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Testosterone and Anti-Müllerian Hormone (AMH) in lean and overweight Labrador retrievers

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Testosterone and Anti-Müllerian Hormone (AMH) in lean and overweight Labrador retrievers

Testosteron och anti-mülleriskt hormon (AMH) hos normalviktiga och överviktiga labrador retrievers

Elina Andersson

SUMMARY

In both humans and dogs, obesity is a serious disorder with increasing prevalence. Overweight individuals are predisposed to several co-morbidities, such as orthopedic disorders, and shorter life expectancy.

In humans, obesity is also associated with reduced fertility. Obese men have lowered serum concentration of the sex hormones testosterone and Anti-Müllerian Hormone (AMH). These sex hormones play an important role in fertility since testosterone supports spermatogenesis and promotes libido, while AMH is a marker of Sertoli cell maturation.

An association between body condition score (BCS) and serum concentrations of testosterone and AMH has not previously been investigated in male dogs. The aim of this study was to investigate the relationship between BCS and serum concentration of testosterone and AMH in clinically healthy, intact male Labrador retrievers.

Twenty-eight show-type Labrador retrievers participated in the study. The dogs were grouped as lean or overweight according to the BCS, a nine-point scale. Twelve dogs were classified as lean (BCS 4-5) and 16 were classified as overweight (BCS 6-8). Blood samples were collected and testosterone and AMH were measured by enzyme-linked immunosorbent assays (ELISAs). Median concentration and inter-quartile range was 9.6 ng/mL (7.4-14.1) for AMH and 9.7 nmol/L (6.3-15.4) for testosterone. The AMH concentration ranged between 5.2 and 22 ng/mL. The testosterone concentration ranged between 1.6 and 20.2 nmol/L.

The hypothesis of this study was that overweight dogs have lower testosterone and AMH serum concentrations than lean dogs, but the results showed that serum concentrations of testosterone and AMH did not differ significantly between lean and overweight dogs. This could be due to that the BCS was too low in the overweight dogs (only mild overweight), or that the sample population was too small and the power of the study thus limited.

To conclude, in this study BCS had no impact on serum concentration of testosterone or AMH in male dogs.

SAMMANFATTNING

Övervikt är en allvarlig sjukdom med ökande prevalens hos både människor och hundar. Överviktiga individer lider ofta av flertalet sekundära hälsoeffekter, exempelvis ortopediska sjukdomar. Överviktiga individer riskerar också kortare livslängd än normalviktiga individer.

Hos människa är övervikt också associerat med nedsatt fertilitet. Överviktiga män har lägre serumkoncentration av könshormonerna testosteron och anti-mülleriskt hormon (AMH). Dessa könshormoner spelar en viktig roll för fertiliteten, då testosteron stödjer spermatogenesen och ökar könsdriften, medan AMH är en markör för sertolicellsmognad.

Sambandet mellan hull och serumkoncentration av testosteron och AMH har inte tidigare undersökts hos hanhundar. Målet med den här studien var att undersöka sambandet mellan hull och serumkoncentration av testosteron och AMH hos kliniskt friska, intakta hanhundar av rasen labrador retriever.

I studien deltog 28 labrador retrievers av utställningstyp. Hundarna klassificerades som normalviktiga respektive överviktiga enligt den niogradiga BCS-skalan. Tolv hundar klassificerades som normalviktiga (BCS 4-5) och 16 klassificerades som överviktiga (BCS 6-8). Blodprov samlades in varpå testosteron och AMH analyserades genom enzymkopplade immunadsorberande analyser (ELISA). Mediankoncentrationen och kvartilavståndet var 9,6 ng/mL (7,4-14,1) för AMH och 9,7 nmol/L (6,3-15,4) för testosteron. AMH-koncentrationen varierade mellan 5,2 och 22 ng/mL. Testosteronkoncentrationen varierade mellan 1,6 och 20,2 nmol/L.

Hypotesen för denna studie var att överviktiga hundar har lägre serumkoncentration av testosteron och AMH än normalviktiga hundar, men resultaten visade att serumkoncentrationen av testosteron och AMH inte skilde sig signifikant mellan normalviktiga och överviktiga hundar. Resultaten kan bero på att de överviktiga hundarna bara var lindrigt överviktiga (vilket gör skillnaden mellan normalviktiga och överviktiga hundar liten), eller att studiepopulationen var för liten och att studiens styrka (power) därför blev begränsad.

Sammanfattningsvis hade hull i denna studie inte någon inverkan på serumkoncentrationerna av testosteron och AMH hos hanhundar.

CONTENTS

INTRODUCTION

Obesity is the most common nutritional disorder in cats and dogs (Hoenig, 2010). The prevalence of obesity in dogs has increased over the last decades, from 20-30 % in the 70's to around 40 % at the beginning of the twenty-first century (Mason, 1970, Edney and Smith, 1986, McGreevy et al., 2005, Colliard et al., 2006, Lund et al., 2006).

A major concern regarding the obese state in dogs is the association with secondary health conditions such as orthopedic disorders (e.g. osteoarthritis and ruptured cruciate ligament) (Impellizeri et al., 2000, Lund et al., 2006). Obese dogs also suffer from shorter life expectancy (Kealy et al., 2002).

In humans, obesity is associated with several co-morbidities (Hubert et al., 1983, Must et al., 1999) and reduced fertility (The Rotterdam group, 2004, Cabler et al., 2010). In men, obesity is associated with lowered serum concentration of the sex hormones testosterone and Anti-Müllerian Hormone (AMH) (Fejes et al., 2006, Håkonsen et al., 2011, Pietiläinen et al., 2011, Pellitero et al., 2012, Robeva et al., 2012). The sex hormones play an important role in fertility since testosterone supports spermatogenesis and AMH reflects Sertoli cell maturation (Rey et al., 1993, Rajpert-De Meyts et al., 1999, Feldman and Nelson, 2004). An association between body condition score (BCS) and serum concentrations of testosterone and AMH has not been investigated in male dogs. The objective of this study was to investigate the relationship between BCS and serum concentration of testosterone and AMH in clinically healthy male Labrador retrievers, a dog breed prone to obesity (Colliard et al., 2006, Lund et al., 2006, Raffan et al., 2014).

The hypothesis was that overweight dogs have lower testosterone and AMH concentrations than lean dogs. If this statement holds true, it will give us yet another reason to control body weight and avoid obesity in companion dogs. It could also influence dog breeding towards a slimmer, and thus healthier, body ideal.

LITERATURE REVIEW

Obesity

The definition of obesity is "accumulation of excess body fat" (Toll et al., 2010). Obesity occurs when energy expenditure is less than energy intake, which leads to excess storage of triglycerides in adipose tissue (Hoenig, 2010). Animals in ideal body condition have 15 to 20% body fat.

In both humans and dogs, the term "obesity" and "overweight" is distinguished from one another. In humans, the Body Mass Index (BMI) is commonly used to classify overweight and obesity. BMI is defined as a person's weight in kilograms divided by the square of his height in meters (kg/m^2). According to the World Health Organization (WHO), a BMI greater than or equal to 25 is overweight, while a BMI greater than or equal to 30 is obesity (WHO, 2015).

Instead in dogs, Relative Body Weight (RBW) may be used in defining overweight and obesity. RBW is defined as the animal's current weight divided by its estimated optimal weight. RBW for overweight dogs is between 10-20% above optimal weight. RBW for obese dogs is >20% above optimal weight (Toll et al., 2010). In another study, an excess of approximately 20 to 25 % above ideal bodyweight is regarded as obesity (Laflamme, 1997).

Obesity in humans

In humans, obesity is a global health problem with increasing prevalence. In 2003-2004, a study conducted on 4431 adults in the US reported the prevalence of obesity to 31.1 % among men and 33.2 % among women (Ogden et al., 2006). An international meta-analysis published in 2014 showed similar results; the proportion of male overweight had increased from 28.8 % in 1980 to 36.9 % in 2013. The same pattern was seen in women, where overweight had increased from 29.9 % in 1980 to 38.0 % in 2013 (Ng et al., 2014). According to WHO, more than 1.9 billion adults were overweight in 2014, of whom 600 million were obese (WHO, 2015).

A serious concern regarding obesity in humans is its association with several co-morbidities. Two big studies conducted on 5 209 and 16 884 individuals respectively, showed that obesity is a risk factor for cardiovascular disease, type 2 diabetes mellitus, high blood pressure, gallbladder disease and osteoarthritis (Hubert et al., 1983, Must et al., 1999). In another smaller study, obesity was also associated with pancreatitis, chronic fatigue and insomnia (Patterson et al., 2004).

Moreover, obesity is associated with subfertility in both men and women. In general, obesity affects male fertility in three different ways; by altered semen parameters (e.g. decreased sperm concentration, abnormal sperm morphology and abnormal sperm motility), altered profile of sex hormones, and by physical disorders such as erectile dysfunction (Cabler et al., 2010). In women, obesity is associated with e.g. polycystic ovary syndrome (PCOS), a syndrome causing chronic anovulation (The Rotterdam group, 2004).

Obesity in dogs

In both cats and dogs, obesity is the most common nutritional disorder, and is in fact regarded as a pandemic (Hoenig, 2010). The prevalence of obesity is increasing in dogs. It was 20-30 % in the 70's and increased to around 40 % at the beginning of the twenty-first century (Mason, 1970, Edney and Smith, 1986, McGreevy et al., 2005, Colliard et al., 2006, Lund et al., 2006).

Like in humans, the obese state in dogs is associated with several secondary health conditions such as orthopedic disorders. In an epidemiological study conducted on 21 754 dogs during one year in the U.S., obese adult dogs were more likely to be diagnosed with ruptured cruciate ligament (odds ratio 2.1). Moreover, the prevalence of osteoarthritis was 4.2 % in obese and 4.0 % in overweight individuals, compared to 2.4 % in normal weight individuals (Lund et al., 2006). In another study, nine dogs with hind limb lameness due to osteoarthritis were fed a restricted-calorie diet for 10 to 19 weeks. At the end of the study, both body weight and hind limb lameness were significantly decreased (Impellizeri et al., 2000). Kealy et al. (2002) also report that obese dogs suffer from shorter life expectancy, in a study where restrictively fed Labradors had significantly longer median life span compared to dogs fed ad libitum.

In dogs, breed is a risk factor for obesity. Retrievers, and particularly Labrador retrievers have an increased risk according to several studies (Edney and Smith, 1986, Colliard et al., 2006, Lund et al., 2006, Corbee, 2013). Interestingly, results from a recent study suggests that Labrador retrievers may have a mutation in the POMC gene, responsible for normal energy homeostasis, making them more prone to obesity (Raffan et al., 2014). In Sweden, the Swedish kennel club reported that the Labrador retriever breed as a whole decreased significantly in weight between 2005 and 2013. However, the Labrador retriever breed in Sweden is divided into two types; the show type and the working gun dog type (The Swedish Labrador Club, 2014), hence the results probably depend on the fact that the much lighter working gun dog type is increasing in its popularity in Sweden (Andersson Linda, DVM, Swedish Kennel Club, personal communication, 2015).

Today, several methods are available for classification of overweight and obesity in dogs. (Mawby et al., 2004), of which one is the Body Condition Score (BCS). BCS is a semi-quantitative assessment of body composition. By visual observation and palpation of the ribs, the dog scores into a nine-point scale (Table 1). In 1997, Laflamme et al. validated BCS against dual energy X-ray absorptiometry (DEXA), which is the gold standard method for determination of body fat and lean body mass in dogs. Body Condition Scoring (BCS) is since then commonly used among practitioners as a tool for obesity assessment in dogs (Laflamme, 1997, Mawby et al., 2004).

Table 1. *The Body Condition Score system in dogs (adapted after Laflamme, 1997).*

Body Condition Score	Designation
1	Emaciated
2	Very thin
3	Thin
4	Underweight
5	Ideal
6	Overweight*
7	Heavy
8	Obese
9	Grossly obese

* Dogs with BCS 6 or above had palpable fat deposits.

The male reproductive system

The main function of the male reproductive tract is to produce and store male gametes (spermatozoa), and to produce and secrete male sex hormones (androgens). The androgen produced in the greatest quantity is testosterone.

Hormonal regulation

The hormones involved in the regulation of the male reproductive system are part of the hypothalamic-pituitary-gonadal axis. From hypothalamus, a gonadotropin releasing hormone (GnRH) is transported to the anterior pituitary, from where the two gonadotropic hormones follicle stimulating hormone (FSH) and luteinizing hormone (LH) are released. FSH and LH then exert their effect on the male gonads.

The hypothalamic-pituitary-gonadal axis is regulated through a negative feedback control system, where GnRH secretion is inhibited by both testosterone from the gonads, and by FSH/LH from the anterior pituitary (Figure 1).
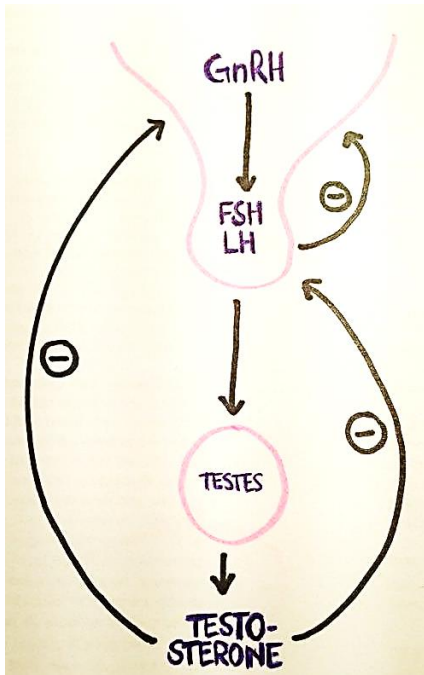
Figure 1. *The hypothalamic-pituitary-gonadal axis and its negative feedback system.*

Spermatogenesis

The production of spermatozoa (spermatogenesis) takes place in the testes. By fibrous septa, the testes are divided into several lobules. These lobules contain the seminiferous tubules, which collect the spermatozoa once they are produced. The production occurs by a series of divisions and subsequent differentiation from sperm stem cells (spermatogonia) located in the epithelium of the seminiferous tubules. The epithelium also contains Sertoli cells, which support, protect and nourish the developing spermatogenic cells. Moreover, the Sertoli cells help in regulation of the differentiation of spermatogonia.

In male dogs, FSH is the gonadotropic hormone that is responsible for stimulation of the Sertoli cells, which in turn affect spermatogenesis and promote the production of mature spermatozoa. FSH also stimulates the Sertoli cells' own development and function (Feldman and Nelson, 2004).

Testosterone

In the intertubular space, between the seminiferous tubules, clusters of Leydig cells are located. Leydig cells are steroid-producing cells, responsible for the formation of testosterone. The testosterone production is triggered by LH mediated stimulation of the Leydig cells (Feldman and Nelson, 2004).

Testosterone is a cholesterol derived hormone, with a hydroxyl group connected to its steroid skeleton. In the blood, testosterone is transported bound to sex hormone binding globulin (SHBG) protein. In some tissues, testosterone is converted to dihydrotestosterone, which has approximately twice as much biological potency as testosterone (Feldman and Nelson, 2004). Testosterone can also be aromatized to oestrogen in many tissues, especially in adipose tissue.

Testosterone has several functions:

- Together with FSH, it promotes spermatogenesis.
- It maintains secretory and absorptive activities in the male sperm duct system.
- It promotes growth and maintenance of the prostate gland.
- During fetal development, testosterone is responsible for differentiating the external genitalia into the penis and the scrotum. Testosterone also converts the Wolffian duct (the precursor structure for the male internal genitalia) into the male reproductive duct.
- It promotes development of primary sex characteristics, which is growth of the internal and external genitalia that distinguishes males from females.
- It promotes development of secondary sexual characteristics (traits that distinguish males from females), such as increased muscle mass, increased hair growth, increased secretion of female attracting pheromones and stronger sex drive (libido).

Plasma concentration of testosterone fluctuates during the day, because GnRH and subsequently both FSH and LH, is secreted in regular and brief pulses (Feldman and Nelson, 2004). In male dogs, after a peak in GnRH, plasma concentration of LH and testosterone peak approximately 15 and 60 minutes later, respectively.

Testicular testosterone concentrations are 50 to 100 times greater than concentrations in blood (Feldman and Nelson, 2004). Therefore, blood concentrations may not reflect alterations in testicular testosterone, nor be a useful diagnostic aid for evaluating spermatogenesis. However, measurement of blood testosterone provides information on the functional status of the hypothalamic-pituitary-gonadal axis.

The relationship between testosterone and male fertility

The definition of fertility is the ability to produce a viable offspring, while infertility is the lack of that ability (NE, 2015). Infertility in dogs can be either acquired or congenital, with normal or decreased libido.

Infertility is associated with several abnormalities in spermatogenesis, e.g. azoospermia (complete absence of spermatozoa in the ejaculate), oligospermia (decrease in the total number of spermatozoa per ejaculate), teratozoospermia (increased numbers of abnormal spermatozoa per ejaculate) and asthenozoospermia (decreased motility of spermatozoa in an ejaculate) (Feldman and Nelson, 2004).

Both libido and sperm production are dependent on an intact hypothalamic-pituitary-testicular axis, and testosterone promotes libido and spermatogenesis. Therefore, testosterone plays an important role in maintaining fertility. Decreased libido in an infertile dog may result from destruction of the Leydig cells, and decreased testosterone production within the testes (Feldman and Nelson, 2004). Lower blood testosterone concentrations can be expected in most dogs with decreased libido. Dogs with normal libido and the ability to mate rarely have plasma testosterone concentrations below 0.4 ng/ml, regardless of fertility.

Anti-Müllerian Hormone

Anti-Müllerian Hormone (AMH) is a dimeric hormone of the TGF-β family, found only in gonadal somatic cells (Wilson et al., 1993). During testicular development, AMH is secreted from immature Sertoli cells. Together with testosterone secreted from the Leydig cells, AMH causes the fetus to develop as a male. During early development, AMH is responsible for the regression of the Müllerian ducts, the precursor structure for the female internal genitalia (Sjaastad et al., 2010). Since FSH is responsible for stimulation of Sertoli cells, FSH also regulates AMH secretion (Lukas-Croisier et al., 2003).

Secretion of AMH in men is maintained high until puberty (Josso and di Clemente, 1999, Rajpert-De Meyts et al., 1999). After puberty, expression of AMH in human gonadal tissue decreases, which is believed to reflect terminal maturation of the Sertoli cells (Rajpert-De Meyts et al., 1999). The same relationship has been seen in dogs. In an immunohistochemical study by Banco et al., AMH expression was high in fetal and young puppies, but diminished in puppies from 45 days of age (Banco et al., 2012). In canine Sertoli cell tumours and testicle degeneration (a risk factor for tumour development), AMH is instead increased (Banco et al., 2012, Giudice et al., 2014, Holst and Dreimanis, 2015).

The relationship between AMH and male fertility

Infertile adult men with dysfunctional hypothalamic-pituitary-gonadal axis, such as hypogonadotropic hypogonadism (impaired secretion of FSH, LH and testosterone) had higher AMH plasma concentrations than normal men (Young et al., 1999). The opposite results were shown in another study from 2005. When comparing AMH in fertile and infertile men, serum concentrations of AMH were significantly higher in fertile men (Al-Qahtani et al., 2005).

The association between obesity and reduced male fertility

Obesity is associated with subfertility in men, by causing altered semen parameters (e.g. decreased sperm concentration, abnormal sperm morphology and abnormal sperm motility), altered profile of sex hormones, and physical disorders such as erectile dysfunction (Cabler et al., 2010).

A large study conducted on 1558 Danish men, reported that high BMI was associated with alterations in semen parameters such as lower sperm concentration and total sperm count. Also, higher BMI was associated with lower percentage of normal spermatozoa (Jensen et al., 2004). Lower sperm concentration in obese men was also seen in a study by Hammoud et al., together with reduced progressively motile sperm count (Hammoud et al., 2008). On the other hand, a meta-analysis from 2010 on studies on humans could not demonstrate a relationship between semen parameters (sperm concentration, total sperm count, semen volume, motility and morphology) and BMI (MacDonald et al., 2010).

Erectile dysfunction causes reproductive difficulties and has been reported in obese men in several studies. In a cross-sectional study of 149 obese men, decreased libido occurred in 22,5

% and erectile dysfunction in 31,7 % (Hofstra et al., 2008). Corona et al (2006) reported an even higher prevalence; out of 236 obese males diagnosed with metabolic syndrome, 96,5 % had erectile dysfunction (Corona et al., 2006).

Sex hormones and fertility among obese men

In the earlier mentioned meta-analysis by MacDonald et al. (2010), strong evidence of a negative correlation between BMI and testosterone, SHBG and free testosterone was shown (MacDonald et al., 2010). Hofstra et al. also reported that low testosterone serum concentrations, consistent with hypogonadotropic hypogonadism, was frequently occurring in male obesity (Hofstra et al., 2008). Low testosterone serum concentrations among obese men has also been reported in other studies, and serum concentrations have been shown to increase after weight loss (Kaukua et al., 2003, Fejes et al., 2006, Håkonsen et al., 2011, Pellitero et al., 2012, Robeva et al., 2012).

Several studies state that oestrogen is the cause of lowered testosterone concentrations in obese men, since they express a characteristic hormonal profile described as "hyperestrogenic hypogonadotropic hypogonadism", i.e. low serum testosterone concentrations caused by increased conversion of testosterone to oestrogen in the increased mass of adipose tissue. The higher concentrations of oestrogen then exert a negative feedback on LH secretion, and testosterone production therefore also decreases (Hammoud et al., 2006, Hofstra et al., 2008). Two studies support this hypothesis; a positive correlation between oestrogen and BMI was seen in the study of Håkonsen et al. (2011), and weight loss in obese men caused a decrease in oestrogen levels (Pellitero et al., 2012).

Regarding AMH, two studies in humans show that serum concentrations are lower in obese patients than in patients with normal body weight (Pietiläinen et al., 2011, Robeva et al., 2012). and one study shows that weight loss increases AMH serum concentrations (Håkonsen et al., 2011).

MATERIAL AND METHODS

Study design

The analyses in this study were conducted at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, in spring 2015. Testosterone and AMH was measured by enzyme-linked immunosorbent assay (ELISA) analysis of already collected blood samples from male Labrador retrievers. The AMH ELISA had earlier been used for dogs (Holst and Dreimanis, 2015) and a brief validation of the testosterone ELISA was performed in this current study. The blood samples were originally collected in 2012-2013 for an accepted, yet unpublished study (Söder et al., in press 2015). Some of them were also used in another study (Hillström et al., 2015). The samples were divided into aliquots, stored in -80 °C.

Animals and inclusion criteria

The study population consisted of 28 intact male show-type Labrador retrievers. The dogs weighed 31,5-48 kg and were 1-9 years old.

To be included in the study, the dog had to be considered healthy by its owner, and pass the clinical health examination conducted at SLU. Furthermore, no history or presence of systemic or organ-related diseases was accepted, nor treatment with antibiotics, non-steroid anti-inflammatory drugs, steroids, deworming drugs or proton pump inhibitors within three months of the examination day.

Conduction of the study

Before arriving to the clinic, all dogs had undergone fasting for 14-17 hours. On arrival, the clinical health examination was conducted by the same veterinarian for all dogs, and the dogs were weighed. To rule out any signs of systemic or organ-related disease, routine analyses of hematology, biochemistry and urine were performed. Subsequently, the fasting serum samples were taken.

All dogs had been classified according to the 1-9 point scale BCS (Laflamme, 1997) by one single veterinarian. The dogs were grouped into lean (BCS 4-5) or overweight (BCS 6-8).

Hormone analysis

Testosterone and AMH were analysed by commercially available human ELISA assays (AMH Gen II ELISA, Beckman coulter, and testosterone ELISA, IBL International, GmBH).

Statistical analysis

The two-sample t-test was used for comparison of body weight and age between groups. The non-parametrical Mann-Whitney U-test was used for comparison of testosterone and AMH concentrations between the lean and overweight dogs, because of non-normal distribution of data. Significance level was set at $P < 0.05$.

Testosterone ELISA validation

A brief validation of the human testosterone ELISA, IBL International, GmBH, was performed for canine serum. The samples used in the validation were originally collected from intact male dogs for a previous study. A total of 27 samples were run on three ELISA-plates, in triplicate the first time and duplicate the last two times.

Intra-assay coefficient of variability (CV) was obtained from analyses of results of five samples with concentrations between 5 and 10 nmol/L, and one sample with a concentration of 15 nmol/L, analysed once in triplicate. Inter-assay CV was calculated after analyzing aliquots of three samples (3-7 nmol/L, 10-14 nmol/L and >17 nmol/L), analysed twice in duplicate.

Linearity upon dilution was studied by diluting (9/10 to 1/10) two samples with dilution media. One of the samples had peaked above the highest calibrator (Dog A) and the other sample had a measured concentration of 8.92 nmol/L (Dog B). After dilution, observed results were plotted against the expected values and a regression fit was performed (Figures 2-5).

Validation results

The intra-assay CV was <6 % for testosterone concentrations of 5-10 nmol/L and 4% for a concentration of 15 nmol/L. The inter-assay CV was 50 % for samples with concentrations of 3-7 nmol/L, 37% for samples of 10-14 nmol/L and 5 % for samples >17nmol/L. After dilution of sample A and B and subsequent construction of simple linear regression models, linearity upon dilution was confirmed (R^2 = 94.8% and 95.6%, respectively). Recovery upon dilution was 45-111% and 74-100%, respectively. In the serial dilution of the canine serum sample A, the ELISA was unable to measure the three highest values correctly (Figure 2-3), even though these values were expected to be below the highest calibrator (16 ng/mL, 55.5 nmol/L).
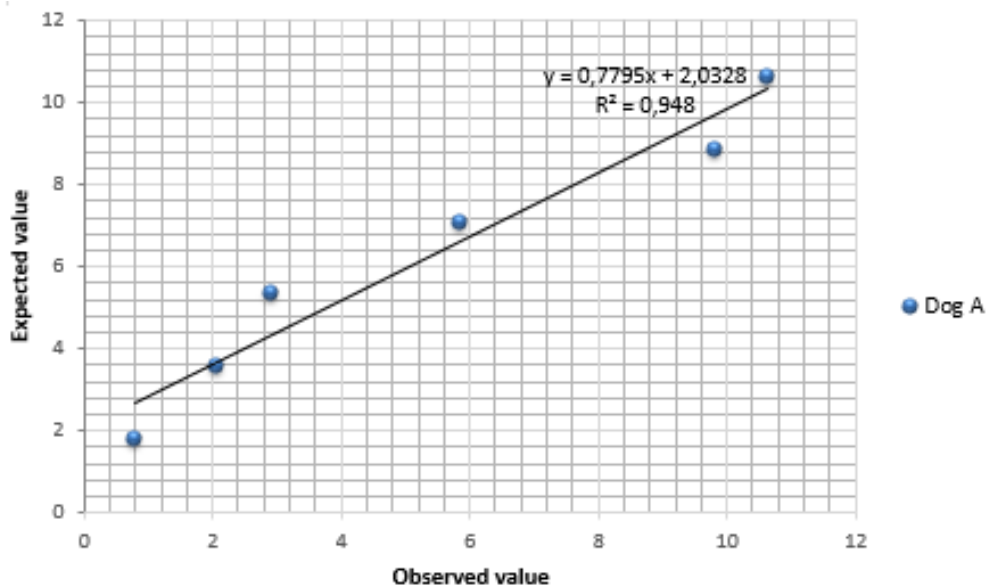

Figure 2. *Serial dilution of a canine serum sample A illustrating adequate linearity with regression fit of measured concentrations of testosterone. The three highest values were excluded in the graph since the ELISA was unable to measure them correctly.*

DILUTION SERIES DOG A				
Name	Dilution	Observed (ng/ml)	Expected	O/E
Dog A	9:10	#VALUE! (too high)	15,94	?
Dog A	8:10	#VALUE! (too high)	14,17	?
Dog A	7:10	#VALUE! (too high)	12,4	?
Dog A	6:10	10,63	10,63	1
Dog A	5:10	9,83	8,86	1,11
Dog A	4:10	5,87	7,09	0,83
Dog A	3:10	2,91	5,32	0,55
Dog A	2:10	2,05	3,54	0,58
Dog A	1:10	0,8	1,77	0,45

Figure 3. *Expected and observed values of testosterone in canine serum sample A. The three highest values were not measured correctly, even though they were expected to be below the highest calibrator.*
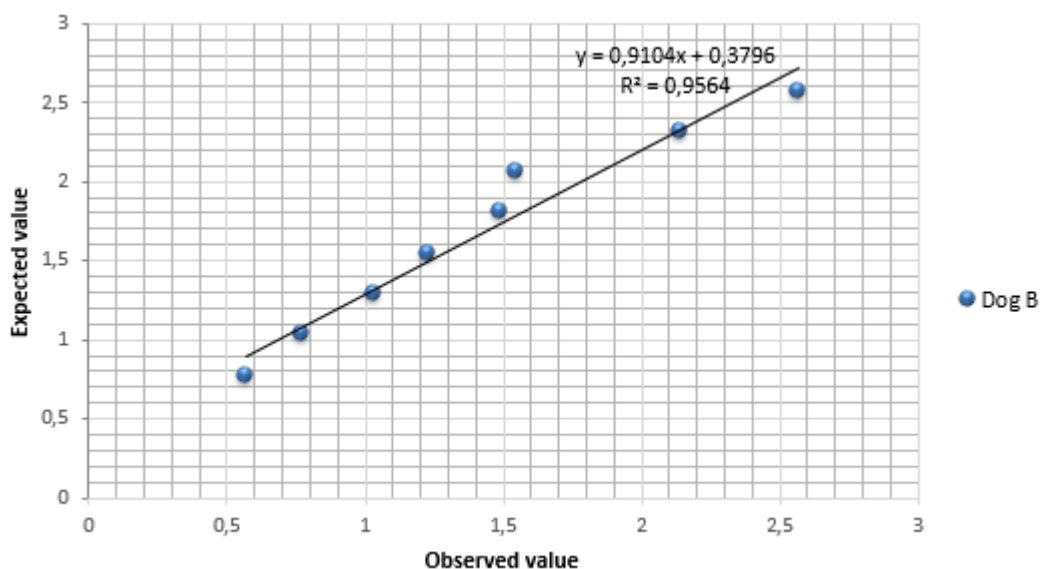

Figure 4. *Serial dilution of canine serum sample B illustrating adequate linearity with regression fit of measured concentrations of testosterone.*

DILUTION SERIES DOG B				
Name	Dilution	Observed (ng/ml)	Expected	O/E
Dog B	10:10	2,57	2,57	1
Dog B	9:10	2,14	2,31	0,93
Dog B	8:10	1,55	2,06	0,75
Dog B	7:10	1,49	1,8	0,83
Dog B	6:10	1,23	1,54	0,8
Dog B	5:10	1,03	1,29	0,8
Dog B	4:10	0,77	1,03	0,75
Dog B	3:10	0,57	0,77	0,74

Figure 5. *Expected and observed values of testosterone in canine serum sample B.*

RESULTS

Parts of the results were presented at the Animal Obesity Congress in Uppsala, Sweden, 14th-16th of June 2015 (Andersson et al., 2015). Twelve dogs were classified as lean and 16 were classified as overweight (Table 2). Body weight differed significantly between groups (P = 0.002). Age did not differ significantly between groups (P = 0.91).

Table 2. *Mean variables (age and weight) ± standard deviation (SD) of the 28 Labrador retrievers included in the study.*

	Lean BCS 4-5 *n*=12	**Overweight** BCS 6-8 *n*=16
Age *(year)*	5.3 ± 1.4	5.2 ± 1.6
Body weight *(kg)*	34.8 ± 2.5	39.5 ± 4.7

Testosterone results from three dogs from the overweight group were excluded due to technical problems. Median concentration and inter-quartile range was 9.6 ng/mL (7.4-14.1) for AMH and 9.7 nmol/L (6.3-15.4) for testosterone. The AMH concentrations in all dogs ranged between 5.21 and 22 ng/mL (Figure 6). The testosterone concentrations in all dogs ranged between 1.58 and 20.16 nmol/L (Figure 7). Serum concentrations of testosterone and AMH did not differ significantly between lean and overweight dogs (P = 0.11 and P = 0.63, respectively).
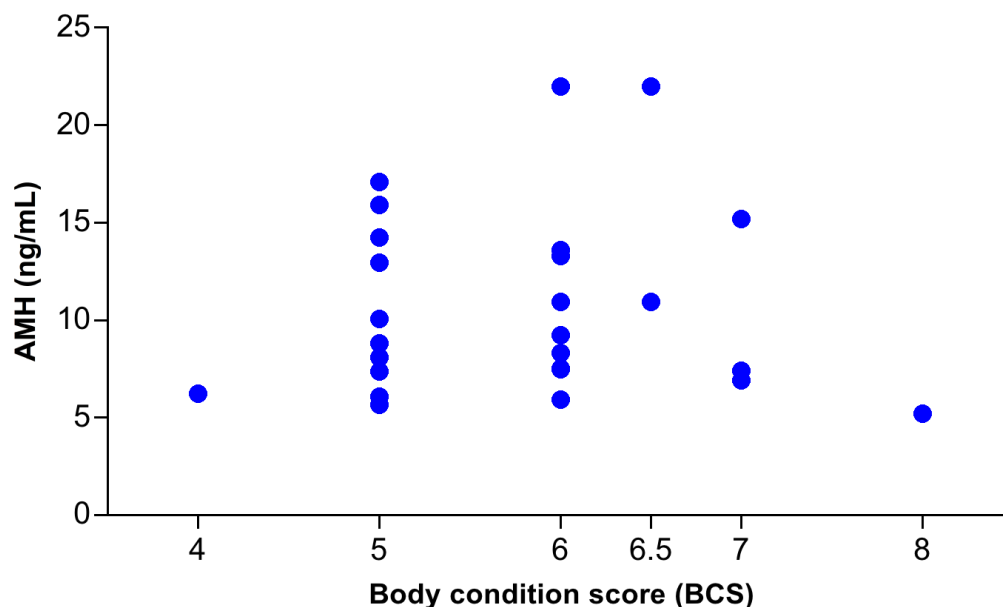

Figure 6. *Serum concentration of Anti-Müllerian hormone (AMH) in lean (BCS 4-5) and overweight (BCS 6-8) male Labrador retrievers.*
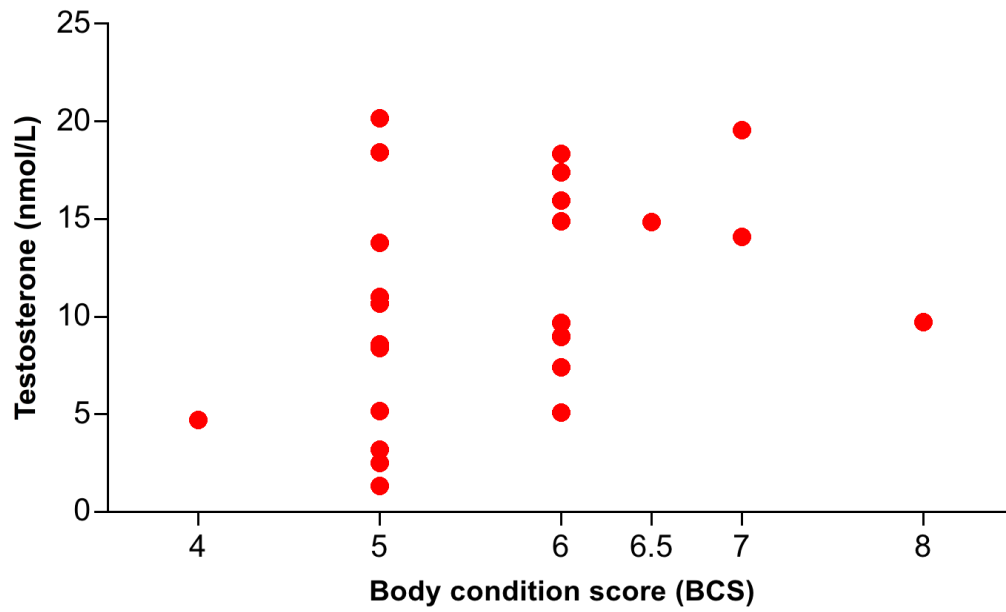
Figure 7. *Serum concentration of testosterone in lean (BCS 4-5) and overweight (BCS 6-8) male Labrador retrievers. Testosterone results from three dogs were excluded due to technical problems.*

DISCUSSION

In this study, serum concentrations of testosterone and AMH did not differ significantly between the lean and overweight group of dogs (i.e. BCS had no impact on serum concentration of testosterone or AMH in male dogs). It is possible that the lack of difference is true. The results could also be due to several other reasons. Firstly, the sample population might have been too small, only 28 dogs, and the power of the study thus limited. In contrast, the human studies referred to in this thesis include considerably larger study populations, with wider spans in BMI, which may reveal even small differences between obese and normal weight groups.

Also, the BCS in the overweight dogs was rather low (only mild overweight). The choice of the Labrador retriever for the study was because it is the most common dog breed in Sweden (SKK Registreringsstatistik, 2014) and because it is a breed prone to obesity (Edney and Smith, 1986, Colliard et al., 2006, Lund et al., 2006, Corbee, 2013, Raffan et al., 2014). Using only one breed also makes the study population more homogenous. Even though body weight differed significantly between groups ($P = 0.002$), we did not manage to recruit really overweight, and at the same time healthy, dogs (only one dog had BCS 8) to the overweight group. The recruiting difficulties may be coupled to feelings of guilt among owners of obese dogs.

In general, one difficulty when writing this thesis, was the lack of canine studies in this field of reproduction and obesity. Therefore, instead much focus has been on human studies for comparison. Our hypothesis was that overweight dogs have lower testosterone and AMH concentrations than lean dogs, because that is what has been proven in men.

The cause of lowered AMH concentrations in obese men is unclear. Some studies found that testosterone has an inhibitory role on testicular AMH secretion in men (Rey et al., 1993, Young et al., 1999). If this is true, and if obese patients really express hyperestrogenic hypogonadotropic hypogonadism as earlier mentioned, high AMH concentrations is to be expected because of the lower testosterone concentrations. This is inconsistent with the more recent results showing that obese men have lowered AMH concentrations. The authors hypothesized that the low AMH concentrations depend on Sertoli cell impairment, caused by the anomalies associated with obesity, such as insulin resistance and higher concentrations of adipocytokines in the adipose tissue (Robeva et al., 2012). It is well known that, to a large extent, the adipocytokines expressed in both human and canine adipose tissue are proinflammatory, such as IL-6 and TNFα (Coppack, 2001, Ryan et al., 2010). Maybe the inflammatory state in obese individuals suppresses the Sertoli cells and hence AMH production. Against this background, we wanted to investigate what applies to male dogs regarding AMH and obesity.

Even though AMH in our study did not differ significantly between the lean and overweight dogs, AMH concentrations were generally high compared to what has been shown in an earlier study on AMH in dogs (Holst and Dreimanis, 2015), using the same ELISA. In the study by Holst and Dreimanis, healthy dogs did not have higher AMH concentrations than 10 ng/mL, and dogs with Sertoli cell tumours (SCTs) had values > 22 ng/mL. Some dogs with non-neoplastic testicular pathologies, or testicular tumours other than SCTs, had AMH values

between 10-22 ng/mL. These results are consistent with other studies that reported expression of AMH in canine SCTs (Banco et al., 2012), and increased expression of AMH in dogs with testicular atrophy (a risk factor for tumour development) (Giudice et al., 2014). As earlier mentioned, AMH is considered to be a useful marker of Sertoli cell maturation. It is likely that with testicular degeneration, the Sertoli cells may regress and become more immature, which in turn causes them to produce more AMH.

In our study, three dogs had AMH concentrations higher than 22 ng/mL, and eleven dogs had AMH concentrations between 10 and 22 ng/mL. It seems unlikely that so many dogs in our study population would have testicular neoplastic changes or testicular degeneration, but unfortunately, in this study the dogs' testicles were only evaluated by palpation, without finding any abnormalities. By palpation only, minor testicular lesions cannot be ruled out, which is a limitation in our study. It would have been better to combine testicular palpation with ultrasonography or histopathological examination, but due to financial and practical circumstances, this was not possible. It would also have been interesting to collect concurrent semen samples and information on libido from the participating dogs, to be able to study sperm morphology and hence further investigate the association between BCS and fertility.

Regarding the testosterone in our study, results from three dogs were excluded due to technical problems; these three samples peaked above the highest calibrator. Unfortunately, we did not have the opportunity to run these samples again after dilution. On behalf of The Council of The Endocrine Society, Rosner et al. (2007) have reviewed the current utility and limitations in measuring testosterone in humans. They conclude that direct assays such as ELISA immunoassays are commonly used for measuring testosterone, but that they have several shortcomings, e.g. the risk of matrix effects, such as interfering proteins or cross-reacting steroids. Because of these problems, Rosner et al. (2007) recommends extraction of such interfering molecules and thereafter mass spectrometry as the gold standard for testosterone measurement in humans. With mass spectrometry, the chemical structure of the testosterone molecule is identified. Liquid chromatography mass spectrometry (LC-MS/MS) has recently been used in a pilot study on pregnant and nonpregnant bitches, where endogenous steroids were measured with promising results (Holst et al., 2015). Because of financial circumstances, the use of extraction and mass spectrometry was not possible in our current study. Holst et al. (2015) also states the LC-MS/MS method should be further validated before it can be used for routine diagnostics of canine samples.

In general, other difficulties with measuring testosterone includes the diurnal secretion pattern of GnRH and therefore also testosterone. To minimize influence of this on our results, all samples were collected at the same time during the day, 08.00-10.00, when testosterone serum concentration peaks, as recommended by The Endocrine Society (Rosner et al., 2007, Bhasin et al., 2013). Also, at least in humans, testosterone concentration varies depending on age and gender. This potential error was minimized in our study since age did not differ significantly between groups and all were intact male dogs. Though, as far as the authors know, what applies to dogs according to time of peak concentration and age and gender fluctuations, has not been investigated.

Human error may also influence the test results in several steps in the analysis process, for example when pipetting the sample into the ELISA wells. Another crucial step is when rinsing the wells with wash buffer after incubation, to wash away samples and enzyme conjugate. To minimize these errors, good laboratory practice and analyse method instructions were followed carefully.

The testosterone ELISA validation

The testosterone molecule structure is well preserved among humans and dogs. Though, since there are a lot of challenges in the measurement of testosterone even in humans, and since matrix effects may vary between species, we decided to do a brief validation of the testosterone ELISA assay for canine serum in our current study. Because the AMH ELISA used in this study had earlier been used in dogs (Holst and Dreimanis, 2015), no validation was performed on AMH.

As Rosner et al. (2007) reports, in humans most trouble lies in measuring testosterone with accuracy in the low ranges of concentration, which is clinically important for humans in diagnosis of e.g. hypogonadism and PCOS. In males, a total testosterone < 200 ng/dl (6.9 nmol/L) is diagnostic of hypogonadism (Rosner et al., 2007). In the validation performed in this study, the ELISA measured these values in the low range of concentration without major problems. Also, most of the dogs had testosterone values above or around 6.9 nmol/L. However, in the serial dilution of samples from dog A the ELISA was unable to correctly measure the three highest values of testosterone, even though these values were expected to be below the highest calibrator (< 16 ng/ml, 55.5 nmol/L). The inter-assay CV for low and medium concentrations was high (37-50%), whereas the CV was 5% for the highest concentrations.

In general, the values in the low range of concentration are not clinically as important in dogs as in humans. Blood testosterone concentrations may be helpful in differentiating intact versus castrated versus bilaterally cryptorchid dogs. The basal concentration of testosterone in normal dogs alters between 1 and 5 ng/ml (3.47 and 17.35 nmol/L), meanwhile in a cryptorchid dog between 0.1 and 2 ng/ml (0,35 and 6,94 nmol/L) (Feldman and Nelson, 2004).

It is undesirable that the ELISA shows lack of accuracy in the high range of testosterone concentration. Because of this, if one is to use this IBL human testosterone ELISA for canine purposes, it can be recommended to dilute peaking samples to secure more correct measurement of the testosterone concentrations. This is preferably done by running duplicates of the samples, where each sample is run in 1:1 and also by dilution to 1:2. E.g., since the peaking values in this study were expected to be below the highest calibrator, a peaking sample should in reality not exceed 16 ng/ml (55.5 nmol/L). If this peaking sample is diluted to 1:2, it should be 8 ng/ml (27.8 nmol/L), and then be measured correctly since it then is well under the highest calibrator.

In conclusion, the testosterone validation showed that the IBL human testosterone ELISA is acceptable, though not optimal, to use for canine study purposes. To optimize the validation procedure, we should have included more ELISA runs, and thereafter validated our ELISA

against the gold standard mass spectrometry analysis method. For financial and practical reasons, only a limited validation was performed in the present study.

CONCLUSION

In the present study, serum concentrations of testosterone and AMH did not differ significantly between lean and overweight dogs. This may be correct, but also limitations of the study and technical aspects could have influenced the results.

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REFERENCES

Al-Qahtani, A., Muttukrishna, S., Appasamy, M., Johns, J., Cranfield, M., Visser, J., Themmen, A. & Groome, N. (2005). Development of a sensitive enzyme immunoassay for Anti-Müllerian hormone and the evaluation of potential clinical applications in males and females. *Clinical Endocrinology*, 63(3):267-273.

Andersson, E., Söder, J., Höglund, K., Hagman, R., Wernersson, S., Holst, B. S. (2015). Testosterone and Anti-Müllerian hormone (AMH) in lean and overweight male Labrador retrievers. *Acta Veterinaria Scandinavica*, doi 10.1186/1751-0147-57-S1-P1. [2015-09-25].

Banco, B., Veronesi, M., Giudice, C., Rota, A. & Grieco, V. (2012). Immunohistochemical evaluation of the expression of Anti-Müllerian hormone in mature, immature and neoplastic canine Sertoli cells. *Journal of Comparative Pathology*, 146(1):18-23.

Bhasin, S., Cunningham, G. R., Hayes, F. J., Matsumoto, A. M., Snyder, P. J., Swerdloff, R. S. & Montori, V. M. (2013). Testosterone therapy in adult men with androgen deficiency syndromes: an endocrine society clinical practice guideline. *The Journal of Clinical Endocrinology & Metabolism*, 95(6):2536–2559.

Cabler, S., Agarwal, A., Flint, M. & Du Plessis, S. S. (2010). Obesity: modern man's fertility nemesis. *Asian Journal of Andrology*, 12(4):480-489.

Colliard, L., Ancel, J., Benet, J.-J., Paragon, B.-M. & Blanchard, G. (2006). Risk factors for obesity in dogs in France. *The Journal of Nutrition*, 136(7):1951S-1954S.

Coppack, S. W. (2001). Pro-inflammatory cytokines and adipose tissue. *Proceedings of the Nutrition Society,* 60(3):349-356.

Corbee, R. J. (2013). Obesity in show dogs. *Journal of Animal Physiology and Animal Nutrition*, 97(5):904-910.

Corona, G., Mannucci, E., Schulman, C., Petrone, L., Mansani, R., Cilotti, A., Balercia, G., Chiarini, V., Forti, G. & Maggi, M. (2006). Psychobiologic correlates of the metabolic syndrome and associated sexual dysfunction. *European Urology*, 50(3):595-604.

Edney, A. & Smith, P. (1986). Study of obesity in dogs visiting veterinary practices in the United Kingdom. *Veterinary Record*, 118(14):391-396.

Fejes, I., Koloszar, S., Zavaczki, Z., Daru, J., Szöllösi, J. & Pal, A. (2006). Effect of body weight on testosterone/estradiol ratio in oligozoospermic patients. *Systems Biology in Reproductive Medicine*, 52(2):97-102.

Feldman, E. C., & Nelson, R. W. (2004). *Canine and Feline Endocrinology and Reproduction*. 3. ed. St. Louis: Saunders.

Giudice, C., Banco, B., Veronesi, M., Ferrari, A., Di Nardo, A. & Grieco, V. (2014). Immunohistochemical expression of markers of immaturity in Sertoli and seminal cells in canine testicular atrophy. *Journal of Comparative Pathology*, 150(2-3):208-215.

Hammoud, A. O., Gibson, M., Peterson, C. M., Hamilton, B. D. & Carrell, D. T. (2006). Obesity and male reproductive potential. *Journal of Andrology*, 27(5):619-626.

Hammoud, A. O., Wilde, N., Gibson, M., Parks, A., Carrell, D. T. & Meikle, A. W. (2008). Male obesity and alteration in sperm parameters. *Fertility and Sterility*, 90(6):2222-2225.

Hillström, A., Hagman, R., Söder, J., Häggström, J., Ljungvall, I. & Kjelgaard-Hansen, M. (2015). Validation and application of a canine-specific automated high-sensitivity C-reactive protein assay. *Journal of Veterinary Diagnostic Investigation*, 27(2):182-190.

Hoenig, M. (2010). Obesity. In: Rijnberk, A. & Kooistra, HS. (eds), *Clinical Endocrinology of Dogs and Cats*. 2. ed. Hannover: Schluetersche, 297.

Hofstra, J., Loves, S., van Wageningen, B., Ruinemans-Koerts, J., Jansen, I. & de Boer, H. (2008). High prevalence of hypogonadotropic hypogonadism in men referred for obesity treatment. *The Netherlands Journal of Medicine*, 66(3):103-109.

Holst, B. S. & Dreimanis, U. (2015). Anti-Müllerian hormone: a potentially useful biomarker for the diagnosis of canine Sertoli cell tumours. *BMC Veterinary Research*, 11:166.

Holst, B. S., Kushnir, M. M. & Bergquist, J. (2015). Liquid chromatography-tandem mass spectrometry (LC-MS/MS) for analysis of endogenous steroids in the luteal phase and early pregnancy in dogs: a pilot study. *Veterinary Clinical Pathology*, 44(4):552-558.

Hubert, H. B., Feinleib, M., Mcnamara, P. M. & Castelli, W. P. (1983). Obesity as an independent risk factor for cardiovascular disease: a 26-year follow-up of participants in the Framingham Heart Study. *Circulation*, 67(5):968-977.

Håkonsen, L. B., Thulstrup, A. M., Aggerholm, A. S., Olsen, J., Bonde, J. P., Andersen, C. Y., Bungum, M., Ernst, E. H., Hansen, M. L. & Ernst, E. H. (2011). Does weight loss improve semen quality and reproductive hormones? Results from a cohort of severely obese men. *Reproductive Health*, 8:24.

Impellizeri, J. A., Tetrick, M. A. & Muir, P. (2000). Effect of weight reduction on clinical signs of lameness in dogs with hip osteoarthritis. *Journal of the American Veterinary Medical Association*, 216(7):1089-1091.

Jensen, T. K., Andersson, A.-M., Jørgensen, N., Andersen, A.-G., Carlsen, E. & Skakkebæk, N. E. (2004). Body mass index in relation to semen quality and reproductive hormones among 1,558 Danish men. *Fertility and Sterility*, 82(4):863-870.

Josso, N. & di Clemente, N. (1999). TGF-β family members and gonadal development. *Trends in Endocrinology & Metabolism*, 10(6):216-222.

Kaukua, J., Pekkarinen, T., Sane, T. & Mustajoki, P. (2003). Sex hormones and sexual function in obese men losing weight. *Obesity research*, 11(6):689-694.

Kealy, R. D., Lawler, D. F., Ballam, J. M., Mantz, S. L., Biery, D. N., Greeley, E. H., Lust, G., Segre, M., Smith, G. K. & Stowe, H. D. (2002). Effects of diet restriction on life span and age-related changes in dogs. *Journal of the American Veterinary Medical Association*, 220(9):1315-1320.

Labrador retrieverklubben (2014-01-01). *Om rasen.* http://www.labradorklubben.se/ [2015-10-05]

Laflamme, D. (1997). Development and validation of a body condition score system for dogs. *Canine practice*, 22(4):10-15.

Lukas-Croisier, C., Lasala, C., Nicaud, J., Bedecarrás, P., Kumar, T. R., Dutertre, M., Matzuk, M. M., Picard, J.-Y., Josso, N. & Rey, R. (2003). Follicle-stimulating hormone increases testicular Anti-Müllerian hormone (AMH) production through Sertoli cell proliferation and a nonclassical cyclic adenosine 5′-monophosphate-mediated activation of the AMH Gene. *Molecular Endocrinology,* 17(4):550-561.

Lund, E. M., Armstrong, P. J., Kirk, C. A. & Klausner, J. S. (2006). Prevalence and risk factors for obesity in adult dogs from private US veterinary practices. *International Journal of Applied Research in Veterinary Medicine*, 4(2):177-186.

MacDonald, A. A., Herbison, G. P., Showell, M. & Farquhar, C. M. (2010). The impact of body mass index on semen parameters and reproductive hormones in human males: a systematic review with meta-analysis. *Human Reproduction Update*, 16(3):293-311.

Mason, E. (1970). Obesity in pet dogs. *Veterinary Record*, 86(21):612-616.

Mawby, D. I., Bartges, J. W., d'Avignon, A., Laflamme, D. P., Moyers, T. D. & Cottrell, T. (2004). Comparison of various methods for estimating body fat in dogs. *Journal of the American Animal Hospital Association*, 40(2):109-114.

McGreevy, P. D., Thomson, P. C., Pride, C., Fawcett, A., Grassi, T. & Jones, B. (2005). Prevalence of obesity in dogs examined by Australian veterinary practices and the risk factors involved. *Veterinary Record*, 156(22):695-702.

Must, A., Spadano, J., Coakley, E. H., Field, A. E., Colditz, G. & Dietz, W. H. (1999). The disease burden associated with overweight and obesity. *The Journal of the American Medical Association*, 282(16):1523-1529.

Nationalencyklopedin (2015-01-01). *Fertilitet.* http://www.ne.se/uppslagsverk/encyklopedi/l%C3%A5ng/fertilitet [2015-10-07]

Nationalencyklopedin (2015-01-01). *Infertilitet.* http://www.ne.se/uppslagsverk/encyklopedi/l%C3%A5ng/infertilitet [2015-10-07]

Ng, M., Fleming, T., Robinson, M., Thomson, B., Graetz, N., Margono, C., Mullany, E. C., Biryukov, S., Abbafati, C. & Abera, S. F. (2014). Global, regional, and national prevalence of overweight and obesity in children and adults during 1980–2013: a systematic analysis for the Global Burden of Disease Study 2013. *The Lancet*, 384(9945):766-781.

Ogden, C. L., Carroll, M. D., Curtin, L. R., McDowell, M. A., Tabak, C. J. & Flegal, K. M. (2006). Prevalence of overweight and obesity in the United States, 1999-2004. *The Journal of the American Medical Association*,, 295(13):1549-1555.

Patterson, R. E., Frank, L. L., Kristal, A. R. & White, E. (2004). A comprehensive examination of health conditions associated with obesity in older adults. *American Journal of Preventive Medicine*, 27(5):385-390.

Pellitero, S., Olaizola, I., Alastrue, A., Martínez, E., Granada, M. L., Balibrea, J. M., Moreno, P., Serra, A., Navarro-Díaz, M., Romero, R. & Puig-Domingo, M. (2012). Hypogonadotropic hypogonadism in morbidly obese males is reversed after bariatric surgery. *Obesity surgery*, 22(12):1835-1842.

Pietiläinen, K. H., Kaprio, J., Vaaralahti, K., Rissanen, A. & Raivio, T. (2011). Circulating Anti-Müllerian hormone levels in adult men are under a strong genetic influence. *The Journal of Clinical Endocrinology & Metabolism*, 97(1):E161-E164.

Raffan, E., Becker, J., Yeo, G. & O'Rahilly, S. (2014). The coding sequence of POMC and obesity and appetite in Labrador retriever dogs. *The Lancet*, doi 10.1016/S0140-6736(14)60349-4. [2015-12-26].

Rajpert-De Meyts, E., Jørgensen, N., Graem, N., Müller, J., Cate, R. L. & Skakkebæk, N. E. (1999). Expression of Anti-Müllerian hormone during normal and pathological gonadal development: association with differentiation of Sertoli and Granulosa cells. *The Journal of Clinical Endocrinology & Metabolism*, 84(10):3836-3844.

Rey, R., Lordereau-Richard, I., Carel, J.-C., Barbet, P., Cate, R. L., Roger, M., Chaussain, J.-L. & Josso, N. (1993). Anti-Müllerian hormone and testosterone serum levels are inversely related during normal and precocious pubertal development. *The Journal of Clinical Endocrinology & Metabolism*, 77(5):1220-1226.

Robeva, R., Tomova, A., Kirilov, G. & Kumanov, P. (2012). Anti-Müllerian hormone and inhibin B levels reflect altered Sertoli cell function in men with metabolic syndrome. *Andrologia*, 44:329-334.

Rosner, W., Auchus, R. J., Azziz, R., Sluss, P. M., & Raff, H. (2007). Utility, limitations and pitfalls in measuring testosterone: an Endocrine Society position statement. *The Journal of Clinical Endocrinology & Metabolism*, 92(2):405-413.

Ryan, V. H., German, A. J., Wood, I. S., Hunter, L., Morris, P. & Trayhurn, P. (2010). Adipokine expression and secretion by canine adipocytes: stimulation of inflammatory adipokine production by LPS and TNFα. *Pflügers Archiv - European Journal of Physiology*, 460(3):603-616.

Sjaastad, Ø. V., Sand, O. & Hove, K. (2010). *Physiology of Domestic Animals*. 2. ed. Oslo: Scandinavian Veterinary Press.

Svenska Kennelklubben (2014-01-01). *Press: Registreringsstatistik 2014.* http://www.skk.se/om-skk/det-har-ar-skk/press/ [2015-11-24]

Söder, J., Wernersson, S., Hagman, R., Karlsson, I., Malmlöf, K. & Höglund, K. (in press 2015). Metabolic and hormonal response to a feed-challenge test in lean and overweight dogs. *Journal of Veterinary Internal Medicine*.

The Rotterdam ESHRE/ASRM-Sponsored PCOS Consensus Workshop Group (2004). Revised 2003 consensus on diagnostic criteria and long-term health risks related to polycystic ovary syndrome. *Fertility and Sterility*, 81(1):19-25.

Toll, P. W., Yamka, R. M., Schoenherr, W. D. & Hand, M. S. (2010). Obesity. In: Hand, Thatcher, Remillard, Roudebush & Novotny (eds), *Small Animal Clinical Nutrition*. 5. ed. Topeka: Mark Morris Institute, 501-542.

Wilson, C. A., di Clemente, N., Ehrenfels, C., Pepinsky, R. B., Josso, N., Vigier, B. & Cate, R. L. (1993). Mullerian inhibiting substance requires its N-terminal domain for maintenance of biological activity, a novel finding within the transforming growth factor-beta superfamily. *Molecular Endocrinology*, 7(2):247-257.

World Health Organization (2015-01-01). *Obesity and overweight.* http://www.who.int/mediacentre/factsheets/fs311/en/ [2015-09-23]

Young, J., Rey, R., Couzinet, B., Chanson, P., Josso, N. & Schaison, G. (1999). Antimüllerian hormone in patients with hypogonadotropic hypogonadism. *The Journal of Clinical Endocrinology & Metabolism*, 84(8):2696-2699.