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Influence of Lysine and Methionine on first antler growth in *Dama dama*

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Declaration

I hereby declare that I have done this thesis entitled Influence of Lysine and Methionine on first antler growth independently, all texts in this thesis are original, and all the sources have been quoted and acknowledged by means of complete references and according to Citation rules of the FTA.

In Prague, 27 April 2018

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Veit NY

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Abstract

Amino acid supplementation in cervids is an almost unexplored research area. Very few studies have been conducted, and those were based on very low sample size leading to inconclusive results. In this study, we show the first trial on a large herd of fallow deer (*Dama dama*), and also the first one focusing on the effects on antlers. Lysine (Lys) and Methionine (Met) are the two amino acids considered as limiting for ruminants. Among them, Lys may have a good potential for antler growth as it is the main component of collagen and bone tissue precursor. However, several studies in other ruminants have shown greater effects when simultaneously supplementing Lys+Met. Two experiments based on Lys and Met supplementation during first antler growth were conducted during two consecutive years. Each experiment involved 45 yearling fallow bucks randomly distributed in three groups: Exp.1: *Pasture*, *Pasture+Barley* (0.2 kg per animal and day), and *Pasture+Barley+Lys* (5 g per animal and day); Exp.2: *Pasture+Barley* (0.5 kg per animal and day), *Pasture+Barley+Lys* (9 g per animal and day), and Pasture+Barley+Lys+Met (3 g per animal and day). The Lys (9 g) and Lys+Met treatments had a certain positive effects on the external antler characteristics and especially in the burr perimeter. In the second experiment, Cu content in the amino acids supplementation groups were lower than the control group, thus also no significant differences of the mechanical and structural properties. However, general mechanical properties and other chemical compositions tend to improve when more inclusive amino acids supplemented. Zinc was very low of all experimental groups. Therefore, amino acids could have direct effects on antler growth (lower Zn means that animals grow antlers with no physiological constraints). In general, the result suggests a more intense positive effect of amino acid supplementation in situations when the animals have a lower performance.

Key words: Antler structure; Body condition; Chemical composition; Fallow deer; Mechanical properties; Ruminally Protected Amino Acids.

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List of the abbreviations used in the thesis

AA: Amino Acid

ADG: Average daily gain

CBA: Cortical Bone Area

CBD: Cortical Bone Diameter

CS: Concentrate selector

GR: Grass eater

H: Hypothesis

IM: Intermediate feeder

IUCN: International Union for Conservation of Nature

RPAA: Ruminally Protected Amino Acids

MJME/W: Mega Joule-Metabolisable energy per Weight

1. Introduction and Literature Review

1.1. Introduction

Deer farming is now growing significantly worldwide. Among other cervids, fallow deer (*Dama dama*) is the most common farmed species (in numbers) in Europe, especially for venison production (Hoffman & Wiklund 2006). Aside from meat production, antlers are also interesting for the production of velvet, trophies and predicted to be an important source for studying medical science in the future (Klein 1992; Landete-Castillejos et al. 2007*a*; Wu et al. 2013).

Antlers are chemically composed of 35%-45% of collagen and protein (Dobrowolska 2002). During antler growth, deer requires high amount of protein up to 16% (Dryden 2016). However, as ruminant, microbial protein alone can be not enough for supporting growth and production (Merchen & Titgemeyer 1992; Kung & Rode 1996). Specific amino acid (AA) requirements are necessary for protein synthesis, and thus the loss of essential AA through ruminal digestion could be a problem for the protein synthesis needed for antler growth. In particular, the two known limiting AA for ruminants are lysine (Lys) and methionine (Met) (Rosenberg 1957; McDonald et al. 2011). For antler growth, Lys supplementation seems especially promising since it is the main component of collagen and thus precursor of bone tissue (McDonald et al. 2011). However, very few studies on AA supplementation have been carried out on cervids, especially on fallow deer.

Only one study of Mendoza-Nazar et al. (2012) studied methionine supplementation in subadult red deer antlers at different concentrations, but found no effects on antler beam length, brow tine length or number of tines. Moreover this study is limited in sample size, and thus, the results are hardly conclusive. Hence, study on the supplementation of these limiting amino acids on first antler growth of fallow deer with more different levels and on bigger sample size should be conducted for deeper insight.

1.2. Fallow deer biology

Fallow deer was distributed throughout Mediterranean area, west Eurasia and speculated to be brought to England and Europe by Romans (Goss 1983; Stachowicz et al. 2014). Fallow deer belongs to Ruminantia, family Cervidae. It is even-toed ungulate. There are two species in this family, including European fallow deer (*Dama dama*) and Persian fallow deer (*Dama mesopotamica*) (Chakanya et al. 2015). Persian fallow deer is typically found in the Middle East while the European fallow deer is mostly abundant in Europe and small part of Asia particularly in Turkey. Persian fallow deer is now included in the IUCN Red List of Threatened Species (Werner et al. 2015). They are physically dimorphic. Persian fallow deer has smaller antlers and the palmate located close to the base while European fallow deer palmate is proximate the tip of antlers (Chapman & Chapman 1980). In addition, Persian fallow deer is larger in body size, which is almost 16% larger for bucks (Stachowicz et al. 2014). Fallow deer is known as the most beautiful deer. Obviously, male deer distinguish from female by presence of antlers and the larger body size as shown in Figure 1 (Goss 1983). Typical colours are red, brown, white, and black (Goss 1983; Stachowicz et al. 2014).

Figure 1. Fallow deer (*Dama dama*) buck with hard antlers.

Fallow deer gives birth to single fawn after the gestation around 8 months. The rutting season is in September and October while deer are around 16 months. Female fallow deer has oestrous cycle for 24 to 26 days. Females and fawns normally live in one group and only meet males during rutting season. Fallow bucks attract the female by their urine, groans vocalisation and behaviour to form harem or lek to perform mating (Goss 1983). Persian fallow deer was known for its importance in game hunting. However, the estimate population in 2013 was only around 371, particularly in Iran (Werner et al. 2015). In contrast, European fallow deer is now known as the most common farmed species in Europe especially for venison production (Hoffman & Wiklund 2006).

1.3. Deer antler

Antler is a unique outside dead bone of cervids that no other mammals possess. There are vast varieties of antler shapes and sizes ranging from smallest to gigantic gorgeous structures which are proportionate with their body size. For instance, *Pudu pudu* known as the smallest deer in the world, has antler only around 3 cm, in proportion of its average little body size weighted (around 6 kg).
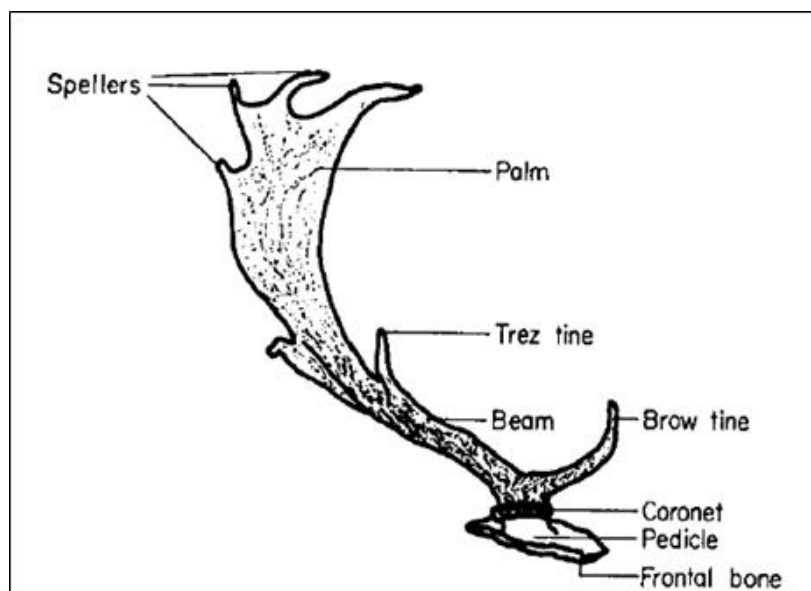
Figure 2. Fallow deer palmate antler extracted from Chapman (1975).

On the other hand some species like red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*), wapiti (*Cervus canadensis*) or reindeer (*Rangifer tarandus*) have huge antler formation. Antlers represent male primacy while most of females do not have them, except reindeer or caribou (Goss 1983). Fallow deer has antler as palmation headpieces and some of their antler parts are called differently from other antler types as shown in Figure 2.

1.3.1. Antlerogenesis

Antlerogenesis is the process of formation and development of antlers. Antlers grow at the apex throughout the extension process of pedicles just like plant growth. It involves pedicle formation, growing antler or "in velvet", velvet shedding, hard antler (ready for the rut), antler casting and the regeneration of new antlers (Goss 1983; Fenessy & Suttie 1985).

Pedicle formation: the initiation of antler growth is from the osteoclast, the tissue which is responsible for bone formation. Primary grown part is pedicle which attaches to the skull of deer. This frontal bone connects the skull to the antler during antler growth which means that antler does not directly grow from the skull (Li & Suttie 2001). Deer starts pedicle growth since foetal life as its primordial pedicle tissues exist since the stage of pregnancy in the *genera* Cervus and Rangifer (Chapman 1975). However, the knob of pedicles starts to form and can be palpable as the fawn initiates first antler growth when they are reaching puberty and the blood testosterone increases (Li & Suttie 2001). This formation part contains special tissue called antlerogenic periosteum. This tissue is considered as the prenatal embryonic retained tissues which self-differentiate and develops to the mature antler and for the whole life antler growth (Kierdorf & Kierdorf 2011). There are two main hormones; testosterone and insulin-like growth factor which help proliferate pedicle generation (Price et al. 2005). The pedicle constitutes mainly spongy bone which then develops to bony structure at the tip due to mineralization. Pedicle stays about few centimetres in young deer. However, in more mature deer, pedicle is shorter to almost unable to see as it is closely attach to the skull (Goss 1983). Pedicle diameter increases when deer is older due to the deposition of concentric layer of bone (Chapman 1975). This part connects to the base of antler which is called antler burr. Antler burr is important for blood transferring and the

starting point of antler growth, casting and regenerating each year (Kierdorf et al. 2003; Kierdorf et al. 2007; Price et al. 2005).

First antlers "in velvet": velvet is the extension of the skin on deer head to cover the antlers Chapman (1975). Velvet antlers are developed with the covering skin from epidermal cells.   It contains numerous blood vessels during antler growth (Li & Suttie 2000; Jeon et al. 2011). The dermis of velvet contains thick collagen fibres. The next layers are called proliferation zone which is the growth zone. This zone consists of a layer called perichondrium which contains reserve mesenchyme cells. Those cells are cartilage matrix which will be mineralized into primary spongiosa and secondary spongiosa. The primary spongiosa is formed by early calcification of collagen fibres. The secondary spongiosa forms cancellous bone in the middle and the compact bone peripherally throughout mineralization (Goss 1983). The differentiation of velvet bone continues through endochondral ossification until the first velvet antlers are fully formed (Price et al. 2005). Velvets remain on deer head up to 3 to 5 months, depending on the species. Velvet is soft, shiny and mainly used in medicinal purposes especially in East Asian countries (Wu et al. 2013). Pedicle formation and velvet antler anatomy is shown in Figure 3 below.
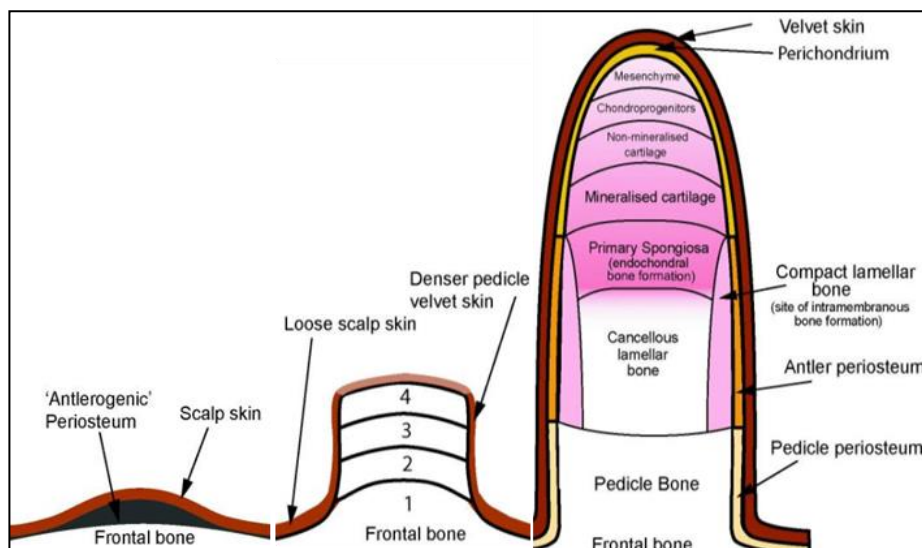

Figure 3. Pedicle formation from frontal bone and velvet antler bone adopted from Price et al. (2005).

Antler shedding: the majority of deer shed their velvet during autumn (Li et al. 2004). When the level of testosterone starts to decrease, the longitudinal growth of velvet ceased (Price et al. 2005; Bartos et al. 2012). Deer normally rub their antlers with hard objects such as tree trunks to remove the covered skin of velvet antlers. After shedding, the antlers remain in hard bony structure which is fully mineralized.

Hard antler: the first set of antlers is normally unbranched and spike. For the next regeneration cycle, more branches structure will develop. Also, some big species have few branches in first antler growth. The rate of antler growth is very fast, approximately 1 mm to 3 cm/day depending on the species (Goss 1983). Hard antlers are mineralized prior rutting and used as weapon during fighting for mating and to get access to food (Lincoln 1992). Hard antlers contain more minerals on the base and decreasing to the tips (Landete-Castillejos et al. 2007*b*).

Casting antler: deer normally cast their antlers during winter or spring before the new fawns are born, for example wapiti, sika deer, red deer and fallow deer (Goss 1983). However, some species such as roe deer grows their antlers during winter. During rutting season the level of testosterone is still high, and then drops rapidly after rutting which causes antler casting (Bartos et al. 2012). After casting, the distal pedicle stump skin is swollen and shiny (Li et al. 2004). Casting antler is caused by osteoclast cells and bone resorption process. It happens between pedicle and antler burr (Kierdorf & Kierdorf 2011).

Antler regeneration: antler regeneration is considered as epimorphic regeneration among mammals. It starts in summer in most species soon after casting of previous antlers. This set of antlers is called the real antlers (Goss 1969). There are three main phases of antler regeneration which includes casting of previous hard antlers, wound healing and the formation of the main beams and brown tines (Li et al. 2004). The regeneration process is initiated by stem cell-based process. Wound healing starts by covering skin and fibroblast deposit collagen and vascularize the pedicle mass. The growth starts from the tips of the pedicle. Most deer species cast their antlers and starts regrow soon after. However, some deer species grow their new antlers after few months. In most of Capreolinae species, antler regeneration occurs soon after casting the old antlers. Odocoileini, on the other hand, pose from one to two months between this regeneration (Wilson & Mittermeier 2011). This prolongation depends on indirect

effects of hormone androgen which is also influenced by environment and nutrients (Goss 1983; Kierdorf & Kierdorf 2011).

1.3.2. Antler composition

Antler is defined as dead bone headpieces of deer and their composition is similar to other bones (Goss 1983). In general, there are two forms of bone in deer antlers; compact bone and cancellous (spongy) bone (Figure 3). This bone changes through deposition of minerals within the cartilaginous matrix through a process called endochondrial ossification (Chapman 1975). As reviewed by Picavet and Balligand (2016), antlers are composed of minerals, collagen, non-collagenous protein and water. Even if minerals seem very important in bone compositions, deer antlers contain up to 44% of collagen (Nowicka et al. 2006). Antler compositions are different depending on deer species (Ceacero 2016), growth stages, nutrition and also the parts of antler (base, middle or top) (Chapman 1975; Landete-Castillejos et al. 2012*a*).

In Velvet: in the state of velvet antler, the covering skin is rich in blood vessels. It supplies from pedicle connected to spongy core and compact bone. Therefore, the antler burr perimeter is really important for the whole antler growth. The composition of velvet antlers is higher in protein at the top while the bottom part is higher in ash content and minerals (Sunwoo et al. 1995; Jeon et al. 2011). Velvet antler of young animals is also abundant in active compounds such as fatty acids and vitamins, while the velvet of older deer contains more mineral (Lee et al. 2007).

Hard antler: hard antlers are mainly composed of minerals 56% (Pathak et al. 2001). There are two categories of minerals such as macro or trace minerals and micro minerals. Macro minerals include Ca, P, Mg, Na, K, S and micro elements are B, Cu, Fe, Mn, Sr, Co, Zn, among others. However, some minerals (Ca, P and Mg) which are considered as the most important due to their functions in bone growth and their big portion in antler (Nowicka et al. 2006). Trace elements (Cu, Mn, Fe and Zn) are the most studied due to their importance in bone growth and indicators of nutritional status of deer (Pathak et al. 2001; Nowicka et al. 2006; Landete-Castillejos et al. 2012*b*). According to Chapman (1975) reviews, fallow deer antlers contain moisture 8%, ash content 48.18%, Ca (18.21%), P (8.68%) and ratio Ca:P is 2.10. Some minerals vary depending on management of deer. This difference in composition could be used to

explain deer physiological constrains and their antler quality. For instance, one study of Landete-Castillejos et al. (2007*c*) found higher Zn in free ranging red deer antlers which indicates the incomplete growth of bone. However, the good nutrient feeding captive deer antlers have higher Na, K and Mg and lower in Zn. Other variations of mineral composition in antlers due to physiological constrain and different feeding nutrition will be detailed in the chapter 1.4.3.

1.3.3. Factors affecting antler growth

As briefly mentioned in the antlerogenesis part, factor influencing antler growth is mainly the androgen hormone, testosterone, which is indirectly induced by photoperiod or growth of animals. Bartos et al. (2012) described the influences of testosterone on antler cycle through time as show in Figure 4. The presence of blood testosterone induces pedicle initiation. It reaches the peak during rutting period and drops afterward, followed by antler casting.
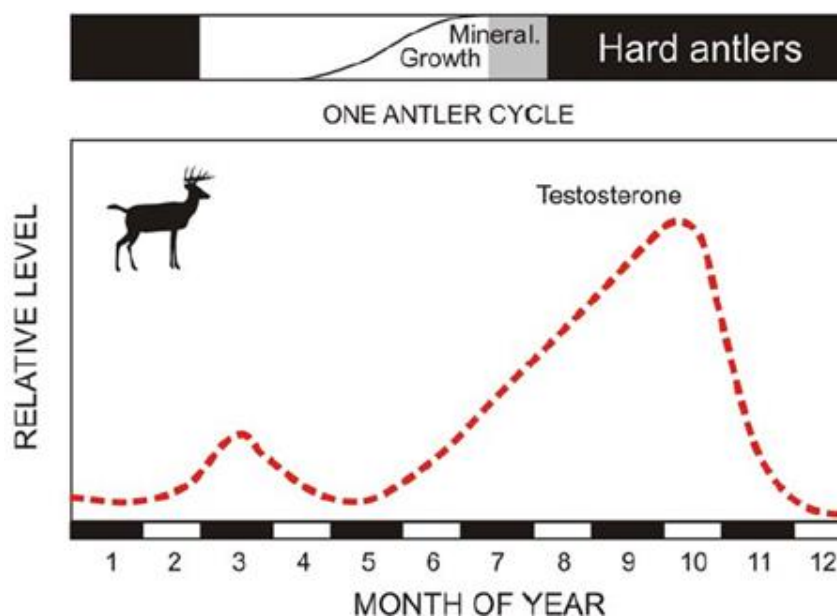

Figure 4. Changes in levels of testosterone during antler cycle extracted from Bartos et al. (2012).

Some manipulations such as castration and changes of photoperiod could affect antler initiation and antler cycles (Bartos et al. 2012). For example, study of Kierdorf et al. (1995) on castration of fallow spikes caused the premature antler casting. And when the fallow bucks in velvet antlers were castrated, the antler remained in velvet form without shedding. Castration of fawns inhibits the pedicle initiation and antler growth. For young deer, testosterone is very important for pedicle initiation. It is also important for antler maturation in older deer. Decreasing day length causes velvet shedding and premature hardening of antler. Day length is independent to first yearling antler. But from the second antler, the cycle is mostly modulated by day length. Deer have more antler cycles when they exposed more than one light cycle (Goss 1983). Other steroid and gonadotrophin hormones are also believed to have some influences (Chapman 1975). Insuline-like growth factor 1 (IGF-1) is a hormone responsible for stimulating antler growth, specifically influencing cartilage growth during velvet antler period (Suttie et al. 1985). Deer need to reach weight of threshold to start pedicle growth, in particularly for yearlings. Thus, nutrients play an important role, both direct and indirect in antler growth (Fenessy & Suttie 1985; Baxter et al. 1999). Poor feeding and management could retard the pedicle formation (Chapman 1975). The details of this nutritional point of view will be discussed in the chapter 1.4.3.

1.3.4. Indicators of antler quality

Quality of antlers can be determined based on external antler characteristics (antler morphometric), internal antler characteristics, mechanical properties and chemical composition assessments. External antler characteristics include length of antler beam, antler weight, number of tines and antler burr perimeter (Cappelli et al. 2017). Antler weight changes through life stages of animals. It increases from yearlings to adults, but tends to decrease during senescence (Dryden 2016). Antler weight can be up to 5% of body weight in spikers (Landete-Castillejos et al. 2012*b*). Yearling stags usually have branchless antlers (Chapman 1975). Therefore, the number of tines in yearling might not be counted as a good antler indicator in most species, including fallow deer. Antler length of the first antler, on the other hand, is correlated with the number of antler tines in the second antler cycle (Schmidt et al. 2001).

As internal antler characteristic measurements; cortical bone thickness, cortical bone diameter and antler density could be included. Measurement of cortical bone thickness and diameter are commonly used for measuring antler bone rigidity level. This is because antler bone is composed of compact bone surrounding trabecular (spongy) bone as shown in Figure 5. The compact bone contains collagen fibrils, which help improving rigidity and mechanical properties of antlers (Krauss et al. 2011). Higher cortical thickness of antler was found in deer fed better diet (Landete-Castilljos et al. 2007*b*). Spongy bone characterizes porous property of antlers. When there is high porosity in the compact bone, it is related to incomplete development of primary osteons. The density of cortical bone can be calculated by the division of dried weight of the antler bar fragment and its volume (Cappelli et al. 2017). Density of antler also differs among deer species and tends to decrease when deer is older. It is lower in the main beam than the antler tips according to Miller et al. (1985) in white-tailed deer (1.5-4.5 years old). The density of complete antlers of red deer stags ranges from 1.2-1.6 kg/dm^3 in which 1.27-1.88 kg/dm^3 correspond to compact bone and 0.34-0.43 kg/dm^3 to spongy bone. In the skeleton the density varies from 1.7 to 2.0 kg/dm^3 (Chapman 1975).
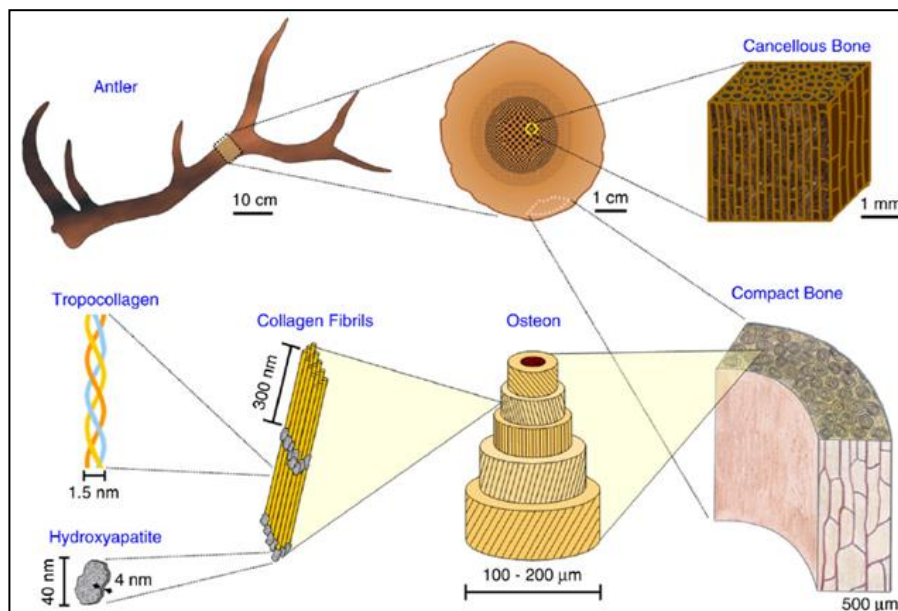

Figure 5. Antler structure of compact bone and spongy bone extracted from Chen et al. (2008).

Based on many studies on red deer antlers, macro minerals (Ca, P, Mg) and micro minerals such as Zn, Mn, K, and Cu are known as good indicators of antler quality (Landete-Castilljos et al. 2007*a*; Landete-Castilljos et al. 2012*a*). Zinc, for instance, indicates the incomplete antler development when it is in high amount in antlers (Landete-Castilljos et al. 2007*c*). Determination of mineral composition in antlers is used for evaluating quality of antler and also indirect effects of nutrient. Last but not least, intrinsic mechanical properties of the antler can be determined by the tree-point bending test for determination Young's modulus of elasticity (*E*), bending strength *(BS)* and work to peak force (*W*), and impact energy absorption (*U*), which indicates the toughness of antler bone (Landete-Castilljos et al. 2010; Cappelli et al. 2017). Moisture content in antler seems also important for mechanical properties of antlers. Comparing to normal bone, dried antlers of red deer (at room temperature) have higher bending strength, impact energy absorption and work to fracture. In contrast, wet antlers have higher work to peak force than skeleton (Currey et al. 2009). The wet antler of red deer has higher work to peak force which helps to reduce breakage antler during fighting (Chapman 1981). The stiffness has relation with cortical bone, density and minerals in antlers too (Landete-Castilljos et al. 2012*a*). One study of Landete-Castilljos et al. (2010) shows that deer antlers growing during hard winter had lower Young's modulus of elasticity, impact energy and work to peak force compared to antler growth during normal winter. This is due to the lack of minerals in plants.

1.4. Nutrition for deer

1.4.1. Digestive physiology and feeding behaviour of fallow deer

Deer are ruminants, and thus their nutrient intake depends on rumination and anaerobic fermentation of microorganisms in their guts. They are more efficient than other kinds of digestive system as they can utilize various feedstuffs due to this symbiotic capacity. Based on feeding categories of ruminants by Hofmann (1989), fallow deer are intermediate feeders as same as reindeer deer and red deer. They graze and browse depending on feed availability but prefer more grazing. This intermediate feeder (IM) switches from grazing to browsing when the plants start to lignify because they avoid the fibre as much as they can. Their digestive anatomy and feeding

behaviour is between concentrate selectors (CS) and grass eaters (GR). They eat less frequency than CS but more often than GR. Shipley (1999) summarized the digestive physiology of CS and GR. Concentrated selectors have larger salivary gland, true stomach (abomasum), liver, cecum and intestine but smaller foregut (rumen, reticulum and omasum) as their feedstuffs are mainly concentrates and low in fibres compared to grass eaters. Feeding activity of fallow deer is more active during morning and evening. They eat much amount of food when it is available and chew later (Matiello et al. 1997). Feed intake also varied with seasonal changes. Feeding behaviours of fallow deer were observed by Appolonio and Di Vittorio (2004). Feed intake was completely stopped for male fallow bucks during rutting season. Therefore, feed supplementation prior the rutting season is really important to maintain the body growth.

1.4.2. Nutrient requirement for deer

Deer require basic nutrients just like other ruminants such as protein, energy, minerals, vitamins, and water (Richardson 2000). The requirements differ across species, sexes and their stages of growth. The main nutrient requirements were reviewed by Dryden (2011). Energy requirements are different depending on stages of life, sex and vary across seasons. Net energy requirements in average during winter to spring for adult and non-lactating deer are 0.39 MJME/W (kg) $^{0.75}$ per day. Adult fallow deer require high energy during winter [0.65 MJME/W (kg) $^{0.75}$ per day] and lesser [0.55 MJME/W (kg) $^{0.75}$ per day] during summer for maintenance. Unlike other ruminants, net energy requirements for deer also include the energy for growing coat of fawns during autumn and for antler development. Main source of dietary energy for ruminants is carbohydrates (celluloses, hemicellulose, starch, pectin). These components supply nutrients both to microorganisms in rumen and also the ruminants themselves. Ruminants can get carbohydrates from plants tissues, grasses, fruits, pastures or concentrate feed (mainly in captivity) (NRC 2007). Another sources of energy are lipids but for deer it is not common (note the absence of gallbladder for fat emulsification; Hofmann 1989). Protein is also used as source of energy when it is in excess amount or there is depletion of energy sources.

The protein requirements are higher during reproduction including lactation and antler growth (16-22%); lower requirement for maintenance which is approximately 4% to 9% (Dryden 2011). Proteins and amino acids are really important for body growth, reproduction, maintenance and lactation (Kung & Rode 1996; Richardson 2000). Aside from growing tissues, proteins also play the main role in bone growth. Malnutrition of protein causes detriment of the production and activity of Insulin-like growth factor 1 (IGF-1) which is important for bone growth (Bonjour et al. 2001). Protein requirements include microbial proteins and dietary proteins that escape from microbial degradation. Final amino acids from that digestion can be absorbed in the small intestine and further be used by ruminants for protein synthesis (NRC 2007). Amino acids are important in protein requirements due to the role as building blocks of protein synthesis. It comprises both essential and non-essential amino acids. Lack of one amino acid, thus protein synthesis cannot be completed (McDonald et al. 2011). Therefore, the description of the limitation of proteins and amino acids will be described in the chapter 1.4.4.

Macro and micro minerals are also essential for deer because they cannot synthesize it. Their primary role is in structural components of organs and tissue particularly bones, skeletons, teeth; functioning in body fluids such as milk composition, osmotic equilibrium; role in metabolisms such as enzymes, co-enzymes or cofactors (NRC 2007). Greater demand of minerals for female deer is during lactation, especially for milk production and contributing to fawns for developing bones and their early growth (Ceacero et al. 2010). This is for sure that the importance during antler growth is inevitable. Deer need macro minerals (Ca, P, Mg, Na, Cl, K, S) and trace minerals (Cu, Zn, Se, I, Mn, Co, Fe). Macro minerals are required in high amount while trace minerals are also important but needed in small amounts (NRC 2007). Calcium and P play most important role in bone, antler growth and also in milk composition (Ceacero et al. 2010). Trace minerals are essential due to their role as enzymes and co-enzymes in metabolic pathways for growth, maintenance and reproduction. Copper is an important trace element for deer to help improving performance and preventing enzootic ataxia (failure of muscular coordination) and osteochondrosis, in case of deficiency (Wilson et al. 2014). Deer need double amount of Cu compared to sheep in their diet which is around 5 mg/Kg DM (Grace & Knowles 2012). Zinc is also really important for nutrient digestibility and production. Dietary minerals from plants depend on the soil characteristics. In animal production, minerals are mainly supplemented in

the form of salt licking (NRC 2007). Supplementation of minerals such as Mn, Cu helps to improve antler cortical thickness, chemical components and mechanical properties (Cappelli et al. 2015; Gambín et al. 2017). Study of vitamins requirement for deer is very limited. Vitamins A, D and E are important for growth and development in deer. Vitamin A is important for ossification of bone and antler growth. Vitamin D could be potential for calcium absorption in mineralization. For vitamin E, the main function could be as the prevention of tissue damaging (Richardson 2000).

Nutrient in feed stuffs is also affected by weather as its indirect effect on compositions of pastures and plants, especially for wild animals or game estate management. For instance, in summer there is more feed availability and better nutrient in plants (Bugalho & Milne 2003). In contrast, animals have feed shortage due to limited nutrients in plants during winter. For this reason, supplementations during winter are needed for adequate deer production. For example, Webster et al. (2001) supplemented barley to red deer stags during winter for compensating the lack of pasture. The result shows that deer increased live weight gain compared to deer fed only grass silage.

1.4.3. Nutrition for antler growth

Antler growth can be affected by the whole stages of growing period (since the foetus, during suckling, weaning and first antler growth). This can affect the spike size of first antler growth. However, in older stags, the bodyweight and antler weight have closer relationship (heavy deer tends to have heavy antlers) than the spike antlers (Dryden 2016). Fawns suckling milk rich in proteins and minerals have higher spike weight (Gómez et al. 2006; Ceacero et al. 2010). During antler growth, deer have specific requirements of nutrients such as proteins, vitamins, energy and especially minerals (Dryden 2016). One of the main nutrients is protein which is required up to 16% for growing antler and approximate 11.5% for initiating pedicle development (Putto et al. 1998; Dryden 2011). However, Dryden (2016) also discussed that a high quantity protein is not always enough to adequate antler growth. In fact, the quality of protein (with adequate amino acids balance) is more important than the amount of protein. One of the limiting effects could be because the proteins are degraded by microorganisms (will be discussed later). Therefore, specific amino acids are more

important than protein supply for antler growth. Among essential amino acids, Lysine would be a good candidate for antler growth due to its contribution to collagen formation and as precursor of bone tissue (McDonald et al. 2011).

The second well known component of diet necessary for antler growth is minerals. Antler growth rate is very rapid, and it requires high amount of minerals (Kierdorf et al. 2000). The peak of minerals demand for antler mineralization from trabecular bone to compact bone in red deer is around 100 days of antler growth (Gómez et al. 2013). The two macro elements; calcium and phosphorus are very important in bone and as well as in antlers (Nowicka et al. 2006). It is important for skeletal mineralization for improving mechanical properties of bone and bony tissue structures such as teeth, skeletons (Nordin 1997). During high peak of mineralization, Ca and P are resorbed from skeletons to antlers (Moen et al. 1999). Phosphorus is known as the most limiting mineral for ungulate herbivores. For deer it is important in alkaline phosphatase involving in minerals deposit during antler growth (Grasman & Hellgren 1993). Study on Irish elk antlers, the largest antlers among cervids, found out that the requirement of Ca and P are approximate 60 g and 30 g deposit in antlers for 60 days growing at high peak of mineralization (Moen et al. 1999). Approximate 20% of minerals are transferred from bone to antlers during antler growth (Landete-Castillejos et al. 2012*b*). Calcium transference from skeleton to antler is higher for young deer (Cowan et al. 1968). Manganese is essential trace mineral in the synthesis of the mucopolysaccharides of cartilage (Leach & Muenster 1962). For example, poor Mn in diet during winter in Spain, caused reduction of cortical thickness (30%), impact energy (27%) and work to peak force (10%) (Landete-Castilljos et al. 2010). Thus, following experiment of Mn supplementation by Cappelli et al. (2015) found increased chemical compositions (Ca, Na, P, B, Co, Cu, K, Mn, Ni, and Se) in adult red deer antlers. Moreover, level of Mn at 80 mg/kg help increasing antler weight in sika deer stags (Bao et al. 2017). Cu also plays an essential role in the maturation of collagen, specifically the synthesis of lysine-derived crosslinks (Hyun et al. 2004).

1.4.4. Limitation of nutrient utilization in deer

Firstly, minerals are known as limited nutrient because deer cannot synthesize them. However, many studies about the effect of minerals supplementation on antler growth have been done, so it will not be discussed in this chapter. Only the main limiting nutrients, protein and amino acids will be discussed.

Deer have limitation of utilizing proteins and amino acids due to digestion process of microorganisms. For ruminant, microbial proteins alone cannot be sufficient for growth and production (Kung & Rode 1996). The proteins or amino acids fed to ruminants are not in the same quality when absorbed in the intestine. The two known limiting amino acids for ruminants are Lysine (Lys) and Methionine (Met) (Rosenberg 1957). Lysine is known as main precursor for collagen as previously mentioned. Methionine, on the other hand, is important for production of cellular components (McDonald et al. 2011). Some studies have proved that supplementation of these limiting amino acids helps improving growth and animal health. Supplementation of Met and Lys helps improving average daily gain of steer fed meat and bone meal and corn and gluten meal (Klemesrud et al. 2000). However, the supplementation has to be done in protected form in order to avoid the degradation through digestion (Dryden 2016). Ruminally Protected Amino Acids (RPAAs) are amino acids which are protected from rumen digestion and thus highly available for absorption in intestine. The protecting methods include heat or chemical treatment; encapsulation by coating material; use of amino acids analogues and esophageal groove closure (Chalupa 1975). Even if supplementation with limiting amino acids is a hot topic for ruminants, most of the supplementation studies have been carried out on monogastricts. It has not been well studied on deer and at all on fallow deer. So far, there are only two studies of Met and Lys supplementation performed on deer. Huang et al. (2015*a*) studied Met supplementation on sika deer calves, finding no increase in final bodyweight or average daily gains (ADG). In the second study they obtained very similar results when supplementing Lys+Met to calves with a poor protein diet, although supplementation did not improve the performance observed in animals with an adequate protein diet (Huang et al. 2015*b*). Only one study (Mendoza-Nazar et al. 2012) focused on the effects of supplementation of different levels of methionine on antler growth of subadult red deer. They found no effects on any external antler characteristics (antler beam

length, brow tine length or number of tines). Unfortunately, these three experiments were performed in small sample size, always 4 animals per group. These limited studies are pioneer of the idea that more exclusive study should be done on this great topic with adequate sample size and more inclusive design.

2.   Aims of the Thesis

The goal of this Thesis was to find out the influence of limiting amino acids supplementation, Lysine and Methionine, on the improvement of first antler growth of fallow deer (*Dama dama*).

Specific objectives of this study were:

- To determine the effect of supplementation of limiting amino acids, Lysine on the improvement of antler characteristics, mechanical properties and chemical composition of first antler growth of fallow deer.

- To evaluate the effectiveness of the different levels of Lysine supplementation on antler characteristics, mechanical properties and chemical composition.

- To determine the effect of combination of limiting amino acids, Lysine and Methionine, on the antler characteristics, mechanical properties and chemical compositions.

The Hypothesis

H1: Lysine supplementation will improve antler characteristics (external and internal antler characteristics), mechanical properties and chemical composition.

H2:   Combination of Lysine and Methionine supplementation will also increase antler characteristics, mechanical properties and chemical compositions.

3. Materials and Methods

3.1. Experimental design

3.1.1. Experimental site and animals

The study was conducted during two consecutive years, 2015 and 2016, at a private deer farm in Mnich village, near Kardašova Řečice town, South Bohemian region, Czech Republic (49.17N, 14.90E; 485 masl) as shown on map Figure A1, Appendix 2. The experiments were carried out on yearling fallow bucks (*Dama dama*) shown in Figure 6. Each year, 45 animals were divided into three groups balanced by weight and ear tagged for identification. All experimental animals were born in the previous spring each year, which means that in first experiment (2015), fawns were born in June 2014 and the second experiment (2016), fawns were born in June 2015. As reported by Czech Hydrometeorological Institute, extreme summer was noticed in 2015 especially in August which temperature was above 35°C especially in South Bohemian and Central Bohemian region (CHMI 2015). Animals were kept in 2 ha paddocks, and raised on good quality extensive grazing pasture from weaning (around January) to the end of the experiments in late autumn (December) except some animals in 2016 that were culled in late winter.

Figure 6. Yearling fallow deer bucks during velvet antler growth.

3.1.2. Feeding and supplementation

Supplementation with Ruminally Protected Amino Acids (RPAAs) started in the beginning of summer (from May) on daily basis distributed on feeding platform to allow access to all animals in group at once, when animals were of one year old, slightly before antler growth starts. The RPAAs were Lysine LysiPEARL™ (in experiment 2015), which was encapsulated by Spray-freezing encapsulation method and LysiGem™ (in experiment 2016). The coating material was Hydrogenated vegetable oil (palm). Ruminally protected methionine was Smartamine® M, which was coated with pH-sensitive polymer. The coating material helps to prevent ruminal degradation of amino acids and makes them highly available for intestinal absorption. The inclusion levels of RPAA were calculated based on the body weight of the animals and the previous study of Mendoza-Nazar et al. (2012), following a 3:1 concentration ratio of Lysine (Lys) and Methionine (Met) (Schwab et al. 2004). During the first experiment (2015), a control group was fed during the antler growth period exclusively on pasture; a second group received 0.2 kg of barley per animal and day; the third one received the same amount of barley and 5 g per animal and day of RPAA Lysine. The purpose of this first trial was to see the effectiveness of Lysine supplementation on antler growth because it is known as the main candidate for collagen precursor in bone tissues.

Table 1. Experimental design along the two years, animals in each experimental group and number of antlers analysed.

Groups	2015	2016
Pasture	15 / 15	
Pasture + Barley[1]	15 / 14	15 / 10
Pasture + Barley + Lys 5 (g/day)	15 / 15	
Pasture + Barley + Lys 9 (g/day)		15 / 9
Pasture + Barley + Lys 9 (g/day) + Met 3 (g/day)		15 / 8
Total antlers used	**44**	**27**

[1] 0.2 kg of barley per animal and day in 2015; 0.5 kg in 2016

In second experiment (2016), a group fed on pasture and barley (0.5 kg per animal and day) was used as control respects the first year; the second group also received level of Lysine was increased to 9 gram and the third group received the combination of Lysine (9 g) and Methionine (3 g). The increase of barley in second experiment to 0.5 kg was to compensate the lower body growth during drought 2015 (CHMI 2015).The composition of groups and experimental designed clearly shown in the Table 1.

Aside from pasture, all groups also received a mineral mixture (Premin Slanisko, VVS Vermerovice s.r.o., Czech Republic). During the second experiment (2016), some animals from each group were culled during winter (February), and these received grass silage to compensate for the lack of pasture; nevertheless, supplementation of silage was well after antlers were fully grown and mineralised. The nutrient compositions of barley, grass silage and pasture are shown in Table A1 in Appendix 1.

3.1.3. Culling

Most animals were culled on late autumn, although a few from each group were culled in late winter in the second year study due to technical farming purposes. Each year, during slaughtering time, the same numbers of animals from each group were culled. In experiment 2015, slaughtering carried during October. In experiment 2016, slaughtered dates were on December 2016 and February 2017 (Late winter). The animals were rendered unconscious by mechanical stunning using captive bolt gun, according to the Czech laws and bled by cutting of neck arteries. The final body mass was measured before culling using a Tru-Test EziWeight (New Zealand) scale with an accuracy of 0.1 kg. Other biometric variables were also measured but not including in this study. Due to the excitable nature of the species, no handling during the experiment was possible. Thus, the only data available was that collected at the start of the experiment (that collected while sorting the groups) or at culling. Body mass was already higher at the beginning of the experiment in the first year [probably due to the worse weather conditions in 2015 (severe drought, CHMI 2015) leading to lower pastures during the early growth in those calves used in the first experiment in 2016]. Body weight and body condition at culling was also not suitable because of different timing between and within each year (due to management purposes). For these reasons,

we decided not to include body mass or body condition as control variable in the statistical analyses described below.

3.1.4. Antler collection

Broken antlers were excluded from the study, since mechanical tests could be performed only in antlers long enough to allow testing. Thus, 44 antlers were fully studied in 2015 but only in 27 animals in 2016. The higher breakage rate in 2016 was probably due to later culling (November for the 2015 experiment; February to January for the 2016 experiment), which increased the chances of breaking the antlers due to multiple reasons.

Antlers were removed after culling just below the burr using a manual saw. Length of both antlers was measured, as so as the burr perimeter. After cutting, antlers were washed, labelled and dried at room temperature (Figure 7) until the mass was constant (approximately four days). Weight was recorded in a precision balance (±0.01 g). Mean values for both antlers were calculated and used in the statistical analyses.

Figure 7. Antler of both sides with identification number during drying.

3.2. Mechanical and chemical analysis

One antler per animal was selected for the rest of analyses, preferentially the left one. A 5-6 cm piece was cut just above the initial pearled part of the antler. Both upper and lower complete transverse cross-sections of the piece were scanned (ScanJet 4370 Photo Scanner, HP Inc., Palo Alto, CA, USA) at 2400 × 2400 dpi and analysed in an image analysis software (ImageJ). The cortical bone thickness was measured at six equally spaced points around the shaft. The area of the cortical section was measured, as so as the areas occupied by trabecular and cortical bone. Mean values were finally calculated for cortical bone diameter (CBD) in cm and in percentage, and cortical bone area (CBA) in percentage.

To calculate the density of the cortical bone, one portion of samples remaining after the mechanical test (with rectangular shape) were dried out for 72 h at 60°C, weighed with a precision balance (±0.01 g) and measured with a precision scale (±0.01 mm). Density was calculated by dividing the dry weight by the volume. Finally, the sample was placed in a muffle furnace (HTC 1400, Carbolite, UK) for 6 h at 480°C, and ash content was calculated as ash weight divided by dry weight.

Another fragment after the mechanical tests was used to determine the mineral content of the antlers. Contents of Ca, P, Mg, Na, K, S, B, Cu, Fe, Mn, Sr and Zn were analysed in a specialized lab (CEBAS-CSIC; Murcia, Spain) by plasma-optical emission spectrometer using a ICAP 6500 DUO Spectrometer/IRIS INTR.EPID II XDL (Thermo Fisher Scientific, Waltham, MA, USA). The ratio of Ca to P was also calculated. Two bars of cortical bone were prepared for each antler in order to test the mechanical properties. The final size of the bars was 4.5 mm wide, 2.5 mm deep, with variable length sufficient for a gauge length of 40 mm. A low-speed circular saw was used for initial cutting of the antler piece in two halves along the longitudinal axis. Surfaces were then abraded using a semiautomatic polishing machine (LaboPol-21, Struers Inc., Ballerup, Denmark) until creating bars of the correct size. Thereafter, samples were placed in Hank's balanced salt buffer solution (BioWhittaker, Belgium) for 48 h, and subsequently the bars were dried at 20°C, 40% relative humidity, for 72 h. This procedure aims to standardize the humidity content. One bar was tested by three-point bending, with the periosteal side in tension, in a Zwick/Roell 500 N machine (Germany) with head speed 32 mm/min, and analysed with the software testXpert II

(Zwick GmbH & Co, Ulm, Germany) (Figure A2, Appendix 3). The mechanical properties measured were Young's modulus of elasticity (E; an estimate of stiffness), bending strength (BS; calculated from the maximum load borne), and the work to peak force (W; determined by the total work done on the specimen up to the greatest load borne and divided by the central cross-sectional area). Further details about these properties can be found in Currey et al. (2009). The second bar was tested for impact work (U), which measures the energy used to break an un-notched specimen by a falling pendulum. This value of U was normalized by dividing by the cross-sectional area of the specimen. Tests were carried out in a CEAST-IMPACTOR II (CEAST S.p.A., Pianezza, Italy) with a hammer of 1 J (Figure A3, Appendix 3).

All mechanical and chemical analysis were carried out in laboratory in the in University of Castilla-La Mancha in Albacete, Spain.

3.3. Data analysis

Firstly, Levene test used to check homogeneity of variances. One-way ANOVAs were used to detect the differences of the treatments in each year. Due to the differences found between years, statistical analyses were done separately for each year. Tukey test was used to find out significant differences among treatments.

MANOVA was used to analyse differences among treatments on the previously described variables, which were grouped as external (antler length, weight and burr diameter) and internal antler characteristics (CBD, CBD%, CBA, ash and density), antler mechanical properties (Young´s modulus, bending strength, work and impact) and chemical composition (Ca, P, Ca/P, Mg, Na, K, S, B, Cu, Fe, Mn, Sr and Zn).

Finally, multivariate General Linear Models studied the effects of body mass at the beginning of the experiment and treatment on the groups of variables previously described for the MANOVA analysis.

The threshold for significance was always considered $P<0.05$. All analyses were performed in SPSS version 20 (IBM, SPSS, USA).

4. Results

4.1. Effect of Lysine on antler growth in the first experiment (2015)

In the first experiment, the RPAA Lysine did not improve any antler characteristics as shown in Table 2.

Table 2. MANOVAs showing lack of significant differences of diet supplementation with ruminally protected Lys on antler characteristics of fallow deer yearlings (Experiment 2015).

Parameters	Pasture	Pasture +Barley	Pasture + Barley+ Lys (5 g/day)	p-value
External antler characteristics (Wilks' λ = 0.943, p=0.886)				
Antler length (cm)	28.9 ± 5.4	26.3 ± 5.9	27.0 ± 6.0	0.461
Antler weight (g)	83.6 ± 18.0	80.2 ± 19.5	81.4 ± 22.6	0.895
Antler burr (cm)	24.0 ± 1.6	24.0 ± 2.3	23.6 ± 3.0	0.897
Internal antler characteristics (Wilks' λ = 0.817, p=0.643)				
CBD (cm)	0.372 ± 0.081	0.340 ± 0.088	0.328 ± 0.166	0.304
CBD (%)	0.420 ± 0.096	0.461 ± 0.074	0.495 ± 0.153	0.300
CBA (%)	0.666 ± 0.071	0.683 ± 0.087	0.617 ± 0.159	0.283
Ash (%)	56.8 ± 2.0	56.6 ± 2.5	55.6 ± 2.8	0.400
Density (kg/dm^3)	1.53 ± 0.17	1.60 ± 0.12	1.54 ± 0.21	0.531
Mechanical properties (Wilks' λ = 0.873, p= 0.716)				
Young´s Modulus (GPa)	10.9 ± 2.2	12.2 ± 1.8	11.7 ± 2.9	0.370
Bending Strength (MPa)	224 ± 41	250 ± 38	245 ± 63	0.327
Work (KJ/m^2)	27.4 ± 5.4	29.0 ± 6.2	29.1 ± 7.1	0.689
Impact (KJ/ m^2)	16.1 ± 3.3	15.3 ± 3.1	15.5 ± 5.0	0.842
Chemical composition (Wilks' λ = 0.219, p= 0.149)				
Ca (g/100g)	16.7 ± 1.0	17.0 ± 1.1	17.0 ± 1.4	0.726
P (g/100g)	11.2 ± 0.8	11.4 ± 1.3	11.6 ± 1.4	0.668
Ca/P	1.490 ± 0.062	1.490 ± 0.085	1.47 ± 0.090	0.754
Mg (g/100g)	0.377 ± 0.024	0.389 ± 0.025	0.372 ± 0.023	0.158
Na (g/100g)	0.540 ± 0.037	0.554 ± 0.031	0.548 ± 0.031	0.407
K (g/100g)	0.0344 ± 0.0086	0.0340 ± 0.0042	0.0344 ± 0.0065	0.991
S (g/100g)	0.233 ± 0.019	0.237 ± 0.014	0.237 ± 0.015	0.771
B (mg/Kg)	0.629 ± 0.120	0.664 ± 0.179	0.624 ± 0.347	0.885
Cu (mg/Kg)	0.378 ± 0.142	0.357 ± 0.085	0.453 ± 0.216	0.239
Fe (mg/Kg)	13.9 ± 9.2	18.5 ± 10.7	15.5 ± 10.5	0.477
Mn (mg/Kg)	16.64 ± 1.24	17.05 ± 0.98	16.56 ± 0.97	0.439
Sr (mg/Kg)	154 ± 17	148 ± 13	147 ± 18	0.533
Zn (mg/Kg)	47.2 ± 5.4	46.3 ± 4.1	46.4 ± 5.1	0.843

Note: CBD: Cortical bone diameter; CBA: Cortical bone area; Ca/P: Ca to P ratio.

The multivariate GLM revealed an effect of body mass on the internal antler characteristics, mechanical properties and chemical composition, which means that larger animals produced better antlers, but not bigger. However, after controlling for the body mass of the animals at the beginning of the experiment, the supplementation treatment only had a marginally significant effect on chemical composition (Table 3).

Table 3. Multivariate General Linear Models showing the effect of body mass at the beginning of the experiment and treatment (different diet supplementation regimes; see text) on the antler characteristics of fallow deer yearlings in the first experiment described (2015).

Multivariate General Linear Models _First Experiment (2015)_		
	Wilk´s λ (p-value)	Pillai´s Trace (p-value)
External Antler Characteristics – Not significant		
Internal Antler Characteristics		
Intercept	0.356 (<0.001***)	0.644 (<0.001***)
Body mass (kg)	0.727 (0.028*)	0.273 (0.028*)
Treatment	(ns)	(ns)
Mechanical properties		
Intercept	0.785 (0.046*)	0.215 (0.046*)
Body mass (kg)	0.643 (0.001**)	0.357 (0.00+**)
Treatment	(ns)	(ns)
Chemical composition		
Intercept	0.002 (<0.001***)	0.998 (<0.001***)
Body mass (kg)	0.412 (0.006**)	0.588 (0.006**)
Treatment	0.335 (0.080†)	0.811 (0.093†)

***, **, * and † indicates significance at p < 0.001, 0.01, 0.05 and 0.01, respectively.

4.2. Effect of Lysine and Methionine on antler growth in the second experiment (2016)

In the second experiment, the RPAAs improved external antler characteristics (Figure 8; Table 4), interestingly, antler burr perimeter (p=0.008).

Table 4 . MANOVAs showing significant differences of diet supplementation on antler characteristics of fallow deer yearlings (Experiment 2016). Significant differences are highlighted in bold.

Parameters	Pasture + Barley	Pasture + Barley+ Lys (9 g/day)	Pasture + Barley+ Lys (9 g/day) + Met (3 g/day)	p-value
External antler characteristics (Wilks' λ = 0.495, *P* = 0.013)				
Antler length (cm)	22.3 ± 7.0	27.2 ± 5.4	27.7 ± 7.2	0.158
Antler weight (g)	55.0 ± 14.9	58.6 ± 13.4	74.5 ± 22.7	**0.061**
Antler burr (cm)	19.6 ± 2.3[B]	21.9 ± 2.4[AB]	23.9 ± 3.1[A]	**0.008**
Internal antler characteristics (Wilks' λ = 0.594, *P* = 0.326)				
CBD (cm)	0.442 ± 0.102	0.441 ± 0.075	0.424 ± 0.126	0.924
CBD (%)	0.496 ± 0.101	0.525 ± 0.088	0.488 ± 0.144	0.754
CBA (%)	0.726 ± 0.106	0.751 ± 0.062	0.726 ± 0.162	0.867
Ash (%)	58.1 ± 7.4	53.7 ± 3.0	56.3 ± 5.0	0.250
Density (kg/dm^3)	1.41 ± 0.26	1.51 ± 0.15	1.63 ± 0.15	0.093
Mechanical properties (Wilks' λ = 0.724, *P* = 0.510)				
Young´s Modulus (GPa)	18.3 ± 4.0	19.2 ± 1.9	22.3 ± 5.0	0.162
Bending Strength (MPa)	223 ± 50	237 ± 22	263 ± 56	0.190
Work (KJ/m^2)	29.2 ± 9.2	31.3 ± 3.7	34.1 ± 7.1	0.359
Impact (KJ/m^2)	14.4 ± 2.8	13.4 ± 3.7	15.3 ± 3.4	0.504
Chemical composition (Wilks' λ = 0.022, *P* = 0.131)				
Ca (g/100g)	25.2 ± 2.1	25.5 ± 1.9	26.1 ± 1.7	0.666
P (g/100g)	11.0 ± 1.0	10.9 ± 1.1	11.3 ± 0.8	0.764
Ca/P	2.230 ± 0.072	2.335 ± 0.066	2.311 ± 0.042	0.420
Mg (g/100g)	0.569 ± 0.058	0.581 ± 0.044	0.608 ± 0.047	0.268
Na (g/100g)	0.683 ± 0.047	0.721 ± 0.059	0.733 ± 0.058	0.137
K (g/100g)	0.0829 ± 0.0059	0.0814 ± 0.0060	0.0819 ± 0.0054	0.849
S (g/100g)	0.199 ± 0.022	0.199 ± 0.015	0.191 ± 0.016	0.614
B (mg/Kg)	0.683 ± 0.371	0.989 ± 1.321	0.707 ± 0.189	0.670
Cu (mg/Kg)	0.480 ± 0.150[A]	0.384 ± 0.116[AB]	0.318 ± 0.075[B]	**0.029**
Fe (mg/Kg)	25.4 ± 25.3	19.9 ± 9.4	18.8 ± 16.1	0.716
Mn (mg/Kg)	26.3 ± 1.7	26.3 ± 1.2	27.0 ± 0.9	0.510
Sr (mg/Kg)	203 ± 29	218 ± 23	226 ± 23	0.174
Zn (mg/Kg)	45.8 ± 4.6	46.2 ± 4.0	47.8 ± 3.1	0.566

Superscripts (A, B, AB) indicate statistical differences between groups.
CBD: Cortical bone diameter; CBA: Cortical bone area; Ca/P: Ca to P ratio.

The supplementation of Lys+Met group shows the highest antler burr perimeter (23.9 ± 3.1 cm) compared to Lysine (5 g) group (21.9 ± 2.4 cm). And the control group was the lowest (19.6 ± 2.3 cm). No significant effects were found on the internal and mechanical antler characteristics. However, the supplementation Lys+Met tends to improve these characteristics compared to the two other groups.
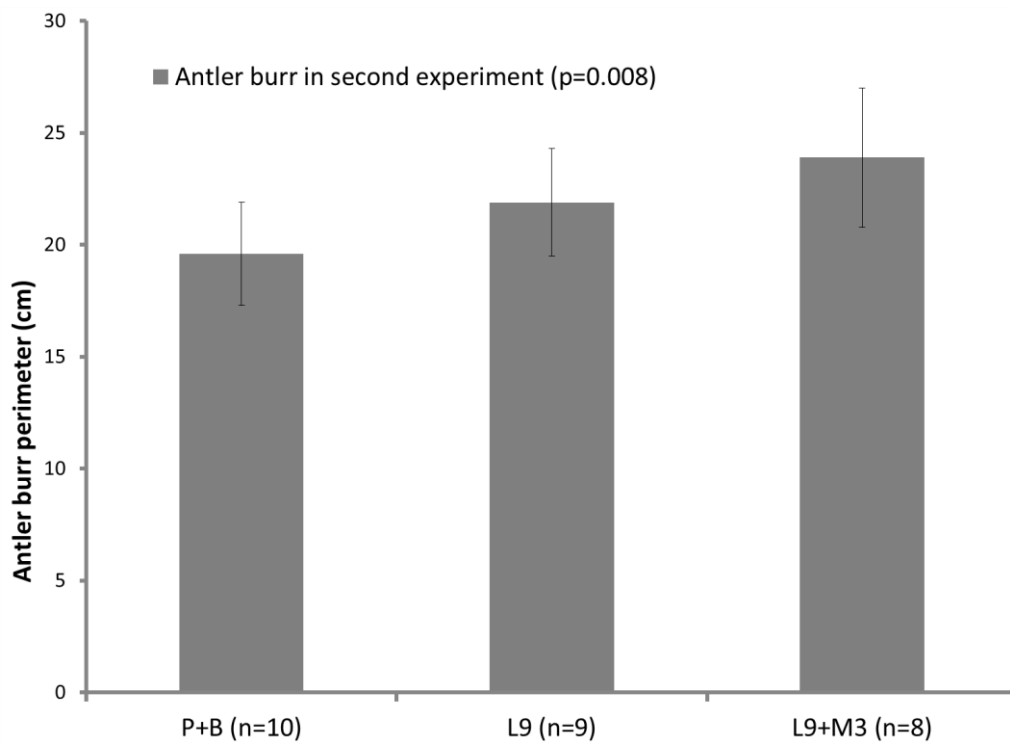

Figure 8. Antler burr perimeter (MEAN±SE) of the three experimental groups in 2016. Tukey test highlighted significant differences between the groups P+B and L9+M3 (see Table 4 for more details). (P-Pasture; B-Barley; L-Lysine; M-Methionine).

Significant differences also found for the content of Copper (Cu), (p=0.029). The highest level was in the control group and decreased successively to the group Lys+Met (Figure 9).

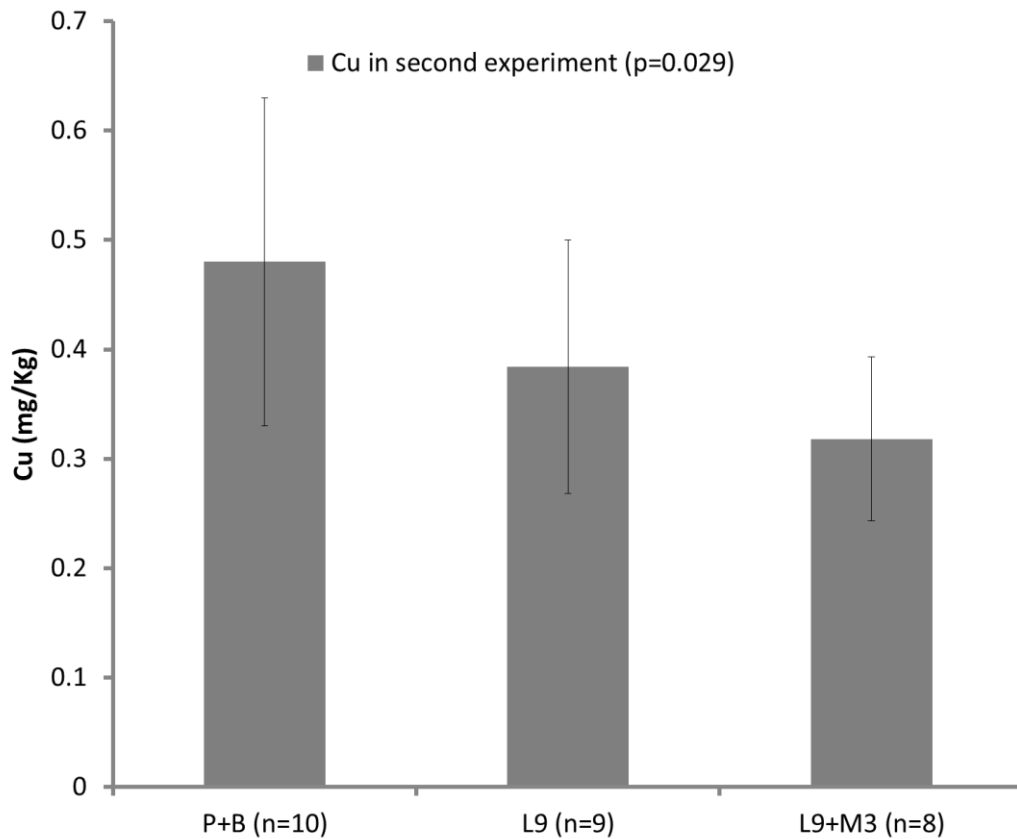
Figure 9. Level of Cu (MEAN±SE) of the three experimental groups in 2016. Tukey test highlighted significant differences between the groups P+B and L9+M3 (see Table 4 for more details). (P-Pasture; B-Barley; L-Lysine; M-Methionine).

In this experiment, multivariate GLM revealed an effect of body mass on the external antler characteristics, and mechanical properties. However, the supplementation treatment had a greater effect than in the first experiment after controlling for the body mass of the animals, significantly affecting the chemical composition and marginally affecting the external antler characteristics as shown in Table 5 below.

Table 5. Multivariate General Linear Models showing the effect of body mass at the beginning of the experiment and treatment (different diet supplementation regimes; see text) on the antler characteristics of fallow deer yearlings in the second experiment described (2016).

Multivariate General Linear Models *Second Experiment (2016)*		
	Wilk´s λ (p-value)	Pillai´s Trace (p-value)
External Antler Characteristics		
Intercept	$0.565 \ (0.024^*)$	$0.435 \ (0.024^*)$
Body mass (kg)	$0.551 \ (0.020^*)$	$0.449 \ (0.020^*)$
Treatment	$0.518 \ (0.084^\dagger)$	$0.556 \ (0.069^\dagger)$
Internal Antler Characteristics – Not significant		
Mechanical properties		
Intercept	$0.829 \ (0.497^{ns})$	$0.171 \ (0.497^{ns})$
Body mass (kg)	$0.590 \ (0.050^*)$	$0.410 \ (0.050^*)$
Treatment	$(^{ns})$	$(^{ns})$
Chemical composition		
Intercept	$<0.001 \ (<0.001^{***})$	$1.000 \ (<0.001^{***})$
Body mass (kg)	$(^{ns})$	$(^{ns})$
Treatment	$0.030 \ (0.033^*)$	$1.566 \ (0.050^*)$

***, **, * and † indicates significance at $p < 0.001$, 0.05 and 0.1, respectively.

4.3. Effect of RPAAs between the two experiments

In both experiments, the level of Zinc (average 46.6 mg/Kg) of all groups was very low (Table 2 and Table 4). The effectiveness of RPAAs was clearly visible that in the second experiment (2016), RPAAs, had more effects on antler characteristics. For instance, we illustrated this significant tendency on the improvement on external antler characteristics (antler weight, antler length, antler burr perimeters) between the two experiments ash shown in Figure A4; Figure A5; Figure A6 in Appendix 4) successively.

Moreover, level of Manganese and Ca contents seem correlated both across years. Within lower Ca in all groups in first year experiment, was also in accordance with the lower level Mn (Table 2 and Table 4). The same for the second experiment, these two minerals were in similar amount. And they were higher than the first experiment.

5. Discussion

Supplementation of Ruminally Protected Amino Acids (RPAAs) has limited effects on antler characteristics. Nevertheless, these effects were found mainly in the second experiment (2016), which is a quite interesting result. At first glance, it may be argued that this is because in the second experiment the supplementation with RPAAs was more adequate (including Met) and with higher levels of inclusion (*i.e.*, 9 *vs* 5 g/day of Lys). However, as indicated previously, the overall performance was greater during the first year probably due to better conditions during early growth. Our only explanation for the differences found between years is that severe drought occurred in 2015 (CHMI 2015), causing lower availability of pastures and leading to poor calf growth already at the start of our experiments. The lower growth in the second year may have constrained antler growth, which on the other hand allows finding greater effects of the RPAAs supplementation in case that, indeed, these are beneficial for antler growth. On the contrary, significant effects on antlers were not found in animals with greater performance (2015 experiment). In fact, our results would be then relatively similar to those observed by Huang et al. (2015*b*). These authors reported that supplementation of Lys+Met increased weight gains in sika deer calves only when they were fed a low quality diet; however, when deer were fed a high crude protein diet there was no effect on body weight or average daily gains.

Low Zn content is considered an indicator of fully completed antler growth. Zinc is involved in bone mineralization through the enzyme alkaline-phosphatase (Hove et al. 1940), and thus, its presence in the antler in high levels is an indicator of inadequate mineralization due to physiological exhaustion (Landete-Castillejos et al. 2012*a*). For instance, red deer antler under poor diet quality, Zinc content is very high (96 mg/kg) (Landete-Castillejos et al. 2012*b*). In both our experiments Zinc content was low (around 46 mg/kg), and very low variability was observed between years, among treatments and intra-treatments, indicating lack of physiological exhaustion. That means that all the animals involved in the experiment could build their antlers under no physiological constraints, which is also supported by the fact that supplementation treatment had no effect on the mechanical properties or the chemical composition of the antlers. This is important to adequately understand our results: if antlers were grown

under no physiological constraint, the differences observed may be indeed caused by the treatments, and not because of the different performance observed between years.

One of our predictions was a positive effect of Lys supplementation on antler growth due to its important influence as component of collagen (McDonald et al. 2011). No effect was found in the first experiment with a concentration of 5 g/day, but it was significant in the second experiment with a concentration of 9 g/day, especially for the external antler characteristics (mainly antler length). Coating material may have also limited the effectiveness of Lys in the first experiment. Lysine coated with dehydrated fatty acid has lower release of nitrogen compared to other coating materials like pH-sensitive polymers (Rossi et al. 2003). Thus, increasing Lys supplementation level in addition to Met produced much greater positive effects in antler weight and burr perimeter. Already Merchen and Titgemey (1992) reviewed the greater effect of supplementing limiting amino acids in mixtures with two or more of them on growth and feed efficiency of growing steers and lambs.

Not only Lys plays a key role in the cartilage formation and further mineralization. Manganese is essential in the synthesis of the mucopolysaccharides of cartilage (Leach and Muenster 1962): rats with low Mn content in diet have bone with low Ca content (Strause et al. 1986), and a supplementation of Mn produced antler with greater Ca content in red deer (Cappelli et al. 2015). Manganese and Ca contents seem correlated both across years and within experiments, but contrary to Landete-Castillejos et al. (2010) or Cappelli et al. (2015) the variability in these minerals did not produce differences in the mechanical properties.

Finally, Cu plays an essential role in the maturation of collagen, specifically the synthesis of lysine-derived crosslinks (Hyun et al. 2004); thus, deficiency increases bone fragility (Opsahl et al. 1982). It has been also recently proposed a potential positive effect of Cu supplementation on antlers (cortical thickness; Gambín et al. 2017). Nevertheless, in our second experiment, supplementation of Lys (9 g/day) and Lys+Met produced a significant decrease of Cu content, and again this had no consequences on antler structure or mechanical properties. Thus, an indirect effect of RPAA supplementation on antler growth mediated by interaction with key minerals can be discarded.

One of the most interesting positive effects is that on antler burr. Antler burr is the connection between antler and pedicle, important for blood supply during antler growth (Goss 1983; Kierdorf et al. 2003, 2007). It is attached to the periosteum, also known as antlerogenic periosteum (Goss 1983), which supplies the major components for the early growth of the antler (Brown 1992). The bigger the burr the better the pedicle, and this has positive long term effects on future antlers (Gómez et al. 2006).

Lysine and Lys+Met supplementation positively affected antler size also after controlling for body characteristics (initial body mass). These effects were more intense again during the second experiment: marginal effect on chemical composition during the first experiment, whilst significant effect on chemical composition and marginally significant effect on external antler characteristics during the second. This suggests that supplementation of amino acids have a direct effect on antlers. However, since we could not use in our analyses the body weight at the end of antler growth we cannot discard the existence of an indirect effect of AAs on antler mediated by positive effects on body characteristics (Gómez et al. 2012). Nevertheless, Mendoza-Nazar et al. (2012) showed positive effects on average daily gains of 4.5 g/day Met supplementation in red deer (although negative effects when supplementing 2.5 g/day), although these were not reflected on positive effect on antler growth. That means that the only two available results on this matter (Mendoza-Nazar et al. 2012 and this study), both suggest that the effects of AAs on antler is direct, and not mediated by body mass.

In summary, our results indicate that RPAAs have limited effects on first antler growth, and these effects are more evident in worst growth conditions and under combined Lys+Met supplementation. As suggested by Mendoza-Nazar et al. (2012) and Huang et al. (2015*a*, 2015*b*), our results (based on a larger sample size) support that there is potential for RPAAs to improve deer production especially in nutritionally poor environments.

6.   Conclusions

The Ruminally Protected Amino Acids, Lysine and Methionine, had significant effects on antler quality. With the increase of the amount and more inclusion of limiting amino acids, external antler characteristics were improved. General antler characteristics, chemical and mechanical properties also tend to improve with more availability of RPAAs. Compared to previous studies, this first trial with more inclusive study showed that RPAAs could be a good candidate for deer production, particularly for improving antler quality. It is really a pioneer for further researches on different sexes, different stages of animals, for purposes of improvement of milk quality, meat quality, or for velvet antler production.

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Appendices

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Appendix 1: Composition of grass silage, pasture and barley in 2015

Table A1. Nutritional composition of the grass silage, pasture and barley supplemented to the animals in dry matter basis.

Composition (%)	Barley	Pasture[*]	Grass silage[**]
Crude protein	11.27	12.74	11.16
Crude fat	2.44	1.91	2.06
Crude fibre	6.68	31.61	34.19
Ash	2.51	8.49	9.18
Nitrogen-free compounds	77.10	45.25	43.44
Lignin	0.83	5.00	6.55
Acid detergent fibre (ADF)	7.26	35.23	21.91
Neutral detergent fibre (NDF)	30.40	65.42	30.32

[*] Mean of three samples collected and the beginning, middle and end of the experiment during the first year.

[**] Grass silage was shortly used only during the winter time in the second year of the experiments.

Appendix 2: Map of study site

Figure A1. Map of experimental place.

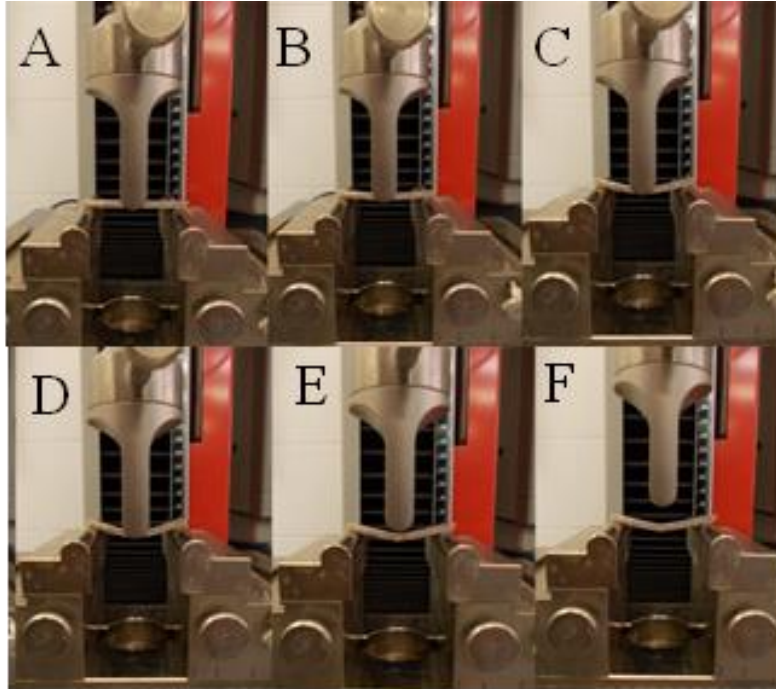# Appendix 3: Mechanical analysis

Figure A2. Process of three-point bending test until the specimen cannot resist (Photo: Jamil Cappelli).

Figure A3. Process of impact test for measuring impact energy absorption to break antler (Photo: Jamil Cappelli).

Appendix 4: The different effects of RPAAs on external antler characteristics (antler weight, antler length, antler burr perimeter)
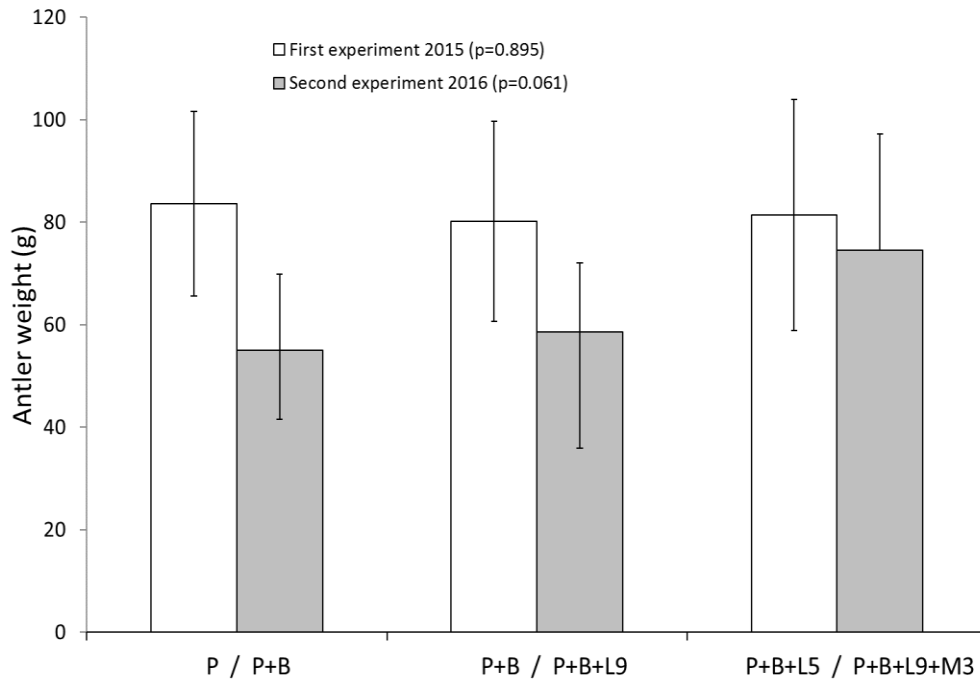

Figure A4. Antler weight (MEAN±SE) of all experimental groups in both experiments. (P-Pasture; B-Barley; L-Lysine; M-Methionine).
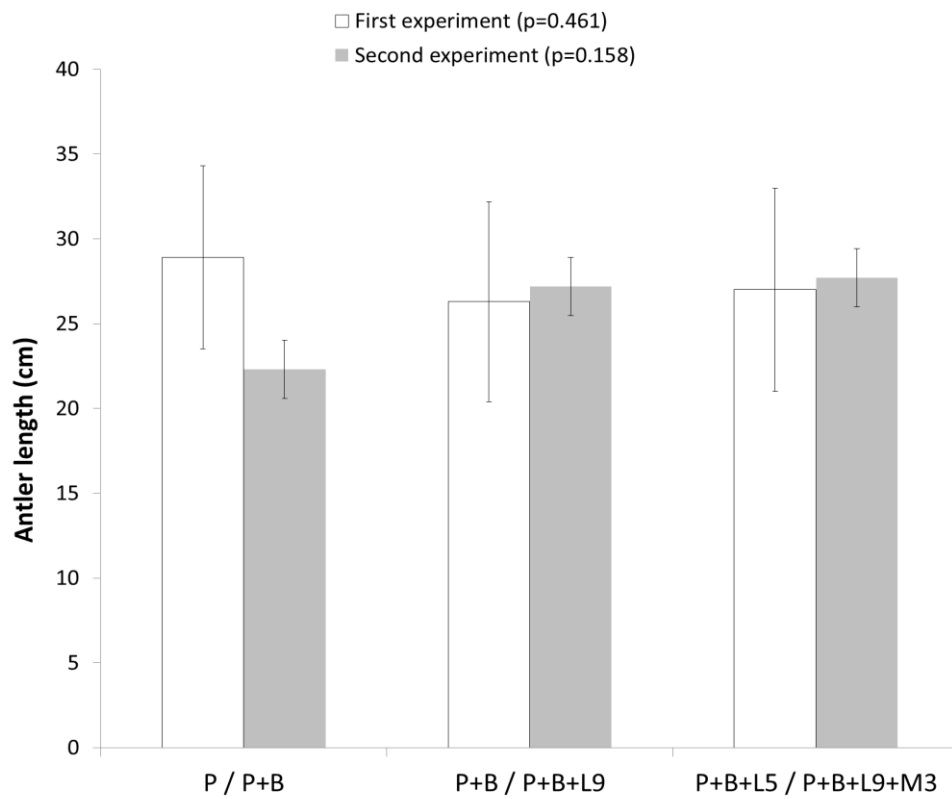
Figure A5. Antler lengths (MEAN±SE) of all experimental groups in both experiments. (P-Pasture; B-Barley; L-Lysine; M-Methionine).
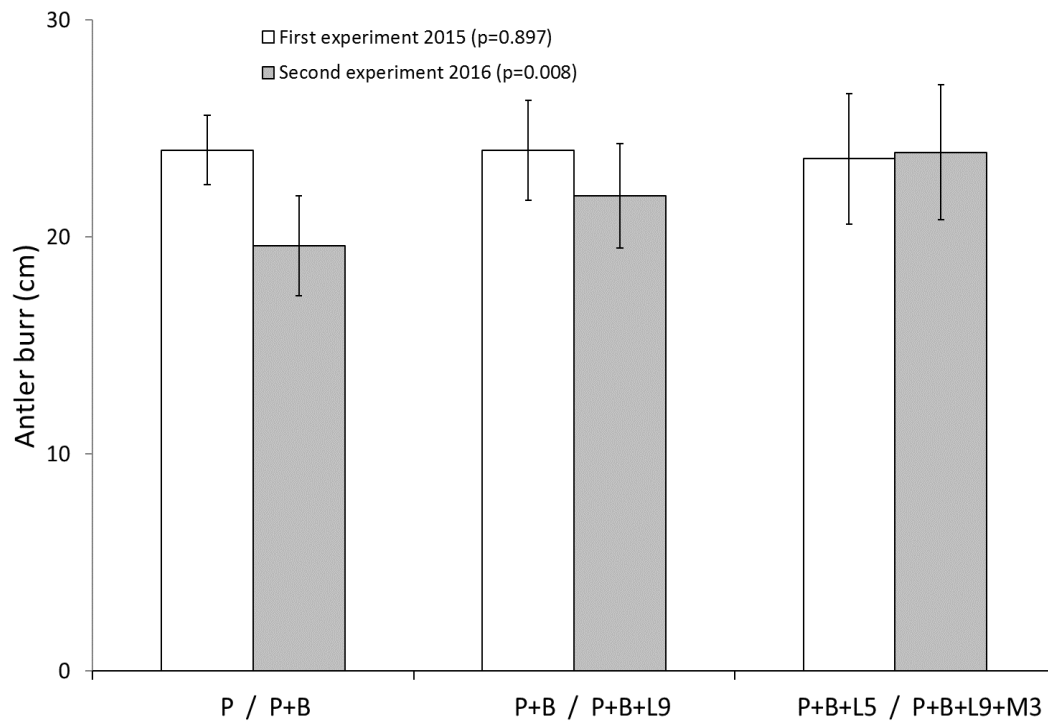
Figure A6. Antler burr perimeters (MEAN±SE) of all experimental groups in both experiments. Tukey test highlighted significant differences between the groups P+B and L9+M3 (see Table 4 for more details). (P-Pasture;

B-Barley; L-Lysine; M-Methionine).