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Investigations on age and breed-associated differences in energy intake, growth rate, body composition, haematological and biochemical values of Labrador Retrievers and Miniature Schnauzers fed different dietary levels of vitamin A

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List of Abbreviations

ALP	Alkaline Phosphatase
ALT	Alanine Aminotransferase
AST	Aspartate Aminotransferase
BW	Body Weight
DE	Digestible Energy
DM	Dry Matter
DXA	Dual-energy X-ray Absorptiometry
EDTA	Ethylenediaminetetraacetic Acid
EI	Energy Intake
F	Female
FEDIAF	Federation Européen de l'Industrie des Ailments pour Animaux Familiers
GE	Gross Energy
GFR	Glomerular Filtration Rate
GLDH	Glutamate Dehydrogenase
HGB	Haemoglobin
HCT	Haematocrit
LAB	Labrador Retriever
M	Male
MCH	Mean Cell Haemoglobin
MCHC	Mean Cell Haemoglobin Concentration
MCV	Mean Cell Volume
ME	Metabolisable Energy
MEI	Maintenance Energy Intake
MS	Miniature Schnauzer
NRC	National Research Council
PLT	Platelet
PME	Predicted Metabolisable Energy
RBC	Red Blood Cell
WBC	White Blood Cell

CHAPTER 1: General Introduction

Balanced nutrition ensuring adequate intakes of energy, protein, minerals and vitamins is essential for the optimal development of young dogs.

An adequate energy supply of growing dogs is critical for the optimal growth rate (Hedhammar et al., 1974; Kealy et al., 1992; Lust et al., 1973; Meyer and Zentek, 2013). The definition of optimal growth however is difficult and comprises both an optimal body condition and an unimpaired health status. For guidance purposes breed specific growth curves have been developed (Hawthorne et al., 2004) as well as predictive equations for the estimation of energy requirements during growth as a function of current puppy weight and expected adult weight (Blanchard et al., 1998; NRC, 2006). Excessive dietary energy can either lead to increased body fat, or alternatively increased and excessive growth leading to skeletal deformities particular in large breeds (Hedhammar et al., 1974; Krontveit et al., 2010; Lust et al., 1973; Meyer and Zentek, 1991; Meyer and Zentek, 1992).

Vitamin A is an essential fat-soluble vitamin that exhibits several biological functions such as support of vision, cellular differentiation, morphogenesis, immune function and growth. Vitamin A deficiency in dogs was one of the first vitamin deficiencies identified and studied. Clinical signs of vitamin A deficiency include anorexia, body weight loss, xerophthalmia, skin lesions and increased susceptibility to infections (NRC, 2006). Exceptionally high dietary intakes (576 µmol retinol (550 000 IU vitamin A)/4184 kJ (1000 kcal) ME) were associated with severe side effects including reduced energy intake, reduced growth rates, pain responses in the carpal and tarsal joints as well as abnormal bone development and premature closure of the epiphyseal plate (Frohring, 1935; Maddock et al., 1949; Wiersig and Swenson, 1967). Morris et al. (2012) have used haematological and biochemical variables, bone specific alkaline phosphatase, cross-linked carboxyterminal telopeptides of type I collagen as well as dual-energy X-ray absorptiometry as markers of safety of different vitamin A intakes. Further investigations looking at the impact of dietary vitamin A on the growth pattern, the body composition as well as on age and breed-associated differences in energy intake, haematology and serum biochemistry are still due. Moreover research in rodents indicates that dietary vitamin A can affect the energy utilisation (Bonet et al., 2012; Zhao et al., 2012); the potential impact on the energy metabolism in dogs and the growth velocity in young dogs specifically however remains unclear.

The first part of thesis focusses on the evaluation of the body weight and body composition development in Labrador Retriever and Miniature Schnauzer dogs during the first

year of life in response to the potential impact of dietary vitamin A intake. This question is of practical relevance due to the common use of liver as raw material in petfood which is known to be a major contributor of dietary vitamin A.

The second part of the thesis deals with the extended analyses of the effects of breed, sex and age and their interaction during the first year of life in Labrador Retriever and Miniature Schnauzer. This aspect is of clinical relevance as longitudinal data from studies in Beagles, Basenjis, German Shepherds and Labrador Retriever (Harper et al., 2003; Lawler et al., 2007; Lowseth et al., 1990; Andersen and Schalm, 1970; Kaspar and Norris, 1977; Pickrell et al., 1974; Fukuda et al., 1989; Strasser et al., 1993) have shown that marked changes in haematological and biochemical variables occurring during the first year of life. Specific data from studies investigating the changes during the growth period are scarcely available and mostly limited to studies in Beagles (Ikeuchi et al., 1991; Ishii et al., 2013; Wolford et al., 1988; Earl et al., 1973; Shifrine et al., 1973). The second part of the thesis therefore contributes to the refinement of breed and age-specific references ranges with data obtained from growing Labrador Retriever and Miniature Schnauzer.

CHAPTER 2: Literature Review

2.1. Energy

Energy from food is derived by the oxidation of fats (lipids), carbohydrates, and proteins. Energy is expressed in either kilocalories (kcal) or kilojoule (kJ): 1 kcal is equivalent to 4.184 kJ (NRC, 2006).

2.1.1. Gross energy (GE)

The GE in a food is defined as the total chemical energy arising from complete combustion of a food in a bomb calorimeter. The heat of combustion can be predicted from the chemical analysis using standard values for the nutrients (NRC, 2006). For pet foods appropriate GE estimates for crude fat, crude protein and carbohydrate (NFE plus crude fibre) are 39.3 kJ/g, 23.8 kJ/g and 17.1 kJ/g respectively (Kienzle et al., 1999; Kienzle et al., 2002; Schrag, 1999).

2.1.2. Digestible energy (DE)

In animal experiments the difference between the GE intake and the GE lost in faeces is used to determine the DE of a food. For this GE of food and faeces is determined by complete combustion in a bomb calorimeter. Alternatively DE can be calculated by multiplication of GE with the percentage energy digestibility. Equations to estimate energy digestibility as a function of fibre have been based mainly on crude fibre (CF) analysis for practical reasons: CF is used in labelling the pet food and the methodology is well established with the added benefit of being cheap and easy to perform (NRC, 2006).

2.1.3. Metabolisable energy (ME)

The ME of a food is the DE less the energy lost in urine and combustible gases. Fermentation losses by gas can be neglected in dogs and cats, therefore only the collection of faeces and urine is required. In order to avoid the use of metabolic cages it is common to collect faeces only and to make a correction for predicted energy losses for protein in urine. A subtraction of 5.20 kJ per g digestible protein or 4.34 kJ per g crude protein respectively is made for dogs using a mean protein digestibility of 83.5% (Kienzle et al., 1998).

2.1.4. Predictive equation for the calculation of ME in pet food

For the calculation of ME (MJ/kg) in the diet used in this study the following 4-step equation for dogs (Kienzle et al., 1998) has been used:

1. Calculation of GE

GE (MJ/kg) = (0.02385 x g crude protein/kg) + (0.03933 x g crude fat/kg) + [0.01715 x (g NFE/kg + g crude fibre/kg)]

2. Digestibility of GE (%):

Percentage % energy digestibility = 91.2 – (1.43 x % crude fibre in dry matter)

3. Calculation of DE:

DE (MJ/kg) = (GE (MJ/kg) x digestibility of GE (%)) / 100

4. Conversion into metabolizable energy:

ME (MJ/kg) = DE (MJ/kg) – (0.00434 x g crude protein/kg)

It has to be noted, that the predictive equation relies on determination of dietary components by validated official methods (Regulation (EC) No 152/2009).

2.2. Energy requirements

2.2.1. Basal metabolic rate

Basal metabolic rate is defined as the energy required maintaining homeostasis in an animal in a post absorptive state (ideally after an overnight fast) that is lying down but awake in a thermoneutral environment to which it has been acclimatized (Blaxter, 1989).

2.2.2. Maintenance energy requirement (MER)

The MER is the energy required to support energy equilibrium (where ME intake equals heat production) over a long period of time (Blaxter, 1989). Thus MER may vary with any factor that affects heat production. It includes energy required for thermoregulation, spontaneous activity and moderate exercise (NRC, 2006).

2.2.3. Daily ME requirements for growth of puppies after weaning

Dietary energy has a key role in determining growth velocity and affects endocrine regulatory mechanisms. Adequate energy supply is a key factor for the nutrition of young dogs as has been shown in several studies with different breeds (Lust et al., 1973; Hedhammar et al., 1974; Kealy

et al., 1992; Meyer and Zentek, 2013). Data on energy intakes show considerable variability (NRC, 2006; Dobenecker, 2008). Over feeding with energy as well as severe under- or over supply of nutrients can predispose for developmental orthopaedic disorders, especially in large breeds (Hedhammar et al., 1974; Lust et al., 1973; Meyer and Zentek, 1991; Meyer and Zentek, 1992; Lawler et al., 2008).

The expected energy intake can be calculated using the equation cited by the NRC (NRC, 2006; Blanchard et al., 1998). This equation is a function of the MER and a factor based on the actual weight and the predicted mature adult weight as follows:

$$ME\ (kcal) = 130 \times BW_a^{0.75} \times 3.2 \times [e^{(-0.87p)} - 0.1]$$

Where:

$p = BW_a/BW_m$

BW_a = actual body weight at time of evaluation (kg)

BW_m = predicted mature adult weight

e = base of natural logarithm ~2.718

2.3. Body composition

Body mass can be subdivided into two or more physiologically distinct components. The traditional two compartment model divides into the fat mass and the fat free mass (Keys and Brozek, 1953; Brozek et al., 1963). This model forms the basis of the majority of the current knowledge of body composition. The assessment of body composition in the form of fat mass and fat free mass provides valuable information about the physical and metabolic status of the individual. The fat mass can be considered to represent a calorie or energy storage depot. Conversely, the fat free mass represents the majority of the metabolic active tissue of the animal. It is a heterogeneous entity consisting predominantly of water, minerals, glycogen, and protein (Elliott, 1991).

2.3.1. Dual-energy X-ray absorptiometry (DXA)

DXA has become a standard procedure for non-invasive evaluation of body composition. It allows precise and accurate measurements of bone and soft tissue body components in live animals by use of low-dose radiation. Accuracy and precision of DXA have been validated in vivo and in vivo in various species including humans, pigs, sheep, rodents, dogs and cats (Lauten et al., 2001; Speakman et al., 2001). DXA was originally developed as a tool to support the diagnosis of osteoporosis in humans allowing to detect smaller changes in bone mineral content than conventional radiography. Other advantages are the speed of measurement and the

low radiation exposure which permits serial analyses with minimal risk to the patient. Therefore DXA has unique advantage for use in nutritional studies and metabolic diseases that affect calcium-phosphorus balance in the body (Lauten et al., 2001).

2.3.2. Body condition score (BCS)

A BCS is a subjective, semi-quantitative method for assessing the animal's body composition, particularly the percentage of body fat, and for estimating the degree of over- and underweight. Different BCS systems have been developed over the years (Laflamme, 1997a; Laflamme, 1997b; German et al., 2006).

A scale of 1 (emaciated) to 9 (grossly obese) has been validated for dogs and cats and has been shown previously to correlate well with body fat mass determined by DXA (Laflamme, 1997a; Laflamme, 1997b).

The assessment of body condition of growing dogs however is more difficult compared to adult animals because a standardized system as for adult animals has not yet been validated. The assessment of ideal body condition is complicated due to the observation that increased energy intake results in increased growth rate, but it is not necessarily leading to a visually detectable change in body conformation (Dobenecker et al., 2011). Therefore BCS in puppies have to be assessed in context of body weight development versus standard growth curves (Hawthorne et al., 2004).

2.4. Vitamin A

2.4.1. Biological Function

Vitamin A is an essential fat soluble vitamin that has functions supporting vision, growth, cellular differentiation, morphogenesis and immune function. All of these functions can be maintained with dietary retinol, retinal or retinal aldehyde since these compounds are interconvertable by animals (Goldy et al., 1996; NRC, 2006).

2.4.2. Absorption and Metabolism

Vitamin A (retinol) is found only in feedstuffs of animal origin, e.g. liver. Feeds of plant origin, e.g. carrots, only contain ß-carotene, a precursor that can be converted into vitamin A by dogs. Beta-carotene and free retinol are taken up by the small intestine enterocytes and largely converted to retinyl esters. These cells then release chylomicrons into the lymph that contain a mixture of triacylglycerols, apolipoproteins and retinyl esters along with unesterified retinol (NRC, 2006).

The dog, unlike non-carnivorous species such as humans or rodents, transports vitamin A in the plasma predominantly in the form of retinyl esters, in both adequate and vitamin A-deprived states (Wilson et al., 1987). In human subjects, retinyl esters are only detected in the plasma in cases of intoxication or following a vitamin A-rich meal (Schweigert and Bok, 2000). The concentrations of retinol found in dog serum are unaffected by dietary vitamin A intake, whereas the concentrations of serum retinyl esters have been shown to parallel the concentrations of vitamin A in the diet (Schweigert and Bok, 2000). In the dog, excess vitamin A is stored in esterified form in lipid droplets contained within the hepatic stellate cells as well as the kidneys (Raila et al., 2000). In addition to the unusual mechanism of vitamin A transport, dogs, unlike humans (Lawrie et al., 1941), excrete vitamin A in the urine (Worden et al., 1955) as both retinol and retinyl esters (Morris et al., 2012; Schweigert et al., 1991).

2.4.3. Effects of vitamin A on energy metabolism and body composition

Research in rodents has shown that retinoic acid, the carboxylic acid form of vitamin A, can reduce body adiposity by enhancing fat mobilisation and energy utilization systemically, in tissues including brown and white adipose tissues, skeletal muscle and the liver. These effects seem to be linked to both multiple genomic and non-genomic mechanisms, notably by impacting on retinoic acid receptor, peroxisome proliferator-activated receptor and liver X receptor signalling, and on p38 mitogen-activated protein kinase and possibly AMP-activated protein kinase activity in key tissues (Amengual et al., 2008; Bonet et al., 2012).

Thermogenesis in brown adipose tissue promotes energy expenditure and is important for thermoregulation and for the control of energy balance (Fruhbeck et al., 2009). The positive effect of retinoic acid on the thermogenic capacity of brown adipose tissue seems to be linked with the induction of uncoupling protein 1 (thermogenin) (Bonet et al., 2000; Felipe et al., 2003). As reported previously (Holloway et al., 1985) functional brown adipose tissue is present at least to 12 month of age in the dog, the effect of retinoic acid on the thermogenic capacity however remains unclear.

Skeletal muscle plays a key role in glucose and lipid metabolism and accounts for ~ 50% of the total energy expenditure in mammals. In mice retinoic acid has been shown to enhance the capability for lipid oxidation in muscle tissue (Amengual et al., 2008). Additionally, it has been shown that muscle uncoupling protein 3 expression responds to both the fat and the vitamin A load of the diet in mice (Felipe et al., 2003), which suggests a role for the novel uncoupling proteins in the handling of lipids as fuel substrates (Samec et al., 1998).

In mice retinoic acid-induced loss of body weight and body fat occurred despite unchanged or even increased energy intake in combination with increases in body temperature (Bonet et al., 2012).

2.5. Haematology

2.5.1. Complete Blood Count and Reference Ranges

The complete blood count describes a haematological profile of tests used to describe the quantity and morphology of the cellular elements in blood and a few substances in plasma. Reference ranges are used to determine if a test result appears normal or abnormal. However reference ranges in haematology and clinical chemistry books and articles may deviate from the reference ranges for a certain institution due to differences in instruments, analytical methods or unique characteristics of the institution's population, e.g. puppies. Therefore reference ranges should be revised every time a lab changes instruments, methods or to account for factors such as age, breed and sex, which can be significant (Willard and Tvedten, 2012).

2.5.2. General age related changes on haematological values during the first 12 month of age

Prominent age related changes occur after birth. At birth haemoglobin (HGB), haematocrit (HCT) and red blood cells (RBC) are around the lower reference range limit for adult dogs, but decline rapidly over about the first 2 month of life. After this, values start to increase, generally reaching adult values by approximately 6 months to 1 year (Meinkoth and Clinkebeared, 2000). At birth canine RBCs are very large with a mean cell volume (MCV) of about 95 femtoliters. The MCV decreases to adult values by 2 to 3 month of age (Andersen and Schalm, 1970; Meinkoth and Clinkebeared, 2000; Willard and Tvedten, 2012).

2.5.3. Overview of studies investigating age and breed related variations in haematological values

Numerous studies have investigated variations in haematological values in various breeds and different ages. An overview of studies investigating changes in growing dogs is provided in **Table 2.1.**

Table 2.1: Overview of studies investigating age and breed related variations in haematological values in growing dogs

Breeds	**Age range assessed**	**Results**	**Reference**
Beagles	2 months to 4.5 years	HCT increased by 43 % from 2 to 8 month of age. HGB and RBC followed same pattern. White blood cells (WBC) decreased with age.	Bulgin et al., 1970
Basenjis	18 days to 10 years	HCT, HGB and RBC progressively increased until 6 month of age. HCT in Basenjis older than 6 month exceeded mean HCT values for general dog populations. WBC of all ages in Basenjis older than 7 weeks exceeded normal values by 20 % or more.	Ewing et al. 1972
Beagles	1 to 56 days	Highly significant ($P < 0.01$) age differences for HCT, HGB, RBC and WBC. HCT, HGB and RBC decreased from birth to 4 weeks and then increased during sixth and eights week. WBC increase until week 6, then starting decrease. Significant ($P < 0.05$) age differences for platelet (PLT). PLT increase until week 6.	Earl et al., 1973
Beagles, German Shepherds and Golden Retrievers	1 day to 58 days	RBC decreased until 10 days of life and then increased until end of trial. HCT and HGB decreased after birth reaching a minimum around 28 to 33 days. MCH and MCV decreased to adult values by 58 days. German Shepherds showed lower RBC, HCT and HGB.	Lund et al., 2000

Table 2.1 (continued)

Breeds	**Age range assessed**	**Results**	**Reference**
Beagles and Labrador Retriever	22 days to 15 years	WBC highest in 3.1 to 8 week old puppies followed by subsequent decrease. Beagles < 1 year of age had higher WBC compared to Labrador Retriever. HCT, HGB und RBC increased during 1st year of life.	Harper et al., 2003
Beagles	8 weeks to 14 months;	HCT, HGB and RBC lower in weanling puppies.	Swanson et al., 2004
Labrador Retrievers	8 weeks to 12 years	HCT, HGB and RBC increased during growth.	Lawler et al., 2007
Greyhounds	5 to 13 months	RBC positively correlated with age; PLT negatively correlated with age; HCT, HGB and RBC above canine reference range from 9 to 10month of age.	Shiel et al., 2007
Beagles	0 to 6 months	HCT, HGB, RBC and mean cell haemoglobin concentration (MCHC) increased; MCV, mean cell haemoglobin (MCH) and WBC decreased from 0 to 2 month. WBC tendency to further decrease with age.	Ishii et al., 2012

2.6. Blood Biochemistry

2.6.1. General age related changes on blood chemical values during the first 12 month of age

At birth, both albumin and globulin concentrations are low compared to older dogs. Following ingestion of colostrum, globulin concentrations increase as a result of absorption of immunoglobulins (Weiss and Wardrop, 2010). Values gradually increase until adulthood (Willard and Tvedten, 2012), which seems to be related to an increase in protein synthesis (and in particular albumin synthesis) as known from research in young rats (Wise and Oliver, 1967; Czajka et al., 1970; Wolford et al., 1988). The lower plasma protein levels in young animals might be explained by greater nutritional demands compared with adult animals because of growth requirements (Weiss and Wardrop, 2010)

The serum total and ionized calcium concentration can be 0.1mmol/L higher in young dogs (i.e. < 12 month old), especially in large and giant breeds, than in adults (Willard and Tvedten, 2012). This seems to be influenced by growth hormone stimulated production of $1,25(OH)_2D_3$ or by decreased clearance of $1,25(OH)_2D_3$ respectively (Goff et al., 1990).

Young animals tend to have higher phosphate concentrations than older animals in consequence of increased renal tubular resorption of phosphate (Russo and Nash, 1980) which is at least in part due to the increased growth hormone levels (Haramati et al., 1990; Mulroney et al., 1989). Phosphate concentration decrease to adult values by 12 month of age (Willard and Tvedten, 2012).

High alkaline phosphatase (ALP) activity in young animals has been linked to high osteoblast activity during growth (Fujise et al., 1988). In bone, ALP is involved in mineralization, possibly by catalysing the formation of phosphate from pyrophosphate (Willard and Tvedten, 2012). Bone ALP activity in puppies drops dramatically within the first 3 months, reaching a magnitude of activity consistent with that of the adult dog by approximately 15 months (Sanecki et al., 1993).

Creatinine values are lower in puppies 2 to 6 month of age due to less muscle mass and higher glomerular filtration rate (GFR) (Willard and Tvedten, 2012).

Urea concentrations above adult values are observed after birth (Kuhl et al., 2000) followed by a significant decrease until 3 months of age (Wolford et al., 1988). Urea concentrations in young animals remain low due to the high anabolic rate (Poffenbarger et al., 1990) which has been associated with the positive effect of growth hormone on tissue net uptake of amino nitrogen and a reduced hepatic conversion of amino nitrogen to urea nitrogen (Grofte et al., 1994; Grofte et al., 1998).

Cholesterol and triglyceride levels in young puppies (≤ 6 weeks) are reported to be higher compared to those measured in later life due to the higher fat content of the milk diet and frequent feeding times in suckling puppies (Wolford et al., 1988; Kuhl et al., 2000).

2.6.2. Overview of studies showing age and breed related changes in biochemical test results

Table 2.2: Overview of studies investigating age and breed related variations in blood chemical values in growing dogs

Breeds	Age range assessed	Results	Reference
Basenjis	18 days to 10 years	Total protein increased with age until 12 weeks. Approximately 30% of the Basenjis older than 6 month had higher plasma protein compared to normal non-breed specific ranges.	Ewing et al., 1972
Beagles	2 to 78 months	Total protein increased between 2 and 78 month. Phosphorus decreased between 2 and 54 month. ALP activity highest in 2 to 3 month old dogs. Cholesterol increased between 11 and 78 month.	Pickrell et al., 1974
Beagles	2 weeks to 1 year	Significant urea decrease between 2 weeks and 3 months. Cholesterol and triglycerides highest during the first 6 to 8 weeks with subsequent decrease. Alanine aminotransferase (ALT) increased during this period and stabilised by 3 month of age. ALP, phosphorus and calcium decreased until 1 year. Total protein gradually increased until 1 year. Aspartate aminotransferase (AST) and creatinine increased until approx. 6 month.	Wolford et al., 1988

Table 2.2. (continued)

Breeds	Age range assessed	Results	Reference
Beagles	6 to 12 month	Total protein gradually increased. ALP and phosphorus decreased. No significant changes of albumin, creatinine, cholesterol and calcium.	Ikeuchi et al., 1991
Beagles, GSDs[a], Golden Retrievers	1 day to 58 days	Total protein and albumin below adult values, increase as of 10 days; lowest values in Golden Retrievers. Urea decreased until 33 days, followed by increase until end of trial; lower urea in GSDs. Creatinine decreased until 10 days, moderate increase until end of trial; values below adult values with lowest values in Beagles. Triglycerides decreased until week 8. Phosphorus increased until 8 to 10 days; values above adult values. ALP highest in 1 to 3 day old puppies, decrease by 8 to 10 days; lowest ALP activity in Beagles. ALT and Glutamate dehydrogenase (GLDH) initial decrease, followed by increase as of day 33. ALT activity lower than in adults. GLDH activity higher than in adults.	Kuhl et al., 2000
Beagles and Labrador Retriever	22 days to 15 years	ALP highest in dogs until week 16, then decrease until 1 year of life. Plasma calcium and phosphorus highest in 3 to 8 week old dogs.	Harper et al., 2003

Table 2.2 (continued)

Breeds	Age range assessed	Results	Reference
		Phosphorus progressively decreased until 1 year of life. Plasma protein significantly increased during first year of life	
Beagles	8 weeks to 14 months	Total protein and creatinine below, Ca, P and ALP above normal range in weanling puppies.	Swanson et al., 2004
Labrador Retrievers	8 weeks to 12 years	Total protein, albumin, ALT, creatinine, urea and cholesterol increased; ALP, calcium, phosphorus and triglycerides decreased in first year of life.	Lawler et al., 2007
Dobermann	2 to 36 month	Total protein, albumin, creatinine, urea, and ALT increased, calcium decreased until 12 month of age. ALP decreased until 36 month. Cholesterol decreased until 24 month.	Mundim et al., 2007
Beagles	0 to 6 months	Total protein, albumin, creatinine, ALT and AST increased, cholesterol, triglycerides calcium and phosphorus decreased. ALP increased from 0 to 3 month, but decreased at 6 month of age.	Ishii et al., 2012

[a] German Shepherd Dogs

Conclusion

The literature survey clearly indicates that considerable breed and age-related variability in energy requirements, haematological and blood chemical values can be observed during growth. Guidance values for the energy requirements and reference ranges for blood parameters are available, however these can't be uniformly applied to all breeds and may even potentially impacted by diet composition, e.g. different vitamin A levels. This has not been investigated in growing Labrador Retrievers and Miniature Schnauzers yet and requires further research.

CHAPTER 3: Aims and Objectives of the Thesis

The impact of vitamin A and its metabolic product retinoic acid on the energy metabolism in dogs is not clear, although data from rodents indicate an influence on both thermogenesis and lipid synthesis via its actions when bound to the retinoic acid receptor. Therefore, we evaluated the development of body weight and body composition and compared observed energy intake with predicted energy intake in response to the potential impact of dietary vitamin A intake. Our hypothesis was that energy intake and accumulation of body fat would be unaffected due to the well-documented high tolerance of dogs to high levels of dietary vitamin A.

In detail, the following objectives were addressed:

1. The possible effects of different dietary vitamin A concentrations on energy intake, growth rate and body composition during growth.
2. Breed related differences of body weight development and body composition.
3. Energy intakes of two breeds during growth.
4. Assess haematological and biochemical data investigating breed, sex and age effects and their interaction during the first year of life.

The results of the current thesis were obtained from a larger study investigating the effects of dietary vitamin A at levels up to 104.8 µmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) ME in growing dogs, and summarized in two manuscripts following in the next chapters (Chapter 4-5).

CHAPTER 4: Energy intake, growth rate and body composition of young Labrador Retrievers and Miniature Schnauzers fed different dietary levels of vitamin A

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Abstract

Research in rodents has shown that dietary vitamin A reduces body fat by enhancing fat mobilisation and energy utilisation; however, their effects in growing dogs remain unclear. In the present study, we evaluated the development of body weight and body composition and compared observed energy intake with predicted energy intake in forty-nine puppies from two breeds (twenty-four Labrador Retriever (LAB) and twenty-five Miniature Schnauzer (MS)). A total of four different diets with increasing vitamin A content between 5.24 and 104.8 μmol retinol (5000–100 000 IU vitamin A)/4184 kJ (1000 kcal) metabolisable energy were fed from the age of 8 weeks up to 52 (MS) and 78 weeks (LAB). The daily energy intake was recorded throughout the experimental period. The body condition score was evaluated weekly using a seven-category system, and food allowances were adjusted to maintain optimal body condition. Body composition was assessed at the age of 26 and 52 weeks for both breeds and at the age of 78 weeks for the LAB breed only using dual-energy X-ray absorptiometry. The growth curves of the dogs followed a breed-specific pattern. However, data on energy intake showed considerable variability between the two breeds as well as when compared with predicted energy intake. In conclusion, the data show that energy intakes of puppies particularly during early growth are highly variable; however, the growth pattern and body composition of the LAB and MS breeds are not affected by the intake of vitamin A at levels up to 104.8 μmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal).

4.1 Introduction

Adequate nutrition is a key factor for the optimal development of young dogs. Vitamin A is an essential nutrient for dogs; however, intake of dietary vitamin A can be variable and is dependent on the natural levels of vitamin A found in some raw materials used in petfood, especially liver. Previously, it has been shown that growing as well as adult dogs can tolerate a wide range of vitamin A levels in their diet[1–3] due to their capacity to increase the levels of retinyl esters in serum[4] and the ability to excrete vitamin A as both retinol and retinyl esters in the urine[3,5–7]. The impact of vitamin A and its metabolic product retinoic acid on energy metabolism in dogs is not clear, although data from rodents indicate an influence of retinoic acid on both thermogenesis and lipid synthesis via its actions when bound to the retinoic acid receptor[7,8]. The main effect seems to be linked with the induction of uncoupling protein 1 (thermogenin) in brown adipose tissue. Mouse data show an increase in lipid oxidation in

muscle tissue by retinoic acid[9], and retinoids have been shown to induce uncoupling protein 3 in the muscle tissue of mice[10].

Data on the safety of dietary vitamin A intake at levels up to 104.8 μmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) have been reported previously with regard to the markers of vitamin A metabolism, haematological and biochemical variables and dual-energy X-ray absorptiometry[3]. In the present study, we evaluated the development of body weight and body composition and compared observed energy intake with predicted energy intake in response to the potential impact of dietary vitamin A intake. Our hypothesis was that energy intake and accumulation of body fat in growing dogs would be unaffected due to the well-documented high tolerance to such levels of dietary vitamin A.

4.2 Experimental methods

The research protocol was evaluated and approved by the WALTHAM Internal Ethics Committee. The protocol has been described in detail previously[3], and the experimental set-up is summarised briefly in the present study.

4.2.1 Animals and Housing

The study was completed by forty-eight puppies from two different breeds, Labrador Retriever (LAB) and Miniature Schnauzer (MS). The puppies were group-housed with their mother until 8 weeks of age. Thereafter, they were housed in pairs in environmentally enriched kennels. The puppies had free access to an attached outdoor area, and participated daily in training and socialisation activities.

All puppies were neutered between week 36 and week 52.

4.2.2 Clinical examination

The puppies underwent physical examination before the start of the trial and every 4 weeks thereafter. Particular attention was paid to the signs of joint or muscle pain. In addition, any illness or injury between the examinations that required veterinary attention was considered as an adverse event. Adverse events were classified into ten categories: poor faecal quality; vomiting; foreign body ingestion; lameness; accident/injury; skin conditions; eye conditions;

ear conditions; dental conditions; urinary conditions. On each occasion, the type and duration of treatment were recorded.

4.2.3 Diet and feeding

The base diet used was a standard dry commercial recipe (Perfect Fit Junior; Mars GmbH) compliant with the 2008 FEDIAF (European Pet Food Industry Federation) recommendations[11] for growth and reproduction.

The predicted metabolisable energy (ME) of each batch of the diet was calculated according to the National Research Council[12], and the results of this calculation were multiplied by the factor 4.184 to convert kcal to kJ. The calculated mean energy density of the diet was 16 640 (SD 582) kJ/kg (n=10 batches). Details about the nutrient composition and feeding regimen have been provided by Morris et al.[3]. Briefly, the maternal bitch was fed the base diet throughout lactation until the puppies were fully weaned to the base diet at 6 weeks of age. Between weeks 8 and 26, the puppies were offered their daily ration in three meals and from week 27 until the end of the trial (week 52 for the MS breed and week 78 for the LAB breed) in two meals. Free access to drinking-water was given at all times.

The allocation of puppies to the diets with four different levels of vitamin A has been described by Morris et al.[3]. In brief, the diets contained the following levels of vitamin A per 4184 kJ (1000 kcal) ME: 5.24, 13.10, 78.60 and 104·8 μmol retinol (5000, 12 500, 75 000 and 100 000 IU vitamin A)/4184 kJ (1000 kcal), respectively.

Although not fully validated in puppies, the body condition score was evaluated weekly using the WALTHAM S.H.A.P.E. (Size, Health And Physical Evaluation) guide[13], which is a seven-category system that uses visual and palpable characteristics to determine the amount of subcutaneous and abdominal fat. Each category is assigned an alphabetical character from A (underweight) to G (obese), with D representing ideal where the outline of the ribs can be felt while applying light pressure when running the fingertips against the direction of the coat.

Feeding allowances were calculated from the amounts consumed during the previous week and adjusted weekly with the aim of maintaining the puppies on standard growth curves with ideal body condition scores[13,14].

4.2.4 Body composition

The puppies of both breeds were scanned at 26 and 52 weeks of age, and the LAB puppies were also scanned at 78 weeks by means of dual-energy X-ray absorptiometry (total body software

package, Lunar Hologic QDR-1000W; GE Healthcare). The puppies were fasted for at least 16 h and sedated with Torbugesic (0.3 mg/kg; Pfizer Animal Health), Medetomidine (MS breed: 20 mg/kg, LAB breed: 5 mg/kg; Pfizer Animal Health) and Midazolam (MS breed: x0.25 mg/kg, LAB breed: 0.20 mg/kg; Roche Limited), and reversed with Atipamezole (0.1 mg/kg; Pfizer Animal Health). Estimates of lean body mass and fat mass were derived using the provided proprietary software package.

4.2.5 Data analysis and statistics

Data were analysed by means of a linear mixed model analysis including the fixed terms sample number (through time), sex, breed and dietary group, and also the baseline measurement of the variable was modelled. The model included the random terms dog and litter (taking account of possible similarities between littermates), with a correlation between successive samples (within an individual dog) accounted for by using an appropriate correlation structure (determined using graphical methods of residuals from a model with an identity correlation structure). Where necessary, data were log transformed to improve the distribution of the data, as assessed by residual plots. Non-significant terms were removed from the model.

Statistical analyses were performed using R2.10.1 (R Foundation for Statistical Computing) using the nlme package[15,16].

For the comparison of the energy intake of puppies in the trial with their predicted energy intake[12,17], a linear mixed model was first fitted to the energy intake data with breed, week and breed*week interaction as fixed factors, litter and dog (nested in litter) as random factors, and an autoregressive (AR(1)) correlation structure to take account of the correlation of successive measurements within a dog.

The expected energy intake based on the equation cited by the National Research council[12,17] was then calculated for each dog at each week of age, taking the adult weight of a dog to be the final weight recorded, which was the weight at week 52 for the MS puppies and at week 78 for the LAB puppies. The mean predicted ME requirement at each week was used to construct a predicted ME curve for each breed. This was then compared with the average actual intake by fitting a linear mixed model (with the same terms as that for the energy intake data) to the difference between the observed energy intake and the predicted energy intake to identify where the predictive equation and the observed energy intake significantly diverged.

For body composition, a linear mixed model was fitted to the data with the factors breed, sex, age, vitamin A group, litter and dog ID (nested in litter). However, as the MS puppies completed the study at an earlier time point, there were no data available for this breed at week

78, which means that fitting breed and age as separate fixed factors with an interaction term in the conventional way was not possible. Therefore, a concatenated variable 'BreedAge' was created that has a level for each combination of breed and age (excluding week 78 for the MS puppies), which was used in the model. The effects of interest around breed and age (testing for breed effects, changes between the successive levels of age, and breed x age interaction effects) were tested using 'planned contrasts' that compared combinations of the levels of BreedAge.

Each body composition endpoint was initially analysed by a model containing all these terms, with litter and dog ID considered as random effects and the others as fixed effects. An autoregressive (AR(1)) correlation structure was used to account for a possible correlation between subsequent time points within a dog. Error variance was initially allowed to differ between the two breeds. Non-significant fixed terms were then subsequently removed (one by one) until a model was reached where all the fixed effects were significant (the exception to this was BreedAge, which was left in the model in order to allow the planned contrasts to be tested). The 'breed-specific' nature of the error variance was also tested for significance and replaced with a single error variance estimate if possible. The resulting model was taken to be the final model.

All endpoints were log-transformed before analysis, and means were exponentiated from the log scale to the original scale for display purposes and to ease interpretation. All endpoints were separately subjected to Bonferroni correction to account for the presence of multiple endpoints; the overall significance level used was 0.05. Data are presented as means and (Bonferroni-adjusted) 95% CI, unless otherwise stated, and *P* values are reported as Bonferroni-adjusted values. The standard error of the mean is not reported as the non-linearity of log transformation (and its inverse) implies that only means and CI can be back-transformed.

4.3 Results

4.3.1 Clinical examination

Poor faecal quality was reported occasionally, with twenty individual puppies being affected. All cases were resolved within 3 days. In the LAB breed, two cases of lameness without any apparent cause were observed; both cases were resolved following treatment in less than 7 days.

4.3.2 Growth

All puppies maintained an ideal body condition score (score D) throughout the trial (data not shown). Body weight increased with time in a breed-specific manner[14] (**Fig. 4.1**). Male MS

puppies were heavier at 2.1 (SD 0.2) kg, compared with female MS puppies at 2.0 (SD 0.2) kg at week 8, which accounted for a difference of 7% between sexes. This difference increased to 16% at the end of the trial in week 52. The final body weight during the observation period was 9.4 (SD 1.2) kg in male MS puppies and 7.9 (SD 1.0) kg in females. At week 8, the average body weight of the male LAB puppies was 4.5 (SD 0.4) kg and the average body weight of females was 4.4 (SD 0.3) kg, which suggested a 2% difference between sexes. At the end of the trial at week 78, this difference increased to 10% with a final body weight of 28.9 (SD 3.1) kg in male LAB puppies and 25.9 (SD 1.6) kg in females. There were no significant differences observed in body weight between the groups treated with different levels of dietary vitamin A at any time point (after accounting for sex and breed) as reported previously[3].

4.3.3 Intake of metabolisable energy

There were no differences in energy intake per kg body weight or per kg metabolic body weight between the groups fed different levels of dietary vitamin A[3] (P<0.05). The mean energy allowance to maintain optimal body condition was 890 kJ/kg body weight$^{0·75}$ per d in the MS puppies at the beginning of the study at week 8, declining to 563 kJ/kg body weight$^{0·75}$ per d at week 52. In the LAB puppies, the mean energy intake declined from 1263 to 599 kJ/kg body weight$^{0·75}$ per d at week 78 (**Fig. 4.2**). The relative energy intake (based on kJ/kg body weight per d) in the MS puppies at 25% of the mature body weight was 1.58 times the maintenance energy requirement at week 52. In the LAB puppies, it was 1.91 times the energy intake compared with week 78. This factor increased to 1.77 in the MS puppies and decreased to 1.65 in the LAB puppies at 50% of mature body weight. The factor decreased in both breeds to 1.33 and 1.18, respectively, at 80% of mature body weight. The energy intake of the LAB breed reached a mature energy requirement of 599 kJ/kg body weight$^{0·75}$ per d at 90% of mature body weight (**Table 4.1**). The energy intake per kg metabolic body weight in the MS breed deviated from the calculated allowance as derived from the predictive equation[12,17] from week 8 to 15 and week 18 to 23 inclusive. Until week 15, energy intake was significantly lower (P<0.01) than the calculated allowance; between weeks 18 and 23 inclusive, energy intake was significantly higher (P<0.05) than the calculated allowance (**Fig. 4.3**). The ME intake of the LAB breed was significantly lower (P<0.01) than the calculated allowance only at week 9. Between weeks 19 and 26 inclusive, energy intake was significantly higher (P<0.05) than the predictions of the National Research Council (**Fig. 4.4**).

4.3.4 Body composition

There was no significant difference in the percentage of body fat between the two breeds of either sex at any of week 26, week 52 and week 78 (week 78 for the LAB puppies only) (**Table 4.2**). The planned contrasts for breed and the BreedAge interaction showed no significant effect between the breeds. Although the overall *P* value for the combinations of the levels of BreedAge and sex showed a significant effect in our model ($P<0.01$), post hoc testing was not able to determine which combination of breed, age and sex was relevant for the effect. This may be because the interaction term has a large number of levels and the post hoc procedure consequently involves a large number of pairwise comparisons; this means that the likelihood of false positives is much increased and the Tukey procedure is necessarily more conservative to account for this. There was no significant difference in the percentage of lean body mass between the MS and LAB puppies at weeks 26 and 52. The percentage of lean body mass in the LAB puppies at week 78 was significantly higher than that in the MS puppies at week 26 (**Table 4.3**).

4.4 Discussion

The aim of the present study was to examine the possible effects of different dietary vitamin A concentrations on energy intake, growth rate and body composition during growth, using MS as a typical breed of small stature and LAB as a large breed. Energy intake was measured during the entire growth phase provided that energy allocation was adjusted to maintain optimal body condition. Body composition was assessed twice during the first year of life for both breeds and at 78 weeks of age for the LAB breed due to the prolonged growth phase. Because of the well-documented high tolerance of dogs to vitamin A intakes at levels up to 104.8 μmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal), we hypothesised that there would be no effect at such levels of vitamin A on energy intake and body composition in growing dogs. This assumption was confirmed in principle by experimental data, even if questions on basic mechanisms of the effects of vitamin A in dogs cannot yet be answered[7-9].

The growth rate of the LAB and MS puppies in the present study was comparable with the data from other studies[14,18–24]. Growth rate is significantly influenced by energy intake. Overfeeding with energy as well as severe under- or over supply of nutrients can predispose for developmental orthopaedic disorders, especially in large breeds[21,25–28]. Lifelong overfeeding has also been shown to have an impact on the occurrence of skeletal and other dysfunctions in adulthood and senescence[29]. Dietary energy has a key role in determining growth velocity and

affects endocrine regulatory mechanisms. The increased biomechanical load seems to increase the risk of musculoskeletal disorders in young dogs[30], although epidemiological studies are not conclusive[28]. In addition to biomechanical effects, high energy intakes affect the endocrine system, such as insulin-like growth factor I and thyroid hormones, which in turn control local growth factors together with the proliferation of chondrocytes and the subsequent mineralisation of the newly formed tissue[31]. The definition of optimal growth rates for dogs is difficult and controversial; however, it should consider not only the expected typical shape of the breed, but also an optimal animal health status. Hence, an adequate energy supply is a key factor for the nutrition of young dogs, as has been shown in several studies with different breeds[21,22,25,32]. Data on energy intakes in specific breeds show considerable variability[12,33], and there are only a few studies reporting energy intake, growth rate and body condition in the LAB breed[18–20,25,34,35]. Unlike the LAB breed, there are no data available for the energy needs of the MS breed in the growth period. Comparing the data collected in the present study with published values of other small breeds, there seems to be a relatively good agreement with the values obtained for Beagles[6,16,22,36–41]. The MS breed reached 98% of the adult body weight at week 48, which appeared in the expected range of 95–100% for this breed[14,22]. The LAB breed reached 93% of the adult body weight, with an expected range of 88–95%, respectively. However, body condition of the dogs has not been clearly defined in many studies. The assessment of body condition of growing dogs is more difficult compared with adult animals because a standardised system as for adult animals[13] has not yet been developed and significant breed-specific influences such as pelvic circumferences[33] exist. Visual assessment is complicated due to the observation that increased energy intake results in increased growth rate, but it is not necessarily leading to a visually detectable change in body conformation[37]. Therefore, body weight is not an accurate measure to assess body fat in growing dogs, and the relationship between the body condition score and body fat has been shown to be more accurate[33].

There was a good agreement between the observed energy intake in the LAB puppies and the expected values calculated by the predictive equation[12,17] up to week 18, but the equation showed an underestimation in energy requirement between weeks 19 and 26 inclusive. The energy intake of the MS puppies was significantly overestimated by the predictive equation[12,17] between weeks 8 and 15, followed by a period of significant underestimation similar to the LAB puppies between weeks 18 and 23 inclusive. The reasons

for such deviations might be breed-specific effects, housing conditions and the activity level of the dogs. In the present study, energy intake was the independent variable and feeding allowances were adjusted to maintain the puppies at an ideal body score. This approach may have led to a shift in growth rates with lower rates in the beginning followed by a compensatory phase.

The total amount of energy consumed until the end of week 52, however, remained for both breeds in a range of ±2% compared with the total predicted energy intake.

Data from beagles and foxhounds have also shown considerable deviations between observed and mathematically derived energy intakes in young puppies of both breeds[37]. In the cited study, the dogs have been fed to achieve growth curves corresponding to theoretically expected age-dependent weight development. This approach may have resulted in significant differences in energy allocation, because the growth capacity of young dogs is determined by individual factors. The dogs used in the present study were maintained under conditions that are to a certain extent similar to private households. The dogs were initially kept in groups and later in pairs, and were exercised according to age. It is often assumed that data from colony dogs differ from values for dogs in private housing. Besides the question of group housing v. individual housing with an anticipated higher activity level, it can be expected that many privately owned dogs have lower energy intakes due to the fact that the level of activity and 'intellectual challenge' is lower. However, variability in housing conditions is substantial. This could have an impact on energy expenditure[26], although no data seem to be available for growing canines. The derived values for energy allowances can be used as guidelines for practical recommendations; however, but it is interesting to see that even in this highly standardised husbandry condition, data vary considerably between individuals. For a 12-month-old MS breed, the difference between the lower and upper CI for ME intake per kg metabolic body weight was 36.3 kJ (±6%) and for the LAB breed, it was 39.7 kJ (±7 %). Therefore, recommendations and nutritional guidelines should take this variability into account.

In addition to growth rate, body composition is an essential feature for the evaluation of growth curves and energy intake. In the present study, no significant differences were found between the two breeds in the percentage of body fat. The observed data on the mean percentage of body fat in the LAB breed were 20.4% at week 26, 20.5% at week 52 d 21.8% at week 78, and were lower compared with other recent findings in growing LAB[42].

In the MS puppies, the mean percentage of body fat was 14.5% at week 26 and 14.9% at week 52. The percentage of body fat for both MS and LAB puppies in the present study was

within the range found previously in beagles and Foxhound-Boxer-Ingelheim Labradors[33]. The mean percentage of lean body mass in the LAB puppies at week 78 was 76.3 %, which corresponds well to the data reported for adult LAB from one previous study[34], where LAB fed ad libitum had a mean percentage of lean body mass of 70% compared with 82% in the food-restricted group. This indicates an adequate energy allocation, which was made on the basis of body condition status.

The possible effects of increased vitamin A intake on growth intensity and body composition were of particular interest because there is some evidence in the literature of an effect of vitamin A and its metabolites on the energy balance of animals[9]. Based on available data on the growth curve as well as on body composition at the age of 26 and 52 weeks, no effect of increased vitamin A intakes was observed in the LAB and MS puppies. Thus, the conclusion seems justified that dogs raised on vitamin A levels up to 104.8 µmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) do not respond to a change in energy expenditure or utilisation. However, the underlying biochemical mechanisms are not clear. The high capacity to bind retinol in the form of retinyl esters in plasma might not result in the disruption of cellular retinoic acid homeostasis, which seems to have a major impact on energy metabolism in other species.

4.5 Conclusion

The present study shows that vitamin A levels up to 104.8 µmol retinol (100 000 IU vitamin)/4184 kJ (1000 kcal) has no apparent effect on energy intake, growth rate or body composition in young dogs. These data confirm the high tolerance of this species to such levels of vitamin A. Data on energy intake, however, showed considerable variability between the two breeds, and also compared with the expected values using the predictive equation cited by the National Research Council, which requires further investigation.

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Authorship

All authors were involved in the design and oversight of the study. P. J. M. was responsible for the conduct of the study. C. S. performed the statistical analyses. T. B. and J. Z. wrote the paper. All authors were responsible for the final content of the manuscript.

Conflict of Interest

T. B., P. J. M. and C. S. are employees of Mars Petcare. The rest of the authors have no conflicts of interest to declare.

Table 4.1: Energy intake by breed at different stages during growth relative to energy intake at adulthood. (Mean values with their SD).

Measure	Breed	% mature BW										MEI (kJ/kgBW$^{0.75}$)	
		25%		35%		50%		80%		90%			
		Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD		
EI relative to maintenance	MS	1.58	0.31	1.52	0.21	1.77	0.33	1.33	0.24	1.13	0.23	563	105
EI (basis kJ/kgBW$^{0.75}$)	LAB	1.91	0.33	1.85	0.30	1.65	0.24	1.18	0.18	1.00	0.21	599	81.5

BW, Bodyweight; EI, Energy Intake; MEI, Maintenance Energy Intake; MS, Miniature Schnauzer; LAB, Labrador Retriever

Table 4.2: Change in total body fat (%) with age in puppies*. (Mean values with their 95 % confidence intervals. Tukey group letters denote significant differences).

Measure	Age (weeks)	Breed	Sex	Mean	95 % CI	
% Fat	26	MS	F	14.3[a]	9.85	20.9
			M	14.7[a]	10.1	21.2
		LAB	F	22.1[a]	15.1	32.5
			M	18.7[a]	12.9	27.0
	52	MS	F	12.9[a]	8.84	18.8
			M	16.8[a]	11.6	24.3
		LAB	F	19.4[a]	13.2	28.4
			M	21·5[a]	14·9	31·2
	78	LAB	F	21·4[a]	14·6	31·4
			M	22·1[a]	15·3	32·0

F, female; M, male; MS, Miniature Schnauzer; LAB, Labrador Retriever

* Data were analysed via linear mixed model.

Table 4.3: Change in percentage lean body mass with age in puppies*. (Mean values with their 95 % confidence intervals. Tukey group letters denote significant differences).

Measure	Age (weeks)	Breed	Mean	95 % CI	
% Lean	26	MS	82.9[b]	78.6	87.5
		LAB	77.2[ab]	72.3	82.3
	52	MS	81.5[ab]	77.3	86.0
		LAB	76.3[ab]	71.6	81.5
	78	LAB	75.2[a]	70.5	80.2

MS, Miniature Schnauzer; LAB, Labrador Retriever

* Data were analysed via linear mixed model

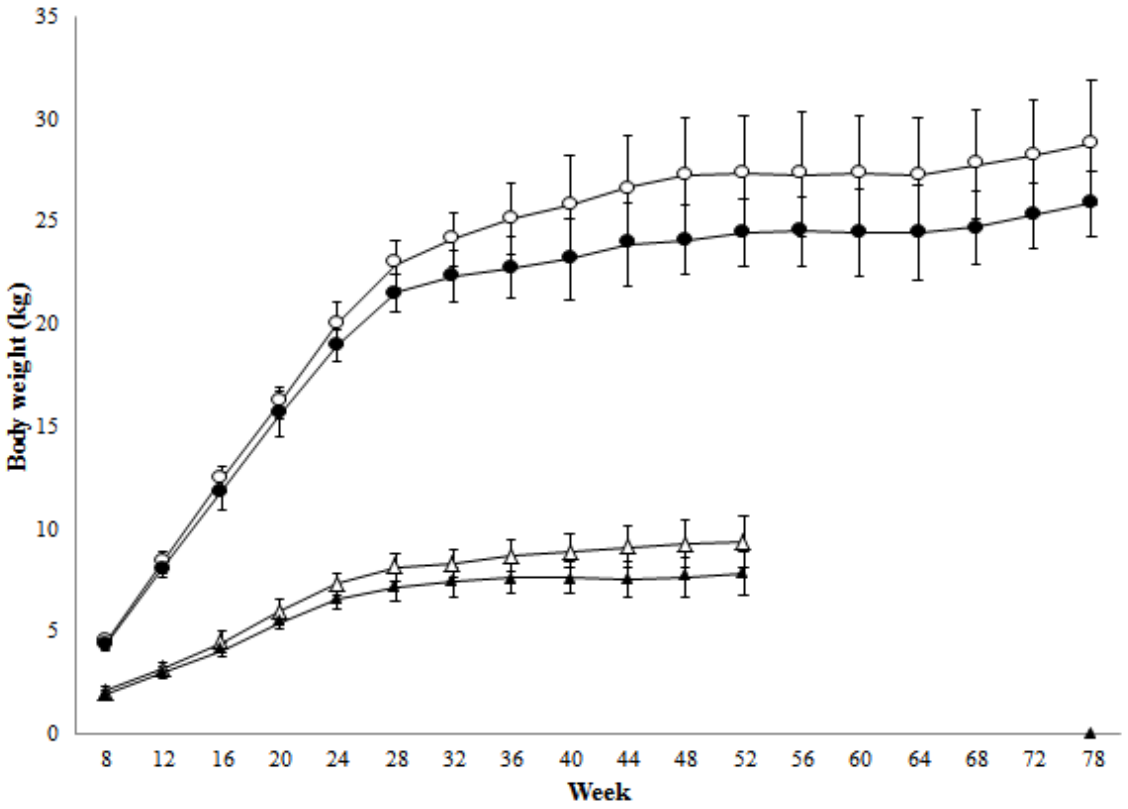

Fig. 4.1: Body-weight development (kg) by sex with age in Miniature Schnauzer (MS) and Labrador Retriever (LAB) puppies Values are means, with SD represented by vertical bars. ○, male LAB; ●, female LAB; △, male MS; ▲, female MS.

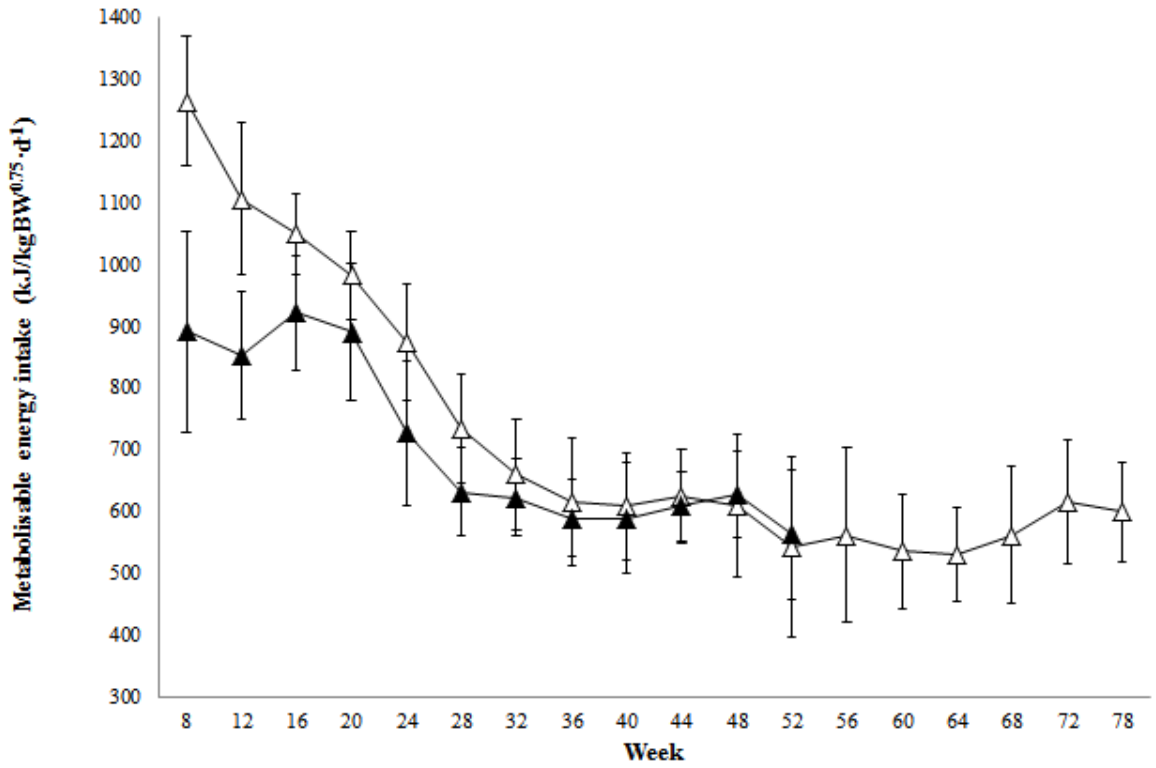

Fig. 4.2: Changes in metabolisable energy intake (kJ/kg body weight (BW)$^{0.75}$ per d) with age in Miniature Schnauzer (MS and Labrador Retriever (LAB) puppies. Values are means, with SD represented by vertical bars. △, MS; ▲, LAB.

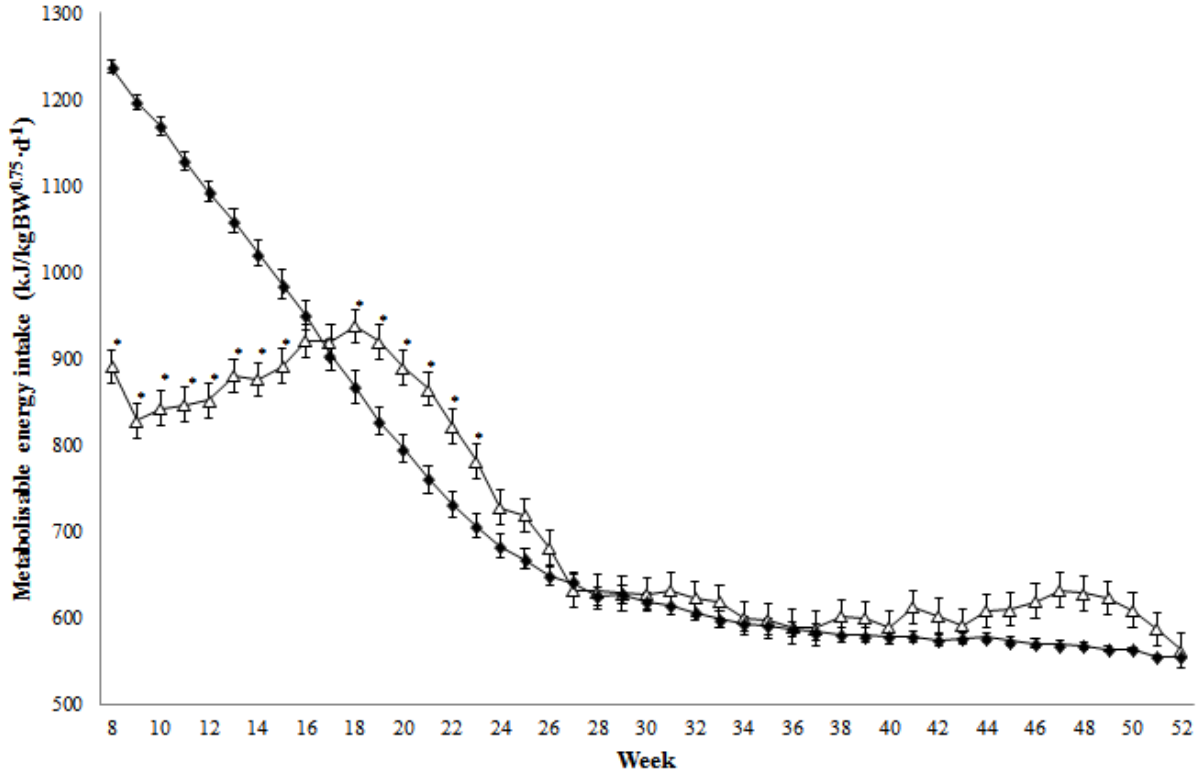

Fig. 4.3: Observed and predicted mean metabolisable energy intakes (kJ/kg body weight $(BW)^{0.75}$ per d) in Miniature Schnauzer puppies from 8 to 52 weeks of age. Values are means, with SEM represented by vertical bars. Mean values were significantly different between observed and predicted ME intakes (P<0.05) during weeks 8 to 15 and 18 to 23 inclusive. It should be noted that the error bars for the observed energy intakes naturally include some element of day-to-day variability which those for the predicted energy intakes do not. △, Observed; ▲, Predicted.

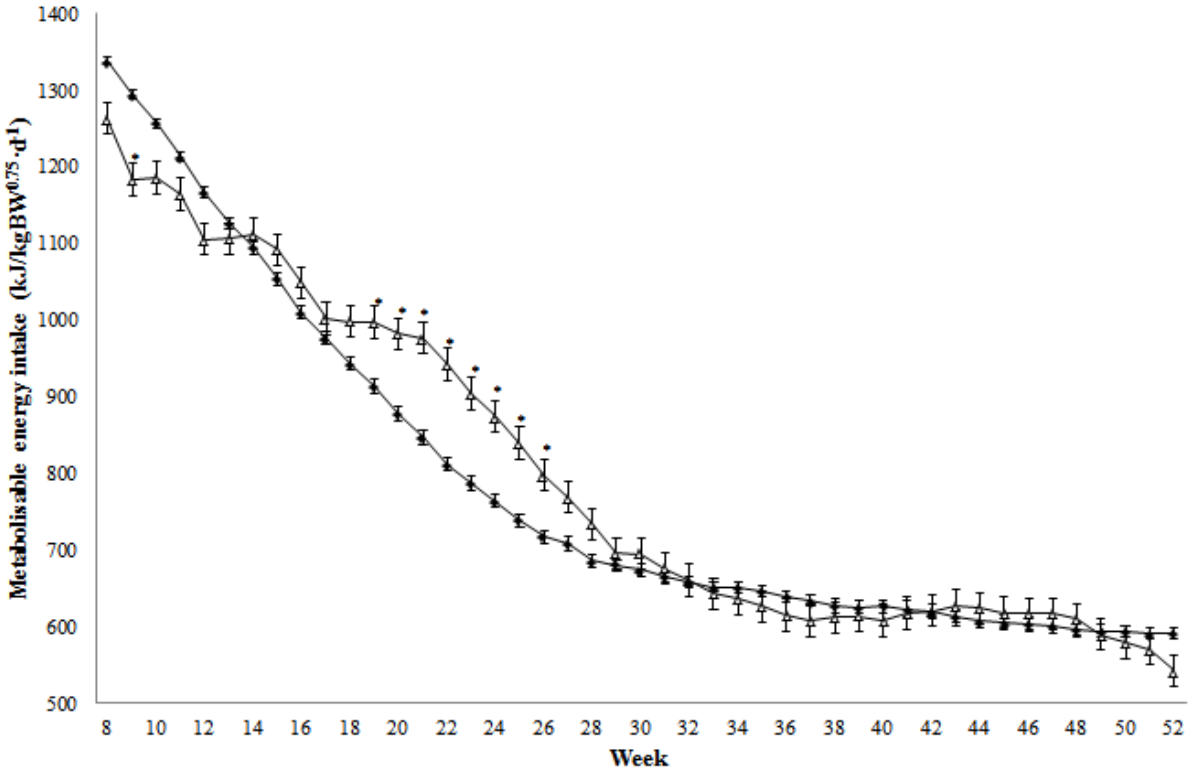

Fig. 4.4: Observed and predicted mean ME intakes (kJ/kg body weight $(BW)^{0.75}$ per d) in Labrador Retriever puppies from 8 to 52 weeks of age. Values are means, with SEM represented by vertical bars. Mean values were significantly different between observed and predicted ME intakes (P<0.05) at week 9 and weeks 19 to 26.inclusive. It should be noted that the error bars for the observed energy intakes naturally include some element of day-to-day variability which those for the predicted energy intakes do not. △, Observed; ▲, Predicted.

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CHAPTER 5: Age-associated and breed-associated variations in haematological and biochemical values in young Labrador Retrievers and Miniature Schnauzer dogs

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Abstract

Breed, sex and age effects on haematological and biochemical variables were investigated in 24 Labrador Retriever and 25 Miniature Schnauzer dogs during the first year of life. Blood samples were taken regularly between weeks 8 and 52. White blood cell and red blood cell counts, haemoglobin concentration, haematocrit, mean cell volume, mean cell haemoglobin, mean cell haemoglobin concentration, platelet count as well as total protein, albumin, calcium, phosphate, alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, glutamate dehydrogenase, total cholesterol, triglycerides, creatinine and urea were evaluated. For all haematological and biochemical parameters, there were significant effects of age on test results. Statistically significant effects for breed and the breed*age interaction on test results were observed for most of the parameters with the exception of haemoglobin. Variations in test results illustrate growth related alterations in body tissue and metabolism leading to dynamic and marked changes in haematological and biochemical parameters, which have to be considered for the interpretation of clinical data obtained from dogs in the first year of life.

5.1 Introduction

Haematological and biochemical profiles are routinely used to monitor the health status in dogs. Established reference intervals are typically derived from data obtained in healthy adult dogs of various breeds (Lumsden and others 1979; Meinkoth and Clinkebeared 2000; Rizzi and others 2006; Schaefers 2013). However, breed-specific differences in haematological and biochemical variables are reported from recent studies in Alaskan Malamutes, Siberian Huskies, Golden Retrievers, English Setters (Sharkey and others 2009), Bernese Mountain Dogs (Nielsen and others 2010), Dachshunds (Torres and others 2014) and Dogues de Bordeaux (Lavoue and others 2014). Breed-specific clinicopathologic characteristics are described for certain breeds such as thrombocytopenia in Cavalier King Charles Spaniels (Pedersen and others 2002), elevated white blood cell (WBC) count in Basenjis (Ewing and others 1972), elevated alkaline phosphatase (ALP) activity in Scottish Terriers (Nestor and others 2006), elevated haemoglobin (HGB) concentration and haematocrit (HCT) in combination with lower WBC and platelet (PLT) counts in Greyhounds (Campora and others 2011; Shiel and others 2007), microcytosis in Shiba Inus (Gookin and others 1998) and hypertriglyceridemia in Miniature Schnauzers (Xenoulis and others 2007). Breed-related differences in dogs from birth to 58 days of age are reported from a large study comparing Beagles, German Shepherds and

Golden Retrievers (Kuhl and others 2000; Lund and others 2000) showing lower initial red blood cell (RBC) count, lower HGB concentration, lower HCT, lower glucose levels as well as lower alanine aminotransferase (ALT) and ALP activities in the German Shepherd dogs.

The effect of age on haematological and biochemical test results has been studied in Basenjis, Beagles, German Shepherds and Labrador Retrievers (Harper and others 2003; Kaspar and Norris 1977; Lawler and others 2007; Lowseth and others 1990; Strasser and others 1993), suggesting that changes are most evident during the first year of life. Haematological and biochemical data in juvenile dogs however are less well documented and were often derived from studies in Beagles (Earl and others 1973; Ikeuchi and others 1991; Ishii and others 2013; Shifrine and others 1973; Wolford and others 1988). Most recently changes in haematological and biochemical variables have been described in Beagle, Borzoi, Labrador Retriever and mixed breed dogs aged up to 60 days (Rortveit and others 2015; Rosset and others 2012). Both studies reported lower values for RBC, HGB, HCT and total protein as well as higher values for creatine kinase and ALP activity compared to adult reference intervals.

The aim of this study was to investigate breed, sex and age effects and their interaction in Labrador Retrievers and Miniature Schnauzer aged 8-52 weeks. A previously published study evaluating the safety of vitamin A in growing dogs (Morris and others 2012) reported on some of the data used in this study. Those data were consolidated with a broader spectrum of haematological and biochemical data for this study.

To our knowledge this is the first study reporting longitudinal haematological and biochemical data obtained from growing Miniature Schnauzers. The systematic evaluation deepens our understanding of changes that should be expected in haematological and biochemical variables during the first year of life and assists the interpretation of clinical data obtained from young dogs.

5.2 Materials and Methods

The research protocol was evaluated and approved by the WALTHAM® Animal Welfare and Ethical Review committee and has been described in detail previously (Morris and others 2012).

5.2.1 Animals and housing

A total of forty-nine dogs, from eight litters, born at the WALTHAM® Centre for Pet Nutrition entered into the study. The dogs were of two breeds, Labrador Retriever (24) and Miniature Schnauzer (25). The dogs were fully weaned by 8 weeks of age. After weaning, the dogs were

housed in pairs in environmentally enriched kennels. The dogs had free access to an attached outdoor area and participated daily in training and socialization activities. All dogs were neutered between week 36 and week 52.

5.2.2 Clinical examination

The dogs underwent physical examination before the start of the trial and in four weekly intervals thereafter. Any blood parameters outside of the puppy reference intervals (Harper and others 2003) were referred to the veterinarian for investigation and re-tests were conducted within 24 h and repeated as required for diagnosis. Only samples from dogs considered healthy at the time of sampling were entered into the database.

5.2.3 Diet and feeding

The base diet was a standard dry commercial recipe (Perfect Fit Junior; Mars GmbH, Verden, Germany) compliant with FEDIAF recommendations for growth and reproduction (FEDIAF 2008) supplemented with various levels of vitamin A up to 104.80 μmol retinol (100 000 IU vitamin A)/1000 kcal ME. Details of the nutrient composition and feeding regime have been provided by Morris et al. (2012). Free access to drinking-water was given at all times. Feeding allowances were calculated from amounts consumed during the previous week and adjusted weekly with the aim of maintaining dogs on standard growth curves with ideal body condition scores as described by Morris et al. (2012).

5.2.4 Blood sample analysis

A 2.8 ml blood sample was taken from the jugular vein following an overnight fast of at least 16 hours at week 8, and at weeks 10, 12, 14, 16, 20, 26, 36 and 52. Blood samples were collected into a syringe and immediately distributed between tubes containing either tri-potassium EDTA, lithium heparin or no anticoagulant. For haematological analysis, a 0.2 ml blood sample was deposited into a tube containing tri-potassium EDTA as an anticoagulant. This was gently mixed on a roller for 10 min at room temperature before automated analysis (Scil Vet ABC, scil animal care, Viernheim, Germany) for WBC and RBC, HGB concentration, HCT, mean cell colume (MCV), mean cell haemoglobin (MCH), mean cell haemoglobin concentration (MCHC) and PLT.

For biochemical analysis, 0.5ml of blood was deposited into a tube containing lithium heparin as an anticoagulant. The tube was mixed gently for 10 s before being stored on ice for a maximum of 30 min. The sample was then centrifuged at 2000 g for 10 min at 4°C. The

resultant plasma was pipetted into a sample cup before automated colorimetric analysis (Olympus AU400; Olympus, Inc., Tokyo, Japan). Concentrations of total protein, albumin, calcium, inorganic phosphate, total cholesterol, triglycerides, creatinine and urea and activities of ALP, ALT, aspartate aminotransferase (AST) and glutamate dehydrogenase (GLDH) were measured.

5.2.5 Data analysis and statistics

Data were analysed by means of linear mixed model analysis including the fixed terms breed, sex, age and breed*age interaction. The model included the random term dog to take account of the correlation between measurements within an individual animal. Residuals were tested for normality using the Shapiro Wilks test. In case of significant non-normality (at the 5% significance level) the optimal Box-Cox transformation was determined and the Shapiro-Wilks test was again performed on the resulting residuals to assess whether the distribution of the data had been successfully normalized. For simplicity, in subsequent calculations, this optimal transform was substituted by the closest standard transformation (out of log, reciprocal, squared reciprocal or square root), this being chosen on the basis of the value of the Box-Cox power parameter. It should be noted that residuals were tested again for normality using the Shapiro Wilks test, before this simpler transformation was accepted as useful. In case of continued non-normality after Box-Cox transformation outliers were identified using residual plots and removed one at a time until data were normally distributed. The model was then run with both outliers included and excluded, and a comparison of the differences between the conclusions conducted. In every case the conclusions were similar – this implied that the outlying observations (all of which were biologically possible) were not influential and could be included in the model without issue. Significant breed*age interactions were investigated with a post hoc multiple comparison procedure (Tukey HSD test).

All endpoints were separately subjected to Bonferroni correction to account for the presence of multiple endpoints; the overall significance level used was 0.05. Data are presented as means with their (Bonferroni-adjusted) 95 % CI unless otherwise stated, and P values are reported as Bonferroni-adjusted P values. The SEM is not presented as the non-linearity of the transformation (and its inverse) implies only the means and confidence intervals can be back-transformed to the original scale, which we felt was necessary to ease interpretation.

Data analyses were performed with commercially available software (Statgraphics Centurion XVI, Version 16.0.07. StatPoint Technologies Inc).

5.3 Results

5.3.1 Clinical examination

A single Labrador Retriever was removed from the study at 9 months of age following diagnosis of a congenital kidney defect. All data from this dog were removed from the analysis.

5.3.2 Haematological and biochemical testing

For all haematological and biochemical parameters, there were significant effects of age on test results. Statistically significant effects for breed and the breed*age interaction on test results were observed for most of the parameters with the exception of haemoglobin.

5.3.2.1 Haematology

In both breeds WBC count decreased over time ($P < 0.01$; Table 5.1) with most marked changes occurring between week 8 and week 14, whilst RBC count, HGB concentration, HCT, MCV and MCH increased with age ($P < 0.01$; Table 5.1). PLT counts varied over time ($P < 0.01$; Table 5.1) starting with an increase up to week 12 followed by a decrease until week 52. Miniature Schnauzers showed higher values for MCV and MCH compared to Labrador Retrievers throughout the trial ($P < 0.01$; Table 5.1). MCHC fluctuated over time in Labrador Retrievers with an initial increase between week 8 and week 16 ($P < 0.01$; Table 5.1). Labrador Retrievers showed higher PLT counts compared to Miniature Schnauzers until week 12 ($P < 0.01$; Table 5.1).

5.3.2.2 Blood biochemistry

Plasma protein and albumin concentrations increased over time ($P < 0.01$; Table 5.2) in both breeds with albumin reaching a plateau at week 26. Miniature Schnauzers showed higher values for both plasma protein and albumin compared to Labrador Retrievers ($P < 0.01$; Table 5.2). Urea and creatinine concentrations increased until the end of the trial in both breeds ($P < 0.01$; Table 5.2). Labrador Retrievers showed higher creatinine levels compared to Miniature Schnauzers from week 26 onwards ($P < 0.01$; Table 5.2). GLDH activity fluctuated over time and remained higher ($P < 0.01$; Table 5.2) in Miniature Schnauzers as of week 36. AST activity fluctuated with age ($P < 0.01$; Table 5.2), initially increasing in both breeds. The ALT activity increased with age in both breeds ($P < 0.01$; Table 5.2) with ALT activity reaching a plateau at week 20 in Miniature Schnauzers. The ALP activity decreased ($P < 0.01$; Table 5.2) over time. The ALP activity in Miniature Schnauzers remained below the ALP activity in

Labrador Retrievers throughout the trial ($P< 0.01$; Table 5.2). Plasma calcium and phosphate concentrations decreased in both breeds until end of the trial ($P<0.01$; Table 5.2) with a marked decrease as of week 20. Plasma phosphate concentrations were higher in Labrador Retrievers compared to Miniature Schnauzers throughout the trial ($P < 0.01$; Table 5.2) but no effect of breed was observed for plasma calcium concentrations ($P = 1.00$; Table 5.2). Cholesterol levels varied over time ($P < 0.01$; Table 5.2) with the highest values in both breeds in week 26. Plasma triglyceride levels fluctuated over time ($P < 0.01$; Table 5.2) with higher plasma triglyceride levels in Miniature Schnauzers between week 20 and week 36 ($P < 0.01$; Table 5.2).

5.4 Discussion

Previously Morris and others (2012) reported a study in growing Labrador Retrievers and Miniature Schnauzers receiving various levels of vitamin A supplementation with respect to markers of vitamin A metabolism, haematological and biochemical variables and dual-energy X-ray absorptiometry. Morris and others (2012) demonstrated that different intakes of vitamin A up to 104.80 µmol retinol (100 000 IU vitamin A)/1000 kcal ME did not affect the haematological and biochemical variables. Therefore we did not consider vitamin A as a factor in our statistical evaluation. We also omitted to calculate reference intervals by age and breed and to compare them with adult reference intervals in consequence of the sample size falling below recommendations for nonparametric reference intervals as laid down in the ASVCP guidelines (Friedrichs and others 2012). However, due to the scarcity of data in growing dogs we consider the data from this study being an important contribution for the deepened understanding of age and breed-related variances that should be expected in haematological and biochemical test results during the growth period.

In this study we found significant effects of age, breed and a breed*age interaction in haematological and biochemical tests in young Labrador Retrievers and Miniature Schnauzers between week 8 and week 52. The age associated changes in haematological and biochemical values reported in this study are in good agreement with findings in juvenile dogs reported in previous studies (Bulgin and others 1970; Ewing and others 1972; Harper and others 2003; Ikeuchi and others 1991; Ishii and others 2013; Lawler and others 2007; Pickrell and others 1974; Wolford and others 1988), confirming the need for age-specific reference intervals for the interpretation of clinical data obtained from young dogs.

No differences were found based on sex, which correlates well with previous reports in growing dogs (Ishii and others 2013; Kuhl and others 2000; Lund and others 2000; Shiel and

others 2007). Differences between male and female dogs, despite being small and probably of minor physiological significance, are reported in studies looking at adult dogs of different breeds (Campora and others 2011; Lawrence and others 2013; Pickrell and others 1974). In addition to sex, the neutering status has been reported to account for small differences leading to higher HGB concentration, MCH and MCHC in neutered dogs (Lawrence and others 2013). These differences were putatively associated with the potential direct impact of sex hormones on erythropoiesis and the indirect impact on the haematopoietic niche of the bone marrow, which might also explain the missing differences in sexually immature dogs.

The effect of neutering on haematological values in this study however remains unclear due to the fact that all dogs in this study were neutered between week 36 and week 52 and no control group of entire dogs was available.

An effect of age was found for WBC count where values rapidly decreased. Within the differential leukocyte counts a plateau was reached between week 10 and week 14. The negative correlation with age is in agreement with findings in previous studies; however some breed-related differences with regards to the time-point of WBC count stabilization are reported. A WBC count decrease until 8 months of age with stabilization at around 16 months of age was reported from a study in Beagles (Bulgin and others 1970). In Basenjis (Ewing and others 1972) WBC counts firstly increased reaching maximal values between 85 and 120 days, with a subsequent decline to values of adult dogs. No correlation with age however was found in Beagle, Borzoi, Labrador Retriever and mixed breed dogs up to 60 days of age, which might be explained by effects of breed and sample size (Rosset and others 2012) or the automated analyser used (Rortveit and others 2015) . A study in growing Greyhounds (Shiel and others 2007) aged between 5 and 11 months reported no correlation with age, which is in good agreement with the early stabilization found in our study. Regardless of these breed-related differences the interpretation of WBC counts in dogs younger than 6 month of age requires careful interpretation as values are likely to be above or at the upper end of the adult reference intervals (Rizzi and others 2006).

RBC count, HGB concentration and HCT significantly increased in both breeds over time. This is in agreement with previous studies (Bulgin and others 1970; Ewing and others 1972; Harper and others 2003; Lawler and others 2007; Rortveit and others 2015; Shiel and others 2007) and relates to the maturation of the erythropoiesis and the positive correlation with age between RBC lifespan and HGB concentration (Bulgin and others 1970).

Despite a steady increase in MCV and MCH until the end of trial all values were comparable to values found in adult dogs. This might be explained by the fact that first samples were taken at 8 weeks of age when the transition from foetal to postnatal erythrocytes has already taken place and MCV as well as MCH values are expected to approach adult values (Bulgin and others 1970; Lund and others 2000; Rortveit and others 2015; Shifrine and others 1973). For MCV and MCH a significant effect of breed was observed with Miniature Schnauzers having significantly higher MCV and MCH throughout the trial. It can be speculated whether the smaller red blood cell size in Labrador Retriever presents an adaptation to the higher metabolic rate (Brenten and others 2014) providing an increased surface area for oxygen exchange (Hawkey 1975).

PLT counts were negatively correlated with age which is consistent with findings in Beagles (Ishii and others 2013). The age effect however was primarily driven by significantly higher PLT values in Labrador Retrievers during the first 3 month of age, whereas the age effect was less prominent in Miniature Schnauzers. Despite this breed effect values for both Miniature Schnauzers and Labrador Retrievers remained within the adult reference intervals throughout the study, which is consistent with previous reports (Rortveit and others 2015; Rosset and others 2012) suggesting a low clinical relevance of the observed differences.

Plasma protein, albumin, and urea concentrations were positively correlated with age, which is consistent with findings in other breeds (Ewing and others 1972; Harper and others 2003; Ishii and others 2013; Rortveit and others 2015). In Miniature Schnauzers total protein and albumin reached a plateau at around 26 weeks of age and remained significantly higher compared to Labrador Retrievers, which might be explained by the higher protein intake per kg absolute bodyweight.

Creatinine significantly increased over time in both breeds, which is in good agreement with a recent study in growing Labrador Retrievers and mixed breeds dogs (Rortveit and others 2015). Labrador Retrievers however showed significantly higher creatinine values compared to Miniature Schnauzers from week 26 onwards. The creatinine increase over time might be a reflection of the increase in lean body mass during growth. It can be hypothesized that both the absolute higher lean body mass and the higher growth velocity (Brenten and others 2014) in Labrador Retrievers contributed to the significantly higher creatinine values compared to values observed in Miniature Schnauzers after 26 weeks of age.

GLDH reference values for growing dogs have not been reported previously. The GLDH values reported in this study are consistent with a recently reported reference interval

for adult dogs (Schaefers 2013), which suggests that this range can be applied to growing dogs until new data become available. Further research will be required however as differences in breed of dog, age range and analytical methodologies may impact the final reference interval.

The increase in AST activity in Labrador Retrievers is in good agreement with previous studies in Beagles reporting an increase in AST activity for up to 6 months of age (Ishii and others 2013; Wolford and others 1988). In our study ALT activity increased steadily over time. An increase in ALT activity was also observed in Beagles, but the ALT activity level in Beagles stabilized much earlier at around 3 month of age (Wolford and others 1988). Whilst ALT is found predominantly in the liver, AST is found in cardiac muscle, skeletal muscle, liver and the kidneys. The age-dependent activities in both enzymes appear to correlate well with tissue growth. The higher AST activity in Labrador Retrievers as of week 26 might be correlated with the higher absolute muscle mass.

ALP levels were negatively correlated with age which corresponds well with previous reports (Harper and others 2003; Ikeuchi and others 1991; Kaspar and Norris 1977). The high ALP activity is thought to be a result of the high bone ALP isozyme activity related to the bone turnover in growing dogs (Kramer and Hoffmann 1997). Labrador Retrievers showed significantly higher ALP activities throughout the trial compared to Miniature Schnauzers, which is in agreement with results from a previous study looking at the differences between Great Danes and Miniature Poodles (Tryfonidou and others 2003). Plasma phosphate levels remained at a high level until 4 months of age in both breeds followed by a subsequent decrease until end of trial, which is consistent with results from previous studies (Ikeuchi and others 1991; Pickrell and others 1974; Wolford and others 1988). The plasma phosphate levels in Labrador Retrievers stayed above the levels in Miniature Schnauzer throughout the trial. The higher plasma phosphate levels in young dogs are described as a result of the growth hormone mediated stimulation on the renal phosphate reabsorption (Haramati and others 1990; Mulroney and others 1989). Plasma calcium levels remained at a plateau until week 26 followed by a decrease until end of trial. The decline however was less distinct in absolute values compared to the decline in phosphate, which might be a consequence of the tight plasma calcium regulation via calcitonin and parathyroid hormone. These findings are in good agreement with a previous study in Beagles (Wolford and others 1988) and a study in Great Danes and Miniature Poodles (Tryfonidou and others 2003). It can be hypothesized that the observed changes over time in plasma calcium levels are driven by the synergistic effect between passive calcium absorption (Tryfonidou and others 2002) and active absorption influenced by growth

hormone stimulated production of $1,25(OH)_2D_3$ or by decreased clearance of $1,25(OH)_2D_3$ (Goff and others 1990) respectively. Two other studies in Beagles however found no correlation in plasma calcium levels with age throughout the first year of life (Ikeuchi and others 1991; Pickrell and others 1974). Due to missing information about the nutritional composition of diets in these studies it can only be speculated whether this observation was associated with nutritional factors. In contrast to ALP activity and plasma phosphate concentrations it appears that plasma calcium concentrations during growth are not influenced by breed-size.

Cholesterol levels increased in both breeds reaching a peak in week 26 followed by gradual decrease until end of trial. This compares well with findings in Beagles aged between 2 weeks and 1 year as reported previously (Wolford and others 1988). In contrast a study looking at Beagles aged between 6 and 12 months (Ikeuchi and others 1991) found no significant correlation with age. Another longitudinal study in Beagles (Pickrell and others 1974) reported increasing cholesterol concentrations only in females as of 11 months. In our study however the sex*age interaction was not significant ($P = 1.00$), which might be a consequence of our limited observation period.

Triglyceride levels in Labrador Retrievers showed less fluctuation during the trial period compared to Miniature Schnauzers. A peak triglyceride concentration was observed in Miniature Schnauzers in week 20 which might be a result of the peak energy intake per kg metabolic bodyweight in Miniature Schnauzers in week 20 as reported previously (Brenten and others 2014) leading to high intakes of dietary fat. Subsequent to this peak both energy intake and triglyceride concentration gradually decreased until end of trial. One study (Ikeuchi and others 1991) found a distinct increase in triglycerides in females and hypothesized a hormonal effect on lipase activity known from studies in rodents (Hamosh and Hamosh 1975). This effect was not found in our study which might be a consequence of the neutering of our dogs between week 36 and 52.

Idiopathic hypertriglyceridemia however is a common clinicopathologic finding described in Miniature Schnauzers. A recent study in 192 healthy Miniature Schnauzers has shown a prevalence of primary hypertriglyceridemia in 32.8% of the dogs (Xenoulis and others 2007) with a significant positive correlation between triglyceride concentration and age in this breed. Ten out of the 17 Miniature Schnauzers remaining after completion of the study at the WALTHAM® Centre for Pet Nutrition were diagnosed with hypertriglyceridemia later on at ages between 4.5 to 6 years. Nine Miniature Schnauzers had mild increases in triglyceride concentrations (109-400mg/dL) and 1 had a moderate increase in triglyceride concentrations

(>400-1000mg/dL). The development of idiopathic hypertriglyceridemia during adulthood as reported here supports the hypothesis that later development of hypertriglyceridemia cannot be excluded in young Miniature Schnauzers with normal triglyceride concentrations (Xenoulis and others 2007).

5.5 Conclusion

The present study shows that maturation and growth in dogs is reflected in changes in haematological and blood biochemical values. The systematic evaluation of the results from this study therefore contributes to the understanding of age-related variances that should be expected in haematological and biochemical test results, which will assist practicing veterinary clinicians with the interpretation of clinical data obtained from young dogs. The early growth phase clearly appears to be most critical and needs to be investigated in more depth.

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Table 5.1: Age and breed specific results of haematological tests in Miniature Schnauzers and Labrador Retrievers. Data are presented as means with their Bonferroni corrected 95 % CI.

			Age									Breed	Age (weeks)	Breed*Age (weeks)
			8 weeks	10 weeks	12 weeks	14 weeks	16 weeks	20 weeks	26 weeks	36 weeks	52 weeks			
			Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean			
Test	**Unit**	**Breed**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	***P***	***P***	***P***
WBC	$x10^3/\mu L$	LAB	13.5^{a}	9.49^{bc}	8.85^{bc}	8.76^{bc}	9.03^{bc}	8.76^{bc}	7.39^{c}	8.00^{c}	8.33^{bc}	1.00	< 0.01	< 0.01
			(11.9-15.0)	(8.41-10.6)	(7.92-9.97)	(7.77-9.78)	(8.00-10.1)	(7.85-9.87)	(6.62-8.25)	(7.17-9.03)	(7.46-9.39)			
		MS	13.2^{a}	10.1^{b}	10.9^{ab}	8.41^{bc}	8.50^{bc}	7.61^{c}	8.17^{bc}	7.92^{c}	9.12^{bc}			
			(11.8-14.7)	(9.03-11.2)	(9.78-12.2)	(7.61-9.39)	(7.61-9.49)	(6.82-8.41)	(7.32-9.12)	(7.10-8.85)	(8.17-10.2)			
RBC	$x10^6/\mu L$	LAB	4.60^{a}	5.09^{b}	4.97^{ab}	5.31^{bc}	5.48^{bc}	5.79^{c}	6.04^{cd}	6.71^{d}	6.46^{cd}	< 0.05	< 0.01	0.36
			(4.44-4.78)	(4.88-5.34)	(4.77-5.20)	(5.06-5.59)	(5.21-5.79)	(5.48-6.15)	(5.69-6.45)	(6.24-7.31)	(6.03-6.98)			
		MS	4.68^{a}	4.81^{ab}	4.96^{ab}	5.21^{b}	5.33^{bc}	5.27^{bc}	5.91^{cd}	6.29^{cd}	6.04^{cd}			
			(4.52-4.86)	(4.63-5.00)	(4.76-5.17)	(4.99-5.46)	(5.09-5.60)	(5.04-5.53)	(5.59-6.29)	(5.91-6.74)	(5.70-6.44)			
Haemoglobin	g/dL	LAB	9.80^{a}	10.9^{b}	10.6^{ab}	11.5^{bc}	12.3^{cd}	12.8^{cd}	13.6^{d}	15.5^{e}	15.1^{de}	0.26	< 0.01	0.06
			(9.43-10.3)	(10.4-11.5)	(10.2-11.1)	(11.0-12.2)	(11.7-13.0)	(12.1-13.5)	(12.8-14.4)	(14.5-16.6)	(14.1-16.1)			
		MS	10.8^{ab}	11.0^{b}	11.4^{bc}	12.1^{c}	12.6^{cd}	12.8^{cd}	14.5^{de}	15.1^{de}	15.1^{de}			
			(10.3-11.3)	(10.5-11.5)	(10.8-11.9)	(11.5-12.7)	(11.9-13.2)	(12.2-13.6)	(13.6-15.4)	(14.3-16.2)	(14.2-16.1)			
HCT	%	LAB	29.9^{a}	32.9^{bc}	32.0^{ab}	34.3^{bc}	35.8^{c}	38.1^{cd}	40.1^{d}	45.4^{de}	43.9^{de}	< 0.01	< 0.01	0.13
			(29.0-31.0)	(31.6-34.4)	(30.7-33.4)	(32.8-36.1)	(34.1-37.7)	(36.1-40.5)	(37.7-42.9)	(42.1-49.6)	(40.9-47.6)			
		MS	32.5^{b}	33.2^{bc}	34.4^{bc}	36.5^{cd}	37.6^{cd}	38.0^{cd}	42.9^{de}	46.2^{e}	45.2^{de}			
			(31.3-33.9)	(31.9-34.7)	(33.0-36.0)	(34.8-38.4)	(35.8-39.8)	(36.0-40.2)	(40.2-46.3)	(42.9-50.4)	(42.1-49.1)			

(continued)

Table 5.1 (continued)

			Age									Breed	Age (weeks)	Breed*Age (weeks)
			8 weeks	10 weeks	12 weeks	14 weeks	16 weeks	20 weeks	26 weeks	36 weeks	52 weeks			
			Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean	Mean			
Test	**Unit**	**Breed**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	***P***	***P***	***P***
MCV	fL	LAB	65.1^{ab}	64.6^{a}	64.5^{a}	65.0^{ab}	65.4^{ab}	65.6^{ab}	66.4^{b}	67.4^{b}	68.1^{bc}			
			(64.2-65.9)	(63.7-65.4)	(63.7-65.3)	(64.0-65.8)	(64.6-66.3)	(64.8-66.4)	(65.4-67.2)	(66.6-68.2)	(67.1-68.9)	< 0.01	< 0.01	< 0.01
		MS	69.4^{c}	69.2^{c}	69.6^{c}	70.1^{c}	70.7^{cd}	72.3^{d}	72.6^{d}	73.4^{de}	75.0^{e}			
			(68.6-70.2)	(68.4-70.1)	(68.7-70.4)	(69.2-70.9)	(69.9-71.6)	(71.4-73.1)	(71.7-73.4)	(72.6-74.3)	(74.1-75.9)			
MCH	pg	LAB	21.3^{a}	21.4^{a}	21.4^{a}	21.7^{ab}	22.4^{b}	21.8^{ab}	22.5^{b}	22.8^{bc}	23.3^{bc}			
			(20.8-21.7)	(20.9-21.8)	(21.0-21.9)	(21.3-22.2)	(21.9-22.8)	(21.3-22.3)	(22.0-22.9)	(22.4-23.3)	(22.8-23.8)	< 0.01	< 0.01	< 0.05
		MS	22.9^{bc}	22.8^{bc}	22.8^{bc}	23.1^{bc}	23.5^{c}	24.3^{cd}	24.4^{cd}	24.1^{cd}	24.9^{d}			
			(22.5-23.4)	(22.4-23.2)	(22.5-23.3)	(22.8-23.6)	(23.0-24.0)	(23.8-24.8)	(23.9-24.8)	(23.6-24.6)	(24.5-25.4)			
MCHC	g/dL	LAB	32.7^{a}	33.1^{ab}	33.3^{ab}	33.4^{ab}	34.2^{b}	33.3^{ab}	33.8^{b}	33.9^{b}	34.3^{b}			
			(32.1-33.2)	(32.5-33.6)	(32.7-33.8)	(32.9-34.0)	(33.7-34.8)	(32.8-33.8)	(33.3-34.4)	(33.4-34.4)	(33.7-34.8)	0.08	< 0.01	< 0.01
		MS	33.1^{ab}	33.0^{ab}	32.9^{ab}	33.1^{ab}	33.3^{ab}	33.7^{ab}	33.6^{ab}	32.9^{a}	33.2^{ab}			
			(32.6-33.6)	(32.5-33.5)	(32.4-33.4)	(32.5-33.6)	(32.8-33.8)	(33.1-34.2)	(33.0-34.1)	(32.3-33.4)	(32.7-33.8)			
Platelet	x10^{3}/µL	LAB	426^{ab}	429^{ab}	498^{a}	441^{ab}	397^{b}	373^{bc}	311^{bc}	286^{c}	309^{bc}			
			(376-476)	(379-478)	(448-547)	(391-491)	(347-446)	(323-422)	(261-360)	(237-336)	(260-359)	< 0.01	< 0.01	< 0.01
		MS	288^{c}	286^{c}	339^{bc}	352^{bc}	317^{bc}	353^{bc}	275^{c}	288^{c}	259^{c}			
			(241-335)	(228-333)	(291-386)	(304-399)	(270-365)	(305-400)	(227-322)	(241-335)	(212-307)			

LAB, Labrador Retriever; MS, Miniature Schnauzer

^{a,b} Mean values within the same test for breeds and weeks; dissimilar superscript letters indicate significant differences within test between breeds and weeks (using the Tukey HSD method)

*Data were analysed using a linear mixed model.

Table 5.2: Age and breed specific results of biochemical tests in Miniature Schnauzers and Labrador Retrievers. Data are presented as means with their Bonferroni corrected 95 % CI.

			Age									Breed	Age (weeks)	Breed*Age (weeks)
			8 weeks Mean	10 weeks Mean	12 weeks Mean	14 weeks Mean	16 weeks Mean	20 weeks Mean	26 weeks Mean	36 weeks Mean	52 weeks Mean			
Test	**Unit**	**Breed**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	**(95% CI)**	***P***	***P***	***P***
Protein	g/L	LAB	46.1^{a}	47.3^{a}	47.7^{ab}	49.8^{bc}	50.2^{bc}	51.0^{bc}	52.8^{c}	54.0^{cd}	55.4^{d}			
			(45.0-47.3)	(46.1-48.4)	(46.5-48.8)	(48.7-51.0)	(49.1-51.4)	(49.9-52.2)	(51.7-54.0)	(52.8-55.1)	(54.2-56.5)	< 0.01	< 0.01	< 0.01
		MS	50.7^{bc}	49.7^{b}	51.6^{bc}	52.1^{c}	53.5^{cd}	55.5^{d}	58.4^{e}	57.9^{e}	57.9^{e}			
			(49.6-51.8)	(48.6-50.8)	(50.5-52.7)	(51.0-53.2)	(52.4-54.6)	(54.4-56.6)	(57.3-59.5)	(56.8-59.0)	(56.8-59.0)			
Albumin	g/L	LAB	22.5^{a}	24.1^{b}	25.3^{bc}	25.5^{bc}	26.2^{c}	26.8^{cd}	28.2^{d}	28.1^{d}	28.4^{d}			
			(21.7-23.2)	(23.3-24.9)	(24.5-26.1)	(24.7-26.2)	(25.4-27.0)	(26.0-27.6)	(27.4-29.0)	(27.3-28.9)	(27.6-29.2)	< 0.01	< 0.01	< 0.01
		MS	26.9^{cd}	25.8^{c}	26.4^{c}	27.1^{cd}	27.9^{d}	28.3^{d}	30.7^{e}	30.7^{e}	30.6^{e}			
			(26.1-27.6)	(25.0-26.5)	(25.6-27.1)	(26.3-27.8)	(27.2-28.7)	(27.6-29.1)	(29.9-31.4)	(29.9-31.5)	(29.8-31.3)			
Phosphate	mmol/L	LAB	2.83^{a}	2.78^{a}	2.84^{a}	2.82^{a}	2.84^{a}	2.72^{ab}	2.57^{bc}	2.18^{d}	1.81^{f}			
			(2.76-2.90)	(2.71-2.86)	(2.77-2.92)	(2.75-2.89)	(2.77-2.92)	(2.64-2.79)	(2.50-2.65)	(2.10-2.25)	(1.74-1.89)	< 0.01	< 0.01	< 0.01
		MS	2.60^{b}	2.51^{bc}	2.43^{c}	2.45^{c}	2.49^{bc}	2.36^{c}	2.00^{e}	1.68^{f}	1.51^{g}			
			(2.53-2.67)	(2.44-2.58)	(2.36-2.51)	(2.38-2.52)	(2.42-2.56)	(2.29-2.43)	(1.92-2.07)	(1.61-1.75)	(1.44-1.58)			
Calcium	mmol/L	LAB	2.80^{ab}	2.79^{ab}	2.79^{ab}	2.83^{ab}	2.80^{ab}	2.77^{b}	2.77^{b}	2.66^{c}	2.61^{c}			
			(2.76-2.84)	(2.76-2.83)	(2.76-2.83)	(2.80-2.87)	(2.76-2.84)	(2.73-2.80)	(2.73-2.81)	(2.63-2.70)	(2.57-2.65)	1.00	< 0.01	< 0.01
		MS	2.86^{a}	2.83^{ab}	2.80^{ab}	2.79^{ab}	2.85^{a}	2.77^{b}	2.77^{b}	2.64^{c}	2.62^{c}			
			(2.82-2.90)	(2.80-2.87)	(2.76-2.84)	(2.76-2.83)	(2.81-2.88)	(2.74-2.81)	(2.73-2.80)	(2.60-2.68)	(2.58-2.65)			
ALP	U/L	LAB	424^{ab}	464^{a}	442^{ab}	432^{ab}	390^{b}	329^{c}	270^{d}	195^{e}	144^{f}			
			(401-448)	(441-488)	(418-465)	(409-455)	(367-414)	(306-353)	(246-293)	(171-218)	(120-167)	< 0.01	< 0.01	< 0.01
		MS	442^{ab}	400^{b}	382^{bc}	375^{bc}	340^{c}	279^{d}	193^{e}	113^{fg}	72^{g}			
			(420-464)	(378-423)	(359-404)	(252-397)	(318-362)	(257-302)	(171-216)	(91.2-136)	(49.4-94.0)			
ALT	U/L	LAB	19.9^{a}	22.0^{a}	24.8^{ab}	26.3^{b}	28.2^{b}	30.9^{bc}	35.5^{bc}	44.7^{cd}	48.9^{d}			
			(17.5-22.6)	(19.3-25.0)	(21.8-28.2)	(23.1-30.0)	(24.8-32.1)	(27.1-35.2)	(31.2-40.4)	(39.3-50.9)	(42.9-55.7)	< 0.05	< 0.01	< 0.01
		MS	28.2^{b}	32.1^{bc}	32.8^{bc}	33.8^{bc}	37.0^{c}	40.9^{cd}	40.9^{cd}	47.0^{cd}	41.3^{cd}			
			(25.0-32.1)	(28.5-36.6)	(29.1-37.3)	(29.7-38.1)	(32.8-41.7)	(35.9-46.1)	(36.2-46.5)	(41.7-53.5)	(36.6-47.0)			

(continued)

Table 5.2 (continued)

			Age									Breed	Age (weeks)	Breed*Age (weeks)
			8 weeks Mean (95% CI)	10 weeks Mean (95% CI)	12 weeks Mean (95% CI)	14 weeks Mean (95% CI)	16 weeks Mean (95% CI)	20 weeks Mean (95% CI)	26 weeks Mean (95% CI)	36 weeks Mean (95% CI)	52 weeks Mean (95% CI)			
Test	**Unit**	**Breed**										***P***	***P***	***P***
AST	U/L	LAB	26.4^{b}	28.6^{bc}	29.4^{bc}	29.5^{bc}	30.7^{bc}	31.8^{c}	32.9^{c}	32.9^{c}	32.2^{c}	0.23	< 0.01	< 0.01
			(24.3-28.8)	(26.2-31.2)	(27.1-32.1)	(27.1-32.1)	(28.2-33.4)	(29.1-34.5)	(29.7-35.5)	(30.3-35.9)	(29.7-35.2)			
		MS	22.0^{a}	27.9^{bc}	29.1^{bc}	28.5^{bc}	30.0^{bc}	27.7^{bc}	26.6^{b}	24.3^{ab}	25.0^{ab}			
			(20.3-24.0)	(25.8-30.6)	(26.6-31.5)	(26.3-30.9)	(27.7-32.5)	(25.3-30.0)	(24.5-29.1)	(22.4-26.3)	(23.1-27.4)			
GLDH	U/L	LAB	4.14a	4.39^{ab}	4.76^{ab}	4.62^{ab}	4.53^{ab}	5.00^{ab}	4.44^{ab}	4.01^{a}	4.01^{a}	< 0.01	< 0.01	< 0.01
			(3.56-4.85)	(3.78-5.16)	(4.10-5.58)	(3.97-5.42)	(3.90-5.26)	(4.31-5.87)	(3.82-5.21)	(3.42-4.66)	(3.46-4.66)			
		MS	4.85^{ab}	5.16^{ab}	5.42^{ab}	5.70^{b}	5.99^{b}	6.62^{b}	5.93^{b}	7.03^{b}	6.11^{b}			
			(4.18-5.64)	(4.44-5.93)	(4.66-6.23)	(4.95-6.62)	(5.05-7.10)	(5.75-7.69)	(5.10-6.89)	(6.05-8.08)	(5.26-7.10)			
Cholesterol	mmol/L	LAB	4.93^{b}	5.44^{bc}	5.67^{bc}	6.38^{c}	6.39^{c}	6.44^{c}	6.76^{c}	6.13^{c}	5.73^{bc}	1.00	< 0.01	< 0.01
			(4.49-5.36)	(5.01-5.87)	(5.24-6.11)	(5.95-6.82)	(5.95-6.82)	(6.00-6.87)	(6.33-7.20)	(5.70-6.57)	(5.30-6.17)			
		MS	5.12^{ab}	4.19^{a}	4.81^{ab}	5.61^{bc}	5.73^{bc}	6.37^{c}	6.87^{c}	6.79^{c}	6.25^{c}			
			(4.71-5.54)	(3.77-4.60)	(4.40-5.23)	(5.20-6.03)	(5.31-6.14)	(5.95-6.78)	(6.48-7.29)	(6.38-7.21)	(5.83-6.66)			
Triglycerides	mg/dL	LAB	55.1^{ab}	55.1^{ab}	50.9^{ab}	56.8^{ab}	66.0^{bc}	54.6^{ab}	47.5^{ab}	43.8^{ab}	42.1^{a}	< 0.01	< 0.01	< 0.01
			(46.5-65.4)	(46.5-65.4)	(42.9-60.3)	(46.1-70.8)	(55.7-78.3)	(46.1-64.7)	(40.0-56.3)	(37.0-51.9)	(35.5-49.9)			
		MS	56.8^{ab}	53.0^{ab}	59.1^{b}	59.1^{b}	54.1^{ab}	83.1^{c}	75.9^{bc}	63.4^{bc}	58.0^{ab}			
			(47.9-66.7)	(44.7-62.2)	(50.4-69.4)	(50.4-70.1)	(46.1-64.1)	(70.8-97.5)	(64.1-89.19	(54.1-75.2)	(48.9-68.0)			
Creatinine	µmol/L	LAB	36.6^{a}	39.5^{ab}	42.3^{b}	47.7^{c}	51.4^{c}	58.5^{d}	73.6^{f}	87.6^{g}	94.3^{h}	< 0.01	< 0.01	< 0.01
			(34.4-38.7)	(37.4-41.7)	(40.1-44.4)	(45.5-49.8)	(49.3-53.6)	(56.4-60.7)	(71.5-75.8)	(85.5-89.7)	(92.2-96.5)			
		MS	38.9^{ab}	40.1^{ab}	44.3^{bc}	47.9^{c}	51.5^{c}	58.7^{d}	67.3^{e}	72.8^{f}	73.7^{f}			
			(36.8-40.9)	(38.1-42.2)	(42.2-46.3)	(45.9-50.0)	(49.5-53.6)	(56.7-60.7)	(65.3-69.3)	(70.7-74.8)	(71.6-75.7)			
Urea	mmol/L	LAB	2.00^{a}	2.50^{ab}	2.82^{b}	3.34^{bc}	3.56^{c}	4.08^{cd}	4.85^{de}	5.39^{e}	6.06^{e}	1.00	< 0.01	< 0.01
			(1.66-2.35)	(2.15-2.84)	(2.47-3.16)	(3.00-3.69)	(3.21-3.90)	(3.74-4.43)	(4.50-5.19)	(5.04-5.73)	(5.72-6.41)			
		MS	2.68^{ab}	2.80^{b}	2.87^{b}	3.27^{bc}	3.77^{c}	4.64^{d}	4.84^{de}	5.44^{e}	5.45^{e}			
			(2.35-3.01)	(2.47-3.13)	(2.54-3.20)	(2.94-3.60)	(3.44-4.10)	(4.31-4.97)	(4.51-5.17)	(5.11-5.77)	(5.12-5.78)			

LAB, Labrador Retriever; MS, Miniature Schnauzer;

[a,b] Mean values within the same test for breeds and weeks; dissimilar superscript letters indicate significant differences within test between breeds and weeks (using the Tukey HSD method)

*Data were analysed using a linear mixed model.

CHAPTER 6: General Discussion and Conclusions

The impact of different dietary vitamin A concentrations on energy intake, growth rate and body composition in Labrador Retriever and Miniature Schnauzer dogs has not been studied up to now. Based on findings demonstrating the high tolerance of dogs to dietary vitamin A with regards to different biomarkers (Morris et al., 2012) it was hypothesized that energy retention evidenced by the accumulation of body fat would be unaffected. Therefore, the development of body weight and body composition was evaluated and the observed energy intake was compared with the predicted energy intake in response to the potential impact of dietary vitamin A intake in the two breeds.

Haematological and biochemical tests were performed on regular basis during the trial to monitor the health status of the puppies. The results of the haematological and biochemical blood tests have been further evaluated for the effects of age, breed and sex. This should allow monitoring changes in haematological and biochemical variables during the first year of life without regard to the potential impact of vitamin A intake.

Dietary energy plays a key role in determining growth and an adequate energy supply is a key factor for the nutrition of young dogs. Overfeeding with energy as well as severe under- or over supply of energy and nutrients can predispose for developmental orthopaedic disorders, especially in large breeds (Lust et al., 1973; Hedhammar et al., 1974; Meyer and Zentek, 1991; Meyer and Zentek, 1992; Lawler et al., 2008). Data on energy intakes however show considerable variability (NRC, 2006; Dobenecker, 2008) and the definition of optimal growth rates is difficult. The determination of “ideal” body condition in growing dogs is complex, not only by definition. Increased energy intake results in increased growth rate, but not necessarily in a visually detectable change in body condition (Dobenecker et al., 2011). Therefore body condition scores in puppies are often assessed in the context of actual body weight versus standard growth curves (Hawthorne et al., 2004). In this study the actual food allowances were calculated from the amounts consumed during the previous week and adjusted weekly so that body weight increased in breed-specific manner (Hawthorne et al., 2004). It was tried to maintain puppies at “ideal” body condition based on an internal scoring system (German et al., 2006). This approach may have biased the outcome.

The possible effect of increased vitamin A intake on growth intensity, energy intake and body composition was of particular interest. Research in rodents has shown that retinoic acid, the carboxylic acid form of vitamin A, can reduce body fat by enhancing fat mobilisation and energy utilisation systemically, in tissues including brown and white adipose tissues, skeletal

muscle and the liver (Amengual et al., 2008). The resulting retinoic acid-induced loss of body weight and body fat occurred despite unchanged or even increased energy intake in combination with increases in body temperature (Bonet et al., 2012).

The effects described in rodents were not found in this study in growing Labrador Retrievers and Miniature Schnauzers fed with different levels of dietary vitamin A.

No differences in energy intake on per kg body weight or per kg metabolic body weight basis were found between the vitamin A treatment groups (Morris et al., 2012). Temporal deviations versus the expected energy intakes (Blanchard et al., 1998; NRC, 2006), i.e. lower and higher energy intakes, were observed during the trial period in both breeds **(Chapter 4, Figure 4.3 & 4.4)**, but these might be related to breed-specific effects, housing conditions or activity level of the dogs. Overall, the total amount of energy consumed until end of trial remained in the range of the total predicted energy intake.

The mean percentage of body fat in Labrador Retriever in this study **(Chapter 4, Table 4.2)** was lower compared with other findings in growing Labrador Retriever (Schoenherr et al., 2010). However, no body condition scores are reported from the cited study and hence these puppies may have had a different energy allocation or higher body condition scores.

Furthermore growth rate **(Chapter 4, Figure 4.1)** and percentage of lean body mass **(Chapter 4, Table 4.3)** of the puppies in this study were comparable with previous data (Hawthorne et al., 2004; Alexander and Wood, 1987; Booles et al., 1994; Chakraborty et al., 1983; Hedhammar et al., 1974; Meyer and Zentek, 2013; Trangerud et al., 2007; Huck et al., 2009; Kealy et al., 2002).

These results are suggesting that unlike to rodents dietary vitamin A does not affect energy utilisation or growth rate in dogs.

This suggestion is further supported by the evaluation of the triglyceride concentration. Hypertriglyceridemia in response to high doses of vitamin A has been reported to occur in both rodents and humans. This effect appears to be a result of enhanced hepatic lipogenesis and VLDL production as well as to defects in VLDL clearance (Bonet et al., 2012). No differences in triglyceride concentrations were observed between the dogs in the different vitamin A treatment groups as reported previously (Morris et al., 2012). However, a mild increase in triglyceride concentration (i.e. 106 – 220 mg/dL) (Xenoulis et al., 2007) was detected in 7 out of the 25 MS in week 20. A high energy intake in MS at this time **(Chapter 4, Figure 4.3)** leading to high intake of dietary fat might explain this mild increase. Subsequently energy intake and triglyceride concentration gradually decreased until the end of the trial. This indicates that dietary vitamin A can be ruled out as underlying cause of this mild temporary

hypertriglyceridemia. Summarising all data on body condition and triglyceride concentrations, it seems that no retinoic acid-induced fat mobilisation and subsequent loss of body fat occurred in this study.

It has to be noted however that idiopathic hypertriglyceridemia is a common clinicopathologic finding described in Miniature Schnauzer. A study in 192 healthy Miniature Schnauzer has shown a prevalence of primary hypertriglyceridemia in one third of the dogs with a positive correlation between triglyceride concentration and age (Xenoulis et al., 2007). More than half of the Miniature Schnauzer remaining at the facility after completion of the study were diagnosed with hypertriglyceridemia later on at ages between 4.5 to 6 years. Although the Miniature Schnauzer have been clinically healthy throughout the trial, it raises the question whether the over proportional high hereditary prevalence for idiopathic hypertriglyceridemia in the Miniature Schnauzer enrolled in the study might have biased the development of the reference ranges because of the subclinical disease.

The haematological and biochemical test results in this study are in good agreement with previously reported results in juvenile dogs (Bulgin et al., 1970; Ewing et al., 1972; Pickrell et al., 1974; Wolford et al., 1988; Ikeuchi et al., 1991; Harper et al., 2003; Lawler et al., 2007; Ishii et al., 2013). The results are intensively discussed in **Chapter 5**.

As described in **Chapter 5** haematological parameters have been assessed regularly between week 8 and week 52 of life. Changes in haematological values however are taking place already after birth. The time between birth and week 8 though has not been evaluated due to the original design of the study (Morris et al., 2012). This presents a flaw in our study design with regards to a comprehensive overview of age-related changes during the first year of life.

After birth an initial rapid decline in RBC, HGB and HCT to values markedly below values found in adult dogs has been reported previously (Earl et al., 1973; Lund et al., 2000). After around 4 weeks of age values for RBC, HGB and HCT start to increase again approaching adult values by approximately 6 months to 1 year (Bulgin et al., 1970; Ewing et al., 1972; Meinkoth and Clinkebeared, 2000; Harper et al., 2003; Lawler et al., 2007; Ishii et al., 2013). Although the initial decline in the first weeks was missed, values for RBC, HGB and HCT in this study gradually increased over time (**Chapter 5, Table 5.1**).

MCV values in neonates are reported to be in the magnitude of about 95 femtoliters due to the very large canine RBCs at birth. Until 2 to 3 month of age the MCV decreases to adult values (Andersen and Schalm, 1970; Meinkoth and Clinkebeared, 2000; Willard and Tvedten, 2012). This corresponds to findings in this study, where from first sampling in week 8 until end

of trial in week 52 all MCV values were comparable to values found in adult dogs. MCHC was comparable between the two breeds, but MCV and MCH values were found to be higher in Miniature Schnauzer. It can be speculated whether the smaller red blood cell size in Labrador Retriever presents an adaptation to the higher metabolic rate **(Chapter 4, Figure 4.2)** providing an increased surface area for oxygen exchange (Hawkey, 1975).

Abnormal bone development and premature closure of the epiphyseal plate have been reported in response to high dietary intakes of vitamin A (Frohring, 1935; Maddock et al., 1949; Wiersig and Swenson, 1967; Rothenberg et al., 2007). This appears to be related to the effect of vitamin A on the differentiation of mesenchymal stem cells into osteoblasts and osteoclasts, and the maturation and mineralisation of cartilage (Jimenez et al., 2001). A reduction in the severity of the effects was observed in a previous study when vitamin A was co-administered with vitamins D and E (Cho et al., 1975), which suggest a potential interaction between vitamin A and D. No differences however were found between the vitamin A treatment groups with regards to biomarkers associated with these processes namely bone ALP, carboxyterminal telopeptides of type I collagen, bone mineral density (BMD) and bone mineral content (BMC) (Morris et al., 2012). The diet used in this study was supplemented with vitamin D at a level recommended for growth and reproduction (FEDIAF, 2008), which might have protected against effects in the skeleton.

Age and breed-related effects for ALP and plasma phosphate were found in this study which are in good agreement with previous reports (Pickrell et al., 1974; Kaspar and Norris, 1977; Bush, 1991; Ikeuchi et al., 1991; Wolford et al., 1988; Kramer and Hoffmann, 1997; Harper et al., 2003). Labrador Retrievers had a higher ALP activity and plasma phosphate levels throughout the trial (**Chapter 5, Table 5.2**). This seems to reflect the absolute higher bone turnover in growing dogs of large breeds which corresponds well to results from a study investigating Great Danes and Miniature Poodles (Tryfonidou et al., 2003). Despite greater BMD and BMC in Labrador Retrievers (Morris et al., 2012) plasma calcium levels were not affected by breed size likely due to the tight plasma calcium regulation via calcitonin and parathyroid hormone. This was also observed in previous studies in Beagles, Great Danes and Miniature Poodles (Wolford et al., 1988; Tryfonidou et al., 2003).

Further age related changes in blood chemical values seem to be linked to general organ and tissue development during the first year of life.

Velocity of body mass development is dependent upon the energy intake. High energy and consequently protein intakes are observed in the early growth phase **(Chapter 4, Figure 4.2)** to satisfy the nutritional demands for the rapid body mass development. Growth

velocity decreased over time in both breeds whilst total protein and albumin increased with age **(Chapter 5, Table 5.2).** This might be explained by the change of the anabolic rate in puppies (Poffenbarger et al., 1990) in combination with a gradual increase in protein synthesis (and in particular albumin synthesis) as known from research in young rats (Wise and Oliver, 1967; Czajka et al., 1970; Wolford et al., 1988). Total protein and albumin levels remained lower in Labrador Retrievers compared to Miniature Schnauzers throughout the trial. Although the percentage lean body mass was not different between the two breeds (**Chapter 4, Table 4.3**) it can be speculated whether the observed difference was a result of the absolute lower protein intake in Labrador Retrievers.

Urea and creatinine values were both positively correlated with age. Low urea concentrations in puppies are related to the positive effect of growth hormone on tissue net uptake of amino nitrogen and a reduced hepatic conversion of amino nitrogen to urea nitrogen (Grofte et al., 1994; Grofte et al., 1998). The increase in urea might be explained by the change of the anabolic rate during growth, whereas creatinine is not influenced by diet or protein catabolism. Normal creatinine values are lower in puppies 2 to 6 month of age due to less muscle mass and higher GFR (Willard and Tvedten, 2012). This is in agreement with findings in this study where creatinine levels gradually increased in both breeds with higher values found in Labrador Retriever as of week 26 **(Chapter 5, Table 5.2)** which seems to reflect the difference in absolute muscle mass.

Conclusion and Perspectives

The impact of vitamin A and its metabolic product retinoic acid on the energy metabolism in dogs is not clear, although data from rodents indicate an influence on both thermogenesis and lipid synthesis via its actions when bound to the retinoic acid receptor. Data obtained from a larger study investigating the effects of dietary vitamin A at levels up to 104.8 µmol retinol (100 000 IU vitamin A)/4184 kJ (1000 kcal) ME in growing dogs were further evaluated in this thesis for the effect on energy intake, body weight development and body composition. The results of the current study suggest that unlike to rodents dietary vitamin A does not affect energy utilization, growth rate or body composition in dogs. The underlying biochemical mechanisms however remain unclear and require further clarification. With regards to haematological and blood chemical parameters the study showed marked age and breed related changes reflecting growth and maturation in puppies. The early growth phase clearly appears to be most critical and needs to be investigated in more depth to ease interpretation of clinical data obtained from young puppies.

CHAPTER 7: Summary/Zusammenfassung

Summary of the Doctoral Thesis:

Investigations on age and breed-associated differences in energy intake, growth rate, body composition, haematological and blood chemical values of Labrador Retrievers and Miniature Schnauzers fed different dietary levels of vitamin A.

Balanced nutrition ensuring adequate intakes of energy, protein, minerals and vitamins is essential for the optimal development of young dogs. An adequate energy supply of growing dogs is critical in controlling the growth rate. Research in rodents indicates that dietary vitamin A impacts energy utilisation; the potential impact on the energy metabolism in dogs and consequently on the energy intake, body composition and growth velocity in growing dogs however remains unclear.

The literature survey in **Chapter 2** indicates that the definition of healthy growth is difficult and controversial. The assessment of ideal body condition of growing dogs is more difficult compared to adult animals because increased energy intake results in increased growth rate, but not necessarily in increased body fat mass. Guidance values for energy intake and body weight development do exist, however considerable variability can be observed between breeds. The overview of studies investigating breed and age related changes in haematological and blood chemical values during growth clearly shows that prominent changes occur. Therefore results obtained from puppies have to be interpreted with care to correctly assess the health status as values may deviate from values found in adult dogs.

Chapter 3 explains the main aims and hypotheses of this thesis. The main work of the current thesis consists of two published manuscripts summarized in **Chapter 4** and **5**. In the first manuscript the possible effects of different dietary vitamin A concentrations on energy intake, growth rate and body composition in Labrador Retriever and Miniature Schnauzer puppies have been evaluated. However, based on the well-documented high tolerance of dogs to dietary vitamin A levels up to 104.8 µmol retinol (100 000 IU vitamin)/4184 kJ (1000 kcal) it was hypothesized that energy intake and accumulation of body fat would be unaffected (**Chapter 4**). This was confirmed by the results of our study.

Given the findings of Morris et al (2012), vitamin A was not considered as a factor in the evaluation of the haematological and biochemical data in the second manuscript (**Chapter 5)**. The main interest of the second manuscript was to increase the knowledge on breed, sex and age effects and their interaction during the first year of life. The evaluation shows

that age and breed-related changes in haematological and blood chemical test results are evident in young Labrador Retriever and Miniature Schnauzer dogs. The results confirm the need for age-specific reference ranges for the interpretation of clinical data obtained from young puppies.

The potential effects of dietary vitamin A as well as growth related alterations in body tissue and metabolism are discussed in **Chapter 6** together with the results obtained from this study. In conclusion, the results of the current study suggest that unlike to rodents dietary vitamin A does not affect energy utilization, growth rate or body composition in dogs. The underlying biochemical mechanisms however remain unclear and require further clarification. With regards to haematological and blood chemical parameters the study showed marked age and breed related changes illustrating growth related alterations in body tissue and metabolism during the first year of life. The early growth phase clearly appears to be most critical and needs to be investigated in more depth to ease interpretation of clinical data obtained from young puppies.

Zusammenfassung der Dissertation zum Thema:

Untersuchungen zum Einfluss von Alter und Rasse auf die Energieaufnahme, Wachstumsrate, Körperzusammensetzung, hämatologische und klinisch-chemische Parameter bei Labrador Retrievern und Zwergschnauzern mit Berücksichtigung der Aufnahme unterschiedlicher Vitamin A-Mengen.

Eine ausgewogene Ernährung mit bedarfsgerechter Versorgung an Energie, Protein, Mineralstoffen und Vitaminen ist essentiell für ein optimales Wachstum von jungen Hunden. Hierbei hat die Energieaufnahme eine zentrale Bedeutung für die Steuerung der Wachstumsrate. Untersuchungen an Nagern haben gezeigt, dass Vitamin A einen Einfluss auf die Energieverwertung hat. Fragen zum möglichen Einfluss auf den Energieumsatz und folglich die Energieaufnahme, Körperzusammensetzung und Wachstumsgeschwindigkeit bei jungen Hunden bleiben derzeit unbeantwortet. Wie in **Kapitel 1** zusammengefasst, besteht auch Forschungsbedarf zum Einfluss von Alter und Rasse, aber auch hinsichtlich der Unterschiede in hämatologischen und klinisch-chemischen Parametern.

Die Literaturübersicht in **Kapitel 2** zeigt deutlich, dass optimales Wachstums schwierig zu definieren ist und kontrovers diskutiert wird. Die Körperkonditionsbeurteilung beim Welpen gestaltet sich schwierig, da eine überhöhte Energieaufnahme in höheren Wachstumsraten resultiert und nicht zwangsläufig in einer veränderten Körperkondition. Richtwerte für die Energieaufnahme und die Gewichtsentwicklung im Wachstum sind vorhanden, allerdings werden erhebliche rassebedingte Abweichungen beobachtet. Studien, die sich mit Alters- und Rasseeffekten auf die Blutparameter befassen, zeigen ebenfalls deutliche Veränderungen der Werte in der Wachstumsphase. Daher müssen Blutwerte von Welpen mit Bedacht interpretiert werden, da Abweichungen von Normalwerten erwachsener Tiere möglich sind.

Kapitel 3 stellt die Hauptziele und Hypothesen der Dissertation vor. Die zwei veröffentlichten Originalarbeiten liegen in Kapitel 4 und 5 vor. Im ersten Beitrag wurde der mögliche Einfluss unterschiedlicher diätetischer Vitamin A Gehalte auf die Energieaufnahme, Wachstumsgeschwindigkeit und Körperzusammensetzung bei Welpen der Rassen Labrador Retriever und Zwergschnauzer untersucht. Aufgrund der gut dokumentierten Toleranz von Hunden gegenüber Vitamin A-Gehalten in der Nahrung von bis 104.8 µmol Retinol (100 000 IU Vitamin A)/4184 kJ (1000 kcal) bestand jedoch die Annahme, dass die Energieaufnahme und Körperfettansammlung unbeeinflusst bleibt (**Kapitel 4**). Dies wurde durch die Ergebnisse dieser Studie bestätigt.

Basierend auf den Ergebnissen von Morris et al (2012), wurde daher im zweiten Beitrag (**Kapitel 5**) Vitamin A als Faktor in der statistischen Auswertung der hämatologischen und blutchemischen Ergebnisse nicht weiter betrachtet. Die Hauptintention des zweiten Beitrags bestand darin, ein tieferes Verständnis zum Einfluss der Rasse-, Geschlechts- und Alterseffekte, sowie zu deren Interaktionen im ersten Lebensjahr zu generieren. Die Auswertung der Ergebnisse hat ein Vorhandensein alters- und rassebedingter Veränderungen der hämatologischen und blutchemischen Werte bei wachsenden Hunden der beiden Rassen gezeigt. Für die Interpretation von klinischen Daten, bei denen die Normalwerte von wachsenden Hunden erhoben wurden, sind daher altersspezifische Referenzwerte notwendig.

In **Kapitel 6** werden die vorher beschriebenen mögliche Effekte von Vitamin A, sowie die wachstumsbedingten Veränderungen im Körpergewebe und Stoffwechsel im Kontext der eigenen Ergebnisse diskutiert. Zusammenfassend zeigt diese Studie, im Gegensatz zum Nager, keinen Hinweis auf einen Einfluss von Vitamin A auf die Energieverwertung, Wachstumsrate oder Körperzusammensetzung beim Hund. Fragen zu den zugrundeliegenden biochemischen Mechanismen bleiben jedoch offen und bedürfen weiterer Klärung. In Bezug auf die hämatologischen und blutchemischen Parameter konnte unsere Studie deutliche alters- und rassebedingte Veränderungen aufzeigen, welche wachstumsbedingte Anpassungen in Gewebe und Stoffwechsel im ersten Lebensjahr reflektieren. Die frühe Wachstumsphase erscheint hierbei am kritischsten und bedarf weiterer tiefgehender Untersuchungen, um die Interpretation klinischer Daten von jungen Welpen zu erleichtern.

Personal Contribution of the Doctoral Student Thomas Brenten to the Published Manuscripts

T.B. was specifically involved in the initiation of the study, coordination of activities between B.K., F.S., J.Z., FEDIAF and WALTHAM during the start-up phase as well as provision and control of the nutritional compliance of the base diet.

Manuscript 1 (Chapter 4):

All authors were involved in the design and oversight of the study. P. J. M. was responsible for the conduct of the study. C. S. performed the statistical analyses. T. B. and J. Z. wrote the paper. All authors were responsible for the final content of the manuscript.

Manuscript 2 (Chapter 5):

All authors were involved in the design and oversight of the study. P. J. M. was responsible for the conduct of the study. T. B. performed the statistical analyses. T. B. and J. Z. wrote the paper. All authors were responsible for the final content of the manuscript.

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Publication List

Publications (peer reviewed)

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Brenten, T., P. J. Morris, C. Salt, J. Raila, B. Kohn, F. J. Schweigert, and J. Zentek. 2016. Age and breed-associated variations in haematological and blood chemical values in young Labrador Retrievers and Miniature Schnauzer dogs.
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2014

Brenten, T., P. J. Morris, C. Salt, J. Raila, B. Kohn, L. Brunnberg, F. J. Schweigert, and J. Zentek. 2014. Energy intake, growth rate and body composition of young Labrador Retrievers and Miniature Schnauzers fed different dietary levels of vitamin A. Br J Nutr 111 (12): 2104-11.

Passlack, N., H. Burmeier, **T. Brenten**, K. Neumann, and J. Zentek. 2014. Short term effects of increasing dietary salt concentrations on urine composition in healthy cats. Vet J 201 (3): 401-5.

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2012

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Eidesstattliche Erklärung

Hiermit erkläre ich an Eides statt, die vorliegende Arbeit selbstständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel verwendet zu haben. Die Arbeit ist in dieser Form noch keiner anderen Prüfungsbehörde vorgelegt worden.

Berlin, den 06.02.2016

Thomas Brenten