative correlation with weight gain at the end of summer rearing period. SAA levels show an increase after the first day of life until the fifth day of life. It could be speculated that reasons for high SAA concentrations could be due to infections or other stressors affecting the neonate, thereby affecting growth development negatively. It is possible that the neonate receives SAA also from the colostrum, but it is not fully understood whether SAA from colostrum transfers from the gut to circulation.
After age of 5 days the SAA levels start decreasing until the second week of life, when the concentration begin increasing again. It could suggest exposure to infections or stress at this point in life. Orro et al. (2006) found that high SAA concentrations during the second week of life had a negative association with weight gain in reindeer calves at the age of four months.

Acute phase response is not yet understood in detail in small ruminants, and the limits of studies performed in relation to acute phase proteins and lambs leave us with too little information to draw exact conclusions of the mechanism of SAA during the neonatal period. According to Coldiz (2002) the activation of innate immune responses is highly draining for the organism, and the alerted immune system might be responsible for draining resources otherwise used for growth and development.

This study also measured the serum GGT activity in the newborn lambs. The hypothesis was that high GGT serum activity during the first weeks of life, would predict a positive correlation with weight gain later in life. Previous studies have concluded that enzyme GGT activity in lamb serum is useful in estimating the status of passive immune transfer in neonates and it seems to correlate positively with serum IgG concentration. In those studies the source of the GGT was determined to be colostrum (Maden et al. 2003; Pauli 1983). However in this study such results were not received. It has been pointed out in previous studies (Pauli 1983) that in lambs GGT levels rise rapidly and the serum clearance is also faster than in calves, the correlation between serum GGT activity and serum gamma globulin concentration is likely to be found only in samples that are taken within the first day of life. Perhaps timing of blood sampling in this study did not fall into the optimal time frame for a correlation between GGT activity and serum gamma globulin concentration to be made. Factors influencing the neonatal lamb during the first week of life seem to predict the overall growth performance during the rearing period.

When it comes to limitations of this study it should be noted that sample size for the lambs at the age of 0 days is very low. In 2012 samples were collected only at days 0 to 14 which also limits the sample size.
In future studies it would be interesting to study the mechanism of serum SAA in greater detail, in order to gain better knowledge of serum SAA function and what effect it has on the development and growth of the neonatal lamb.
7. CONCLUSIONS

In conclusion this study has found a positive correlation between serum albumin and globulins concentration during the first week of life in neonatal lambs and their weight gain during the summer rearing period of approximately three months. There was also indicated a negative correlation between serum amyloid A concentration during the first week of life and the lambs weight gain during the summer rearing period. This supports the hypothesis laid out before the study. The present study could not find a positive correlation between serum enzyme GGT activity and with weigh gain.

Results of present study suggest that the first week of a lamb's life is important and indicate future trend in growth and production. Challenges during the first week of life increase SAA concentration and for some reason it has an effect on weight gain. Still more studies are needed in order to fully understand the mechanism of serum SAA and its exact role in the neonatal development.

This could be valuable information especially for practitioners dealing with heard health, in emphasising for farmers the importance of early neonatal care and welfare. Supporting welfare and health, not purely survival during the first week of life, could optimize the production of lamb meat after the rearing period.
8. SUMMARY

Serum concentrations of globulins, albumin and serum amyloid a (SAA) of neonatal lambs and associations with weight gain during summer rearing period

The present study was undertaken to investigate some biochemical parameters during the first weeks of life and their relations with growth rate over the first months (summer rearing period – aprox. 3 months) of life in neonatal lambs.

The material used in this study was collected from lambs during two lambing seasons in 2011 and 2012. Analysed samples were collected from 322 lambs at one sheep farm in southern Estonia. In 2011 there were 248 samples collected and in 2012 a total of 276 samples. Out of the 322 lambs a total of 524 samples were analysed. Biochemical methods used in analysing the samples were automated colorimetric methods for albumin, total protein and gamma-glutamyl transferase (GGT). The concentration of globulins was calculated by subtracting albumin concentration from total protein concentration from the same sample. To determine blood serum amyloid A (SAA) concentration ELISA-method was used.

All biochemical parameters studied showed market changes in their blood levels during the first 2 weeks of lambs life. Serum globulins, SAA concentrations and GGT serum activity of lambs were highest at birth or shortly after that and then decreased until 2 weeks of age. In contrary lambs serum albumin concentrations were lowest at birth and increased during next 2 weeks. Lambs serum globulins and albumin concentrations at the age of first week had positive association with weight gain during summer rearing period and SAA concentration at the same age period had negative association with weight gain. Associations between serum enzyme GGT activity and weight gain was not found.

Results of present study suggest that the first week of a lambs life is important and indicate future trend in growth.

Keywords: albumin, globulins, GGT, SAA, lambs, weight gain
9. REFERENCES


10. KOKKUVÕTE

Tallede seerumi globuliinide, albumiini ja seerumi amüloid a sisaldus neonataalperioodil ja seos tallede kaaluiibega suvisel kasvuperioodil

Käesoleva lõputöö eesmärgiks oli uurida tallede mõningaid biokeemilisi parameetreid neonataalperioodil ja nende võimalikku seost kaaluiibega 2,5-4,5 elukuul.


Selle uuringu tulemused näitavad, et talledes esimene elunädal on oluline nende edasise kasvu ja arengu seisukohalt.

Märksõnad: Albumiin, APP, GGT, globuliin, kaaluiibe, SAA, talled
I would like to thank my supervisors at the Estonian University of Life Sciences; Professor Toomas Orro PhD and Kristel Peetsalu DVM, for all the support, help and guidance needed to finish my thesis work.

I dedicate this thesis to my children, Frida and Stella. Thank you for enduring these six long years of veterinary studies by my side (I guess you did not have a choice!). At times it was hard, but I hope that this is remembered above all as an adventure. If nothing else may it show you that it is never too late to fulfil a dream.

Behind everything there is always a mother. Thank you Annikki Kilpi for being there for me and making sure that I didn't give up!

Special thoughts go to my father.
Non-exclusive licence for depositing the final thesis and opening it for the public and the supervisor’s (supervisors’) confirmation for allowing the thesis for the defence

Hereby I, Anna Josefina Kilpi
29/05/77

1. grant Eesti Maaülikool, the Estonian University of Life Sciences, a free-of-charge non-exclusive licence to store the final thesis titled **SERUM CONCENTRATIONS OF GLOBULINS, ALBUMIN AND SERUM AMYLOID A OF NEONATAL LAMBS AND ASSOCIATIONS WITH WEIGHT GAIN DURING SUMMER REARING PERIOD**, supervised by Toomas Orro and Kristel Peetsalu. for
   1.1. preservation;
   1.2. depositing a digital copy of the thesis in the archive of DSpace and
   1.3. opening it for the public on the Web until the validity of the term of protection of copyright.

2. I am aware that the author retains the same rights as listed in point 1;

3. I confirm that by being issued the CC licence no rights deriving from the Personal Data Protection Act and the Intellectual Property Rights Act have been infringed.

Author of the final thesis …………………………………………..

(signature)

In Tartu, 26.05.2015.

-------------------------------------------------------------

The core supervisor’s approval for the final thesis to be allowed for defence
This is to confirm that the final thesis is allowed for defence.

Supervisor’s name and signature          Date

Institute of Veterinary Medicine and Animal Sciences

Estonian University of Life Sciences

Supervisor’s name and signature          Date