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Role of leptin in reproductive function of male dog

A study on immunohistochemical detection of leptin and its receptors in the canine testis, epididymis and sperm

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TABLE OF CONTENT

Introduction

Studies investigating leptins role in canine male reproductive function has not previously been done. For that particular reason, this evaluation of a previous pilot study was designed to investigate the presence of leptin and leptin receptors in canine testis, epididymis and spermatozoa.

Obesity is currently the most frequent nutritional disease existing in companion animals, however this increasingly issue is not given proper attention.
In obese animals an increased amount of adipose tissue is found. With an excess of adipocytes, an increased level of leptin will be produced. An interesting question is therefore raised, and it could possibly be of great value if we were capable of finding out whether leptin demonstrates an impact towards the male reproductive function. However, this issue should be investigated by first examining the role of leptin in the function of the male genital tract of healthy dogs in normal condition.

In this research we obtained samples from six healthy and mature dogs. The samples were collected through routinely neutering and manual semen collection. In this thesis, we performed the analysis of results from a previously performed sample collection and immunohistochemical examination. I compared these results to the data in literature in other species.

Testis and epididymis were fixed in formalin and finally embedded in paraffin prior to immunohistochemistry identification. We used an indirect immunoperoxidase method to perform the immunohistochemistry. Rabbit polyclonal antibodies were used for detection of leptin and goat polyclonal antibodies for leptin receptors. Murine ovaries with CL and canine CL were used as positive controls, and a chromogenic agent was applied to make it possible to evaluate the result. The slides were assessed under 100x magnification.

A full sperm-rich portion of semen was collected by manual stimulation. Both epididymal and ejaculated spermatozoa were further purified using the swim-up technique prior to immunocytochemistry identification. The samples were collected, purified and processed and

the immunocytochemical expression of the antibodies was obtained through the same indirect immunoperoxidase method as used in immunohistochemistry.

The Objective of this study is to investigate if it is possible to detect leptin and its receptors in the male reproductive tract of canines with the use of immunohistochemistry and immunocytochemistry.

Survey of literature

1. Male reproductive tract

1.1 Anatomy

The male reproductive organs comprehend paired gonads, the testes producing male gametes and hormones, paired gonadal duct systems each consisting of an epididymis and a deferent duct, a suite of accessory glands, the male urethra, the penis and skin adaptations like the scrotum and prepuce (Dyce et al., 2002).

Testis and epididymis

Tunica albuginea is a common thick capsule enclosing the testis, a composite tubular gland combining endocrine and exocrine components. Each lobule within the testis is made of highly convoluted seminiferous tubules, constituting the exocrine portion of the testis whose product are spermatozoa. Within the tunica albuginea, dense connective tissue modifies to a delicate connective tissue layer arranged with lymph vessels, blood vessels and massed interstitial cells called Leydig cells. These constitute the endocrine tissue of the testis producing steroid androgenic hormones. At the end of the spermatic cords the testes are suspended into the scrotum, each of which consist of the excretory duct of the testis, the ductus deferens and the blood vessels and nerves supplying the testis.

The epididymis is an elongated organ divided into three parts; caput (head), corpus (body) and cauda (tail). It is closely attached to the posterior surface of the testis, and is made up of the convoluted proximal part of the excretory duct system, from the efferent ductules in the testis to epididymal ducts ending in the deferent duct (Bloom et al., 1962; Dyce et al., 2002).

Seminiferous tubules

Within each seminiferous tubule, two cell types are lining the walls, the supporting cells and the spermatogenic cells. The supporting elements are of a solitary somatic cell type, the Sertoli cell, providing mechanical support and protection for the developing germ cells through production of hormones and growth factors. The spermatogenic cells include a number of morphologically distinguishable types: spermatogonia, primary spermatocytes, secondary spermatocytes, spermatids and spermatozoa. They are clearly distinguishable alternating stages in the process of differentiation of the male germ cells (Bloom et al., 1962; Dyce et al., 2002).

1.2 Physiology of the male reproductive system; spermatogenesis and the cycle of seminiferous epithelium

Spermatogenesis is the sequence where the primordial germ cells are transformed from diploid spermatogonia (2n) into haploid (n) spermatozoa within the seminiferous tubules over an extended period of time. The germ cell genotype controls the total duration of spermatogenesis, and it last from 30 to 75 days in mammals (Soares et al., 2009). This process is divided into three phases; the spermatocytogenesis, meiosis and spermatogenesis. These phases are made up of different cell associations, called stages and both the phases and stages are essential to maintain a continuous sperm production. It is dependent on several elements, from intrinsic (germ cells and Sertoli cells) to extrinsic (androgens and retinoic acids) factors (Cheng, 2008).

Numerous concentric layers penetrated by a single type of somatic cells, identified by Enrica Sertoli in 1865, make up the seminiferous epithelium. As mentioned above, the Sertoli cells are present to maintain and nurture the germ cells throughout spermatogenesis. The first step of germ cell is to repeatedly divide through mitosis, followed by meiotic division. It appears in this way to accomplish a duplication of chromosomes, a genetic recombination, to further reduce the chromosomes in forming haploid spermatids finally becoming mature compacted spermatozoa.

- *1st, spermatocytogenesis; mitosis.* Spermatogonia begin as diploid germ cells proliferating by mitotic division to replace themselves and give rise to spermatocytes.
- *2nd, meiosis*. Through another mitosis, B-spermatogonia divide and form two preleptone spematogonia, which is the beginning of the meiotic prophase. They undergo two maturation divisions, where they first reduce the chromosome number to produce secondary spermatocytes followed by spermatids:

  Meiosis I: 4n cells are divided to form secondary spermatocytes (2n). Meiosis II: the division of secondary spermatocytes with 2n forms haploid spermatids (n).
- *3rd, spermiogenesis*. In the latter phase, haploid, spherical spermatids undergo advanced differentiation steps, leading to the formation of elongated mature spermatozoa (Bloom et al., 1962; Cheng, 2008).

The seminiferous epithelium cycle stages are characterized according to the acrosome system and morphology of the spermatid nucleus. Soares, Avelar and França described eight cycles in

the dog, although Chang defined XII stages (divided into stage I-V as the early stage, VI-VIII as middle stage and IX-XII as late stage) in the mouse.

In the dog stage I, II, III and IV were characterized with a few basal type A-spermatogonia present, together with preleptone spermatocytes. Two generations of spermatids, the early round ones and the elongated spermatids was also present. Stage V and VI showed rounded, smaller and more numerous spermatogonia of both type A and B, pachytene spermatocytes and elongated spermatids. Spermatozoa were present in the tubular lumen. Stage VII and VIII presented elongation and growth of spermatids and they formed bundles with their heads pointing towards the tubule. Primary and pachytene spermatocytes were found together with an increased number of type A spermatogonia (Soares et al., 2009; Bernal-Mañas et al., 2013).

2. Physiology of the hypothalamic pituitary gonadal axis

Hypothalamus (HT) secretes gonadotropin-releasing hormone (GnRH) in response of feedback provided by the body. GnRH stimulates the anterior pituitary (P) to produce gonadotropic hormones like follicular stimulating hormone (FSH) and luteinizing hormone (LH) (figure 1). Leydig cells in the testis are the target cells for LH and upon stimulation from LH they will produce testosterone, which has a primary role in steroidogenesis as well as spermatogenesis. Together with FSH, testosterone stimulates the seminiferous tubules and act on them in the way that they support germ cells inducing spermatogenesis. More specifically, FSH targets Sertoli cells in the testis and has its primary role in the spermatogenesis. Leydig cells producing androgens and Sertoli cells producing inhibin constituents the endocrine functions of the testis. Both are stimulated under the pulsatile release of the gonadotropins. Through negative feedback mechanisms, inhibin seems to regulate secretion of FSH as well (figure 1). Androgens possess distinct local purpose but are also responsible for secondary sex characteristics in the manner of maturation if the accessory sex glands, skeletomuscular development, skin characteristics and prenatal differentiations of certain brain and spinal cord nuclei. (Bloom et al., 1962; Dyce et al., 2002)

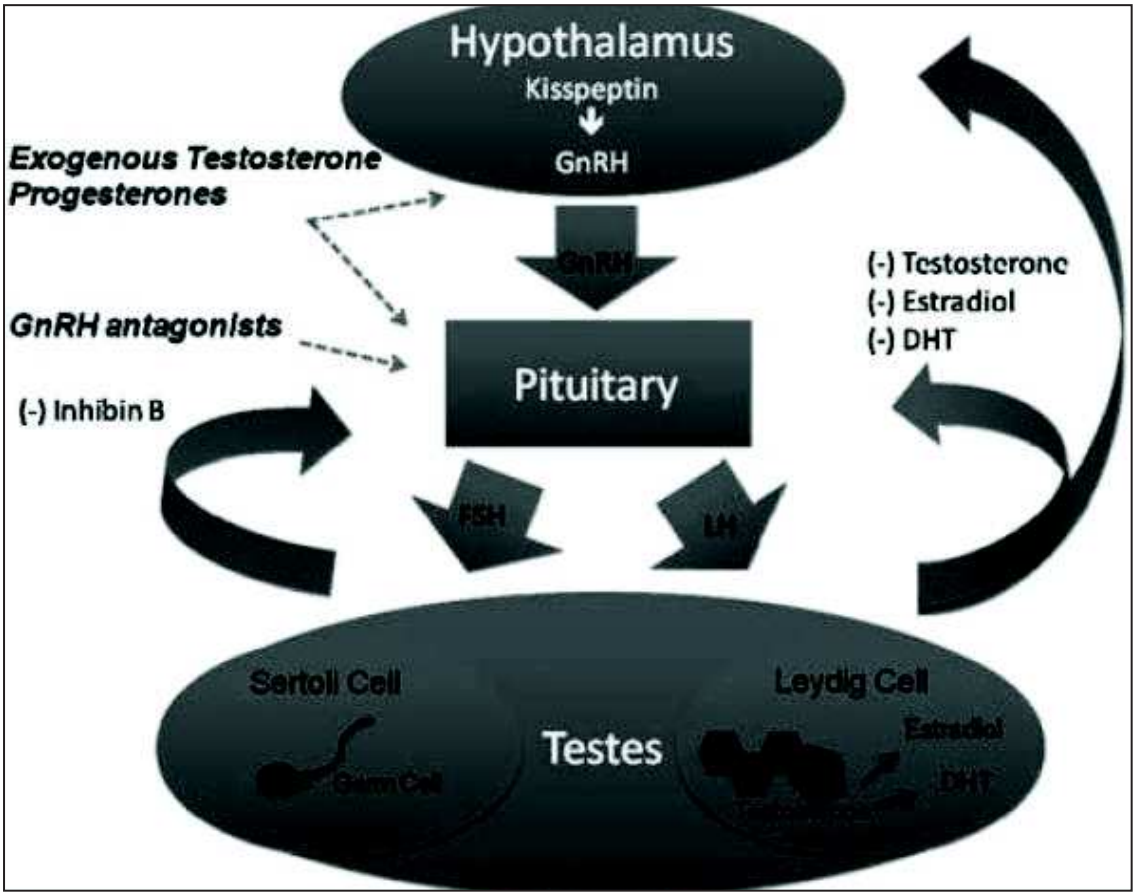

Figure 1. Endocrinology of spermatogenesis. The hypothalamic–pituitary–gonadal axis (Medscape, 2015).

Thus both FSH and testosterone within the testes is required in the production of spermatozoa. The seminiferous tubules are immersed in endogenous testosterone secretion from the interstitial Leydig cells, which promotes spermatogenesis. (Bloom, Fawcett and Alexander, 1962). Testosterone is also essential for the maintenance of the secretory and absorptive activities of the efferent duct, epididymis and ductus deference, for the growth and maintenance of prostate gland and maintaining libido.

Although, the precise role of FSH in promoting spermatogenesis is not clear but its secretion is necessary to maintain germ cell differentiation and development of spermatozoa (Ganong, 1981). FSH additionally facilitates the completion of spermatid maturation by stimulating Sertoli cell development, Sertoli cell function, and the synthesis of androgen- binding protein (Bloom and Fawcett, 1975).

3. The role of leptin

*„**Leptin**- a helical protein secreted by adipose tissue and acting on a receptor site in the ventromedial nucleus of the hypothalamus to curb appetite and increase energy expenditure as body fat stores increase." (TheFreeDictionary.com, 2015)*

The name „leptin" originates from the Greek word „leptos", which means „thin" or „lean" coming from the weight-reducing quality of the hormone (Henson and Castracane, 2003). Leptin, a 16-KD protein secreted by adipose tissue, was primarily seen as a satiety hormone believed to play its key role in regulation of food intake and body weight homeostasis (Smith, Jackson, Foster, 2002). Adipose tissue is acknowledged as an endocrine organ that secretes steroid hormones. Leptin is the prototype adipocyte-secreted hormone or cytokine (adipokine) that was identified as the product of and controlled by, the ob-gene (Yu et al., 1997). It showed action in reducing appetite, increasing energy expenditure through action in the brain and then decreasing body weight and fat mass. The fundamental factors regulating serum leptin levels seems to be caloric intake and the amount of energy stored in adipocytes (Ramos and Zamoner, 2014). Several reports propose leptin being a pleiotropic mediator as it is an important regulator in both a nutritional function (Smith et al., 2002), as well as being involved in the control of different neuroendocrine systems. In humans and experimental animals, leptin has been pointed out in having a major role in the regulation of female development during puberty and fertility with persuasive evidences. The proper function of leptin in the male reproductive system is however more diffusing. Nonetheless, in the last couple of years, data has been collected and studies have been analyzed with an outcome indicating leptin is acting on the hypothalamic-pituitary-testicular axis at different levels (Tena-Sempere and Barreiro, 2002). Leptin provides a stimulatory effect at hypothalamus and pituitary level whilst function as an inhibitor towards the gonads (Moschos *et al*., 2002; Mehr et al., 2012). Yet it is not well understood how it function in the regulatory network controlling the male reproductive system (Tena-Sempere and Barreiro, 2002; Landry et al., 2013). The hormone seems to inhibit testicular steroidogenesis leading to reducing testosterone levels and modulating gene expression.

The very important discovery of the leptin gene, as Zhang et al. described, was made in genetically obese (ob/ob) mice lacking endogenous leptin as a result of a mutation on the gene coding for leptin or the receptor coding for the gene (db/db) (Yu et al., 1997). This mutation

results in failure of producing leptin by adipocytes. The deficit of biologically active leptin due to a mutation in the ob gene, revealed that obesity was not the only result but also infertility of the ob/ob mice (Henson and Castracane, 2003). The mice demonstrated hypogonadotropic- hypogonadism (Tena-Sempere and Barreiro, 2002), atrophic reproductive organs, azospermia and multinucleated spermatids (El-Hefnawy et al., 2000), morbidly obesity, diabetes and infertility (Zhang et al,. 1994; Fekete, 2008). Through administration of exogenous leptin into the reproductively incompetent mice, their sterility defect was eliminated and the fertility was restored. GnRH secretion was stimulated eliciting FSH and LH secretion (Spicer, 2001; Smith et al., 2002). Mice with mutations of the db gene, encoding for leptin receptor, additionally showed obesity and diabetes but did not improve with administration of leptin.

Fascinatingly, ob/ob females showed consistently infertility, while a restricted quantity of ob/ob males were still fertile and had normal reproductive development. This is indicating that different sex have various effect and physiological extent of leptins role, even though it initiates an effect at the reproductive level in both parts (Spicer, 2001).

Studies have not defined whether leptins role in the reproductive system is direct or indirect, however the underlying nature of leptin deficiency causing infertility is suggested to be an indirect effect of leptin via the central neuroendocrine system, like in the females. These findings proved to be the start of future studies on the subject of leptins action of the hypothalamo-pituitary-gonadal axis (Spicer, 2001; Smith et al., 2002).

4. Leptin (canine)

According to a study performed on adult beagles by Iwase et al., back in 2000, an abundant appearance of leptin mRNA was discovered in adipose tissue, but not in any of the other tissues. These results were yet another indication that white adipose tissue is the major site of leptin production, similar to what has been found in other species. They emphasized that there was a limited amount of literature and studies done regarding leptin and its receptors in companion animals. It is a bit surprising given that obesity is one of the major nutritional and metabolic disorders present at small animal practices. Their study involved cloning canine leptin as an attempt to clarify leptins role in obesity and its related diseases. Canine leptin structures were found to be considerably homologous with those of other species (table 1).

Porcine leptin was the one with closest identity. The report stated that recently reports, and as I have mentioned above, had registered a presence of leptin in the placenta of mouse and humans, however they were not able to examine those tissues in the dog.

An induced phosphorylation of both STAT3 and MAPK was recognized by canine leptin as well as of other species, which indicated that similar biological activities were present. Finally they concluded that the canine leptin found in white adipose tissue had a similar molecular structure as to the other mammalian species with similar biological activities (Iwase et al., 2000).

Table 1. Nucleotide and deduced amino acid sequence identities of Canine mature leptin with leptins of other species

Species	Identity (percentage)	
	Nucleotide	Amino acid
Dog	100	100
Cow	89	88
Pig	92	88
Human	88	82
Mouse	84	79
Rat	83	79
Chicken	82	76
Reference		Iwase et al., 2000

5. Leptin receptor (canine)

Leptin receptors (Ob-R) are activated by leptin, and are members of the class 1 cytokine receptor family. They appear in at least five isoforms arising from alternative splicing of mRNA, (Smith et al., 2002; SAYED et al., 2003) and are involved in mediating the role of leptin in the brain and peripheral organs. Leptin receptors are present in numerous tissues, and there are only a minority of tissues that does not express mRNA for at least one of the isoforms (Henson and Castracane, 2003). In almost all the peripheral tissues, including ovary, testis and prostate, these short isoforms of the leptin receptors are distributed, suggesting a direct effect of leptin on them. Various mechanisms regulate leptin secretion. Adipocyte tissue, insulin, glucocorticoids and cytokines stimulate the leptin secretion, whilst inhibition

of leptin release is initiated by free fatty acids, catecholamine, thyroid hormones and cold exposure. Estrogens strengthen leptin production, whereas androgens suppress it, which shows a sexual dimorphism in serum leptin levels (Wauters et al., 2000; Tena-Sempere and Barreiro, 2002; Ramos and Zamoner, 2014).

All isoforms have similar ligand-binding domains consisting of three domains: an extracellular leptin binding domain, a transmembrane domain and an intracellular cytoplasmic tail (Henson and Castracane, 2003). The different ligand-binding domain differs at the C-terminus, the intracellular tail. Leptin receptors are expressed in specific hypothalamic nuclei (Tena-Sempere and Barreiro, 2002). The single long form of the receptor, Ob-Rb (figure 2), contains a long intracellular domain, and is the only isoform having cell-signalling capabilities in this tissue. Signal transduction pathways affected by leptin and leptin receptors interaction are the activation of JAK/ STAT (janus kinase/signal transducer and activator of transcription) via the full length Ob-Rb and MAPK (mitogen-activated protein kinase) via Ob-Rb and also via the truncated Ob-Ra (Smith et al., 2002; Fekete, 2008; Landry et al., 2013). The receptors, mainly Ob-Rb, are expressed in large quantities in the hypothalamic arcuate nucleus, ventromedial, dorsomedial and paraventricular nuclei, and are the predominant signalling form of the receptor. Other structures have short (Ob-Ra, Ob-Rc, Ob-Rf) or no (Ob-Re) cytoplasmic domains. The latter being a soluble protein receptor with properties of binding leptin and regulate its bioavailability (Landry et al., 2013).

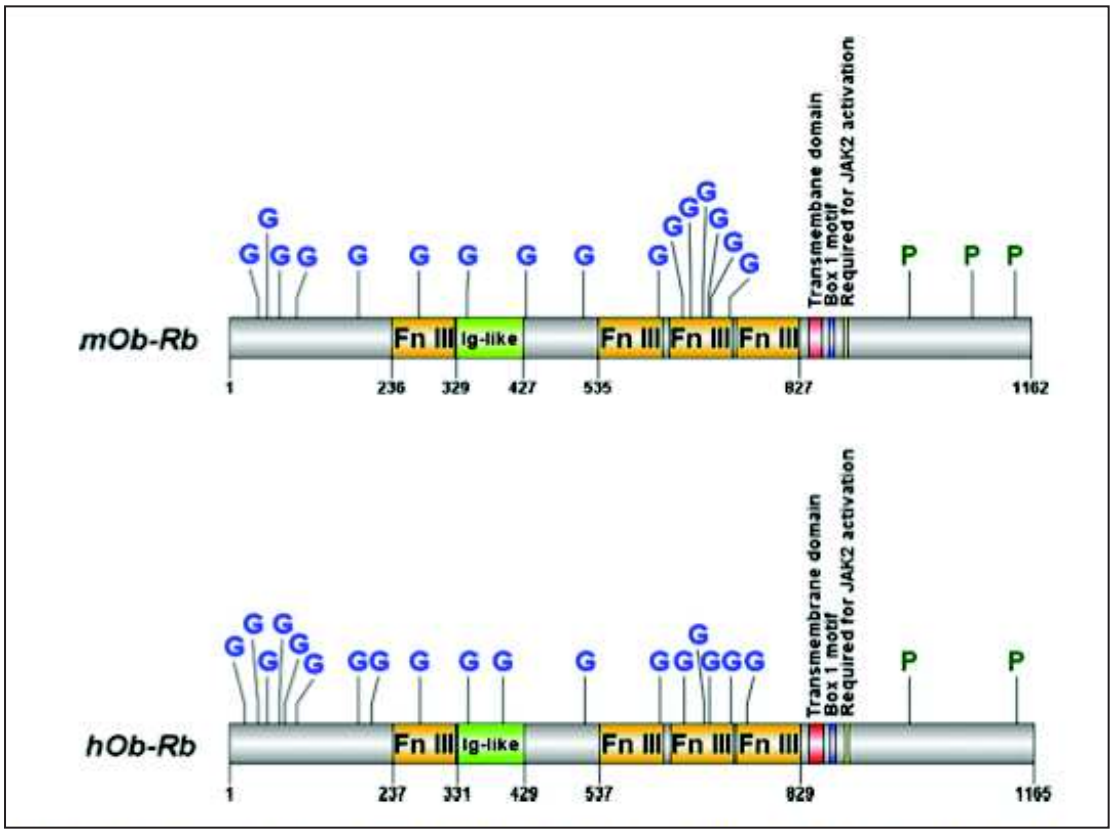

Figure 2. Structures of Ob-Rb, the long isoform. leptin receptor in mouse (top) and human (bottom). Important sites are Fibronectin type III (Fn-III). G stands for Glycosylation and P for Phosphorylation sites (Landry et al, 2013).

6. Role of leptin in male reproductive function

The central role of leptin in female reproduction and fertility is supported by the results provided by various experiments. Absence of leptin, or its biological action, resulted in infertility in the female, whilst administration of leptin presented fertile mice with reproductive cyclicity despite being fasting ob/ob mice (Chehab et al., 1996). Gonadotropin secretion was stimulated by leptin and a blockage of leptin would disturb LH secretion and cyclicity (Tena-Sempere and Barreiro, 2002). Earlier however, leptins role in testicular physiology was not completely understood. Receptors was present in the testis and some reproductive functions had been described as a result of leptins actions in the testis, although it was not fully identified how and why (Ramos and Zamoner, 2014). Through administration of leptin to male mice and rats, gonadotropin-releasing hormone secretion was stimulated. GnRH further stimulated FSH and LH secretion and increased the basal LH secretion in male

mice and rats. This effect shows a recovery in fertility and reproductive function. It is suggested that leptins effect upon infertile, leptin deficient mice, is an indirect effect via the central neuroendocrine system, as seen in the female mice (Barash et al., 1996; Yu et al., 1997; Mounzih, Lu and Chehab, 1997).

However, in obese animals presenting hyperlipidemia, a decreased production of androgens and function of spermatogenesis is observed. Serum testosterone concentrations are decreased as a consequence of an Ob-R mediated inhibition of Leydig cells functioned by leptin (Landry et al., 2013).

6.1 Leptins function in each level of hypothalamic-pituitary-testicle axis

Leptin utilize its action on hypothalamus and the pituitary gland through activating NOS (nitric oxide synthases). Its effect on releasing GnRH, FSH and LH is blocked by NMMA (NG-monomethyl-L-arginine). Via a transport mechanism mediated by Ob-Ra receptors in the choroid plexus, the leptin hormone (like the cytokines) appears to reach the brain via the cerebrospinal fluid into the 3rd ventricle and further into the hypothalamus (Schwartz et al., 1996; Yu et al., 1997).

6.1.1 Hypothalamus function

One of the first tissues leptin receptor mRNA was located in was the hypothalamus. Since then, there has been several studies locating the leptin receptor mRNA in the hypothalamus of rat, sheep, mice and humans. Most studies are done without evaluating the direct effect of leptin on hypothalamus function in vitro.

A study reported that leptin produced a dose-related increase in FSH and LH release (Yu et al., 1997). At low doses of leptin, GnRH secretion increased whilst at high doses leptin decreased the GnRH secretion respectively (Spicer, 2001). A different study in male rats using hypothalamic explants, provided that leptin at very low concentrations reduced the GnRH interpulse interval, thus, increased the GnRH pulse frequency. Whereas the GnRH pulse amplitude was not affected. Accordingly, leptin may possess jointly stimulatory and inhibitory effects on GnRH release. In that case, its effect may possibly be dose dependent and vary with species, culture conditions and (or) sex of species (Yu et al., 1997). We assume

that once leptin reaches a threshold level, it acts as a trigger to initiate hypothalamic-pituitary-gonadotropin secretion.

Leptin mRNA found in the hypothalamus of the rat indicates that, like the pituitary, systemic levels of leptin may not be the exclusive regulator of leptin effects at the level of the hypothalamus. Over the last years studies clearly designate that leptin has an influencing function on reproduction in mammals. The reproductive organs influenced by leptin and have leptin receptors present, include hypothalamus, anterior pituitary gland, gonads, uterus and placenta. It may be species dependent what specific role leptin plays in the function of each of these reproductive organs. Specific quantitative immunoassays are developed for domestic animals, and may help to a define leptins role in domestic animal reproduction (Spicer, 2001).

6.1.2 Pituitary function

Questions whether leptins role on the pituitary is direct or indirect has not been obvious. Nevertheless, in sheep, pigs, mice and rats, leptin receptor mRNA is expressed in the pituitaries, suggesting that direct pituitary effects of leptin may exist (Spicer, 2001). In the in vitro study performed by Yu et al., leptin revealed a potent effect on the pituitary gland. Within 3 hours of incubation FSH and LH release were stimulated. Earlier it hadn't been reported any leptin receptors in the anterior pituitary, but based on the results, receptors must exist on secretary cells, like the gonadotropes (Yu et al., 1997). FSH release from anterior pituitary of rats in vitro, were increased by leptin. In ovariectomized female rats, exogenous leptin decreased FSH secretion. But when leptin was applied IV in ovariectomized ewes, it showed no effect of LH secretion. Hence, different species used and experimental paradigm done influences leptins effect on gonadotropin secretion (Spicer, 2001).

It is presumed that leptin circulates to hypothalamus, diffuses from portal capillaries and into the median eminence in hypothalamus. Remaining leptin in the portal capillaries travels further to the anterior pituitary where it combines with Ob-Rb receptors, which further stimulate FSH and LH secretion (Yu et al., 1997). In the course of fasting, the leptin signal is removed; studies reveal that concentration and pulse frequency of LH and reproductive function declines (Yu et al., 1997; Smith et al., 2002). There is also a cessation of oestrous cycle of mice. However the decrease in LH is prevented when leptin is provided. Similar results have been reported in sheep (Smith et al., 2002).

Conversely, when there is an overproduction of leptin, as in obesity, the consequences to gonadotropin secretion are not clear. Abnormal reproductive circumstances are seen in these cases as well when there is an excess of leptin. Yu et al., revealed in their study that the highest concentration of leptin tested in vitro, decreased LH releasing hormone. Accordingly, leptin show a powerful influence on reproduction, whether it is deficient or in excessive amounts, by eliciting actions on both hypothalamus and pituitary gland (Yu et al., 1997).

6.1.3 Gonadal function

Leptins mechanic role on reproductive processes has shown to be multiphase and being involved at different levels on the hypothalamic-pituitary-gonadal axis (figure 3). It is recognized that leptin enhance its primary action at hypothalamic level as well as initiating a direct effect on pituitary function. Lately, studies have started to focus towards its direct action via peripheral tissue membrane receptors on specific targets, like the gonads, and the Ob-R distribution with its biological effect of leptin (El-Hefnawy et al., 2000; Tena-Sempere and Barreiro, 2002). Tena-Sempere and Barreiro evaluated leptins effect upon testosterone secretion of testes in vitro in rodents. This study was done on pubertal and adult rats, under basal and hCG-stimulated conditions and with increasing concentrations of leptin. In adult testicular sample, leptin inhibited testosterone secretion in both conditions, but in pubertal tissue it remained ineffective. Thus, the pubertal tissue produces 5alpha-reduced androgens instead of testosterone and this is thought to be the explanation (Tena-Sempere et al., 1999). El-Hefnawy et al. additionally discovered in their study that LH/hCG-induced steroidogenesis was down regulated by leptin. However, hCG-induced camp production in cultured rat Leydig cells and cultured rat testis was stimulated (El-Hefnawy et al., 2000). Independently, Fabbri and co-workers presented corresponding results using primary cultures of rat Leydig cells and the murine mLTC-1 clone from Leydig cell origin. Comparative investigation done with in vitro responses of leptin and testosterone secretion, exposed that leptin was able to decrease the hCG-stimulated mRNA expression levels of SF-1, StAR and P450scc enzyme but failed to alter those of 17b-HSD type III in a dose-dependent manner (Landry et al., 2013).

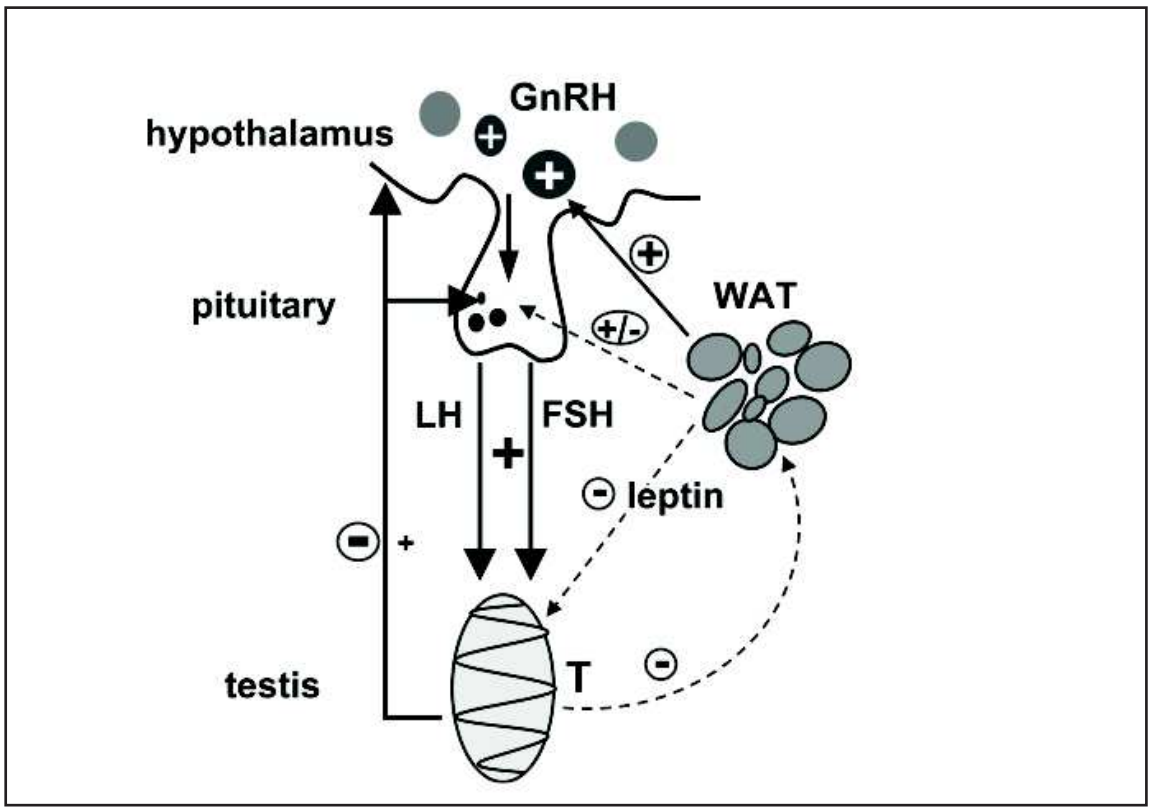

Figure 3. Leptins role in each level of hypothalamic-pituitary-gonadal axis

Model of leptins complex mode of action on several levels of the hypothalamic-pituitary-testicular axis

1. + Primary effect at the hypothalamus, involving stimulation of GnRH release
2. +/- Regulate gonadotropin (LH & FSH) secretion at the pituitary level, where stimulatory or inhibitory responses may arise depending on the prevailing metabolic status.
3. - Direct effects upon testis, which involves inhibition of testosterone (T) secretion, that is likely fully expressed in the presence of significantly elevated leptin levels, as those of obesity. In turn, testicular T is able to directly inhibit leptin secretion by the white adipose tissue (Tena-Sempere and Barreiro, 2002).

Location of Ob-R mRNA evidenced a scattered pattern of expression within adult rat testis tissue, with specific signals detected in Leydig and Sertoli cells (Caprio et al., 1999). As mentioned earlier, the diverse Ob-R subtypes show different functional capacities. Such a complex pattern of processing may therefore result in the generation of different receptor

isoforms with variable signalling ability, ranging from complete (Ob-Rb) to partial (Ob-Ra) or absent biological activities (Ob-Re and others). To fully understand the mechanisms for testicular actions of leptin, this, together with the anticipated interaction between Ob-R isoforms in leptin signalling may be relevant (Landry et al., 2013).

A contribution to the leptin-induced inhibition of testosterone production is though to be a decreased mRNA expression for several steroidogenic genes in adult rat testis.

An activation of the JAK/STAT pathway by leptin signalling is typical in other cells. Interplay between the JAK/STAT pathway and the adenylate cyclase-cAMP-PKA pathway in Leydig cells, alongside being stimulated by leptin and LH, might take part in the regulation of testosterone production under ordinary leptin concentrations (Landry, Cloutier and Martin, 2013). The STAT3 signalling pathway is associated with differentiation pathways in several cell types. And by activation of STAT3, leptin regulates the mouse testicular germ cell proliferation and differentiation (El-Hefnawy et al., 2000). An altered leptin and leptin receptor expression in human testis is associated with dysfunctional spermatogenesis (Ishikawa et al., 2007; Chen et al., 2008; Rago et al., 2009).

Numerous expression profiles done on leptin and its receptor in the testis, indicates it to be species-specific. The conclusion of Caprio and Fabbrinis study was that, in rat testis, Ob-R expression was characteristic of mature Leydig cells and it is functional in adult but not in prepubertal life. However no signal of Ob-R was detected within the tubules and the immunoreactions was always confined to Leydig cells (Caprio and Fabbrini, 2003). Although with adult rats through in situ hybridization, Ob-R mRNA was detected in Sertoli cells. The reason for this is though to be due to a lack of immunoreactivity of Ob-R protein inside the tubules. The signals are not translated in Sertoli cells to a level sufficient to be detected (Landry et al., 2013). El-Hefnawy, Ioffe and Dym found that in testis of 5-days-old and 10-days-old rat testis Ob-R was found mainly on type A- spermatogonia and some in type B-spermatogonia. As they got older, 20-and 30-days-old, Ob-R was expressed mainly on the spermatocytes and some in the lumen of the seminiferous tubules. Adult testes presented receptors on the spermatocytes at stage IX and X of the seminiferous epithelium cycle. No expression, or a very weak expression, was found in the adult interstitial cells (El-Hefnawy et al., 2000).

Leptin receptors and their mRNA have also been expressed in swine and mouse testes. It is indicated through former studies that testicular leptin receptor gene expression is developmentally regulated and sensitive to regulation by LH and FSH. Studies in rats imply

leptins role as primarily inhibitory to gonadal function. Whereas in mice and primates, in contrast, the studies suggest that leptin may not influence testicular steroidogenesis. Supportively to this suggestion, in male rhesus monkeys or Siberian hamsters, leptin infusion has no effect on testosterone. Leptin receptor mRNA was neither present in Leydig cells or Sertoli cells of mice. By the use of immunocytochemical analysis, it was proven that leptin receptors in mice are mainly located on spermatocytes at the stage of sperm clearance. Consequently, it exist species differences in terms of the specific role leptin plays within the testis (Spicer, 2001). In bovines Kawachi et al., published in 2007 their study showing that leptin receptors are found in different tissues, the testis being one amongst them. Ob-Rb had the highest expression in the testis followed by Ob-Ra and the least of Ob-Rc. (Kawachi et al., 2007)

A suppression on steroid gene expression in seen during hyperleptinemia, which leads to a counteraction on the testosterone production mediated by luteinizing hormone. This negative effect on the steroidogenesis in Leydig cells is a consequence of increased leptin levels, which represents a further regulatory mechanism leptin shows towards the reproductive function. Leptin may act as a paracrine agent in the testis since it is able to cross the blood-testis barrier and can therefore regulate testosterone production through acting directly on testicular Leydig cells. Thus, Ob-R is expressed in Leydig cells and leptin is not only secreted in the circulation but also produced in the seminiferous tubules. So in addition to leptins endocrine effect, it is suggested that it holds a paracrine/autocrine effect on reproduction because of leptin and its expression in the gonads (Landry et al., 2013).

In leptin-deficient mice, Bhat et al investigated the changes of testicular morphology and germ cell apoptosis. What they found conducting cross-sections of the testis was reduced tubular area in the seminiferous tubules together with a decrease in pachytene spermatocytes. There was a decrease in quantity of tubules presenting elongated spermatids and mature spermatozoa. Sertoli cell vacuolization and condensation of germ cell nuclei were obvious in the animals and an increase in apoptotic activity in the germ cells, especially with pachytene spermatocytes, was recognized. The vacuolisation indicate that leptin may be involved in the regulation of function of Sertoli cells. Additionally there was a negative relation between leptin levels and the levels of inhibin B (Zorn et al., 2007), which represent a good marker of spermatogenesis, and is secreted by Sertoli cells. Proapoptosis-related genes were furthermore identified in a higher quantity of ob/ob mice than normal control mice. And their results

established a clear association between impaired spermatogenesis, germ cell apoptosis and proapoptitic genes to leptin deficient mice (Bhat, 2006).

6.2 Leptin in function of epididymis and prostate

Rago et al., identified leptin and leptin receptors in the testis and epididymis of young and adult swine in their study with immunohistochemical analysis. The hormone and its receptor have additionally been reported in epididymal epithelial cells in other studies (Mehr et al., 2013). Rago et als work presented a differential cell-type expression pattern of the two proteins in young and adult animals. And based on this, the result suggested that the leptin hormone possibly acted through endocrine or paracrine/autocrine mechanisms in porcine male reproduction. Immature swine expressed leptin and leptin receptors in interstitial compartments. Leptin was not found in the epididymal epithelial tissue, whereas leptin receptors were. In mature testis leptin and leptin receptors were present in both Leydig cells, seminiferous tubules and in epididymal tissue. This is making it a species-specificity of leptin and Ob-R expression in pig testes. Compared to other studies in mammals, where leptin and leptin receptors are present within the seminiferous tubules (mainly in spermatids), in the mature gonads and not the immature. The hypothesis of leptin having a role in the pig cell differentiation is based on these results, which also correlates with previous studies done in rodents and humans. Thus, hollow seminiferous tubules with abnormal multi- nucleated spermatids and few spermatozoa characterize the ob/ob mouse testes, however leptin treatment restored the spermatogenetic arrest (Rago et al., 2009).

6.3 Leptins function in development, maturation, and function of spermatozoa

In several different species, the free leptin hormone has been identified in seminal plasma, from what they believe may be the source, the prostate or seminal vesicles (Jope et al., 2003; Nikbakht et al., 2010; Mehr et al., 2013). Immunohistochemical localization supports this probability (Landry, Cloutier and Martin, 2013). Whether it possibly influences and affects the mechanisms involved in the development of motility of spermatozoa or the physiology of spermatozoa is still indistinguishable. However presence of leptin in seminal plasma and its

receptor on spermatozoa is confirmed in human (Jope et al., 2003), pig and bull spermatozoa, and in ram epididymal spermatozoa and Leydig cells (Mehr et al., 2012; Mehr et al., 2013; Gil et al., 2014). The leptin receptor isoform found in human spermatozoa was found more specifically at the tail (Jope et al., 2003). The study done on leptin receptors in boar spermatozoa identified Ob-Rb on its acrosome, subequatorial area and either on the whole tail or on the midpiece. The receptors seem to be present where leptin from seminal plasma is able to best achieve its effect. (De Ambrogi et al., 2007).

Different results and effects have been published regarding leptins relationship to spermatozoa. Amongst other, Zorn et al suggested in 2007 that no interference between leptin levels and morphology and motility of the sperm were present (Zorn et al., 2007). Although in the study done by Lampiao and Plessis in humans, it was recognized an increased motility and acrosome reaction as a consequence of the presence of leptin. And through an in vitro study, leptins effect on sperm function was observed. Based on this, they believe that a fertility capacity of spermatozoa may be affected by leptin (Lampiao and Plessis, 2008). Similar findings were also provided from Aquila et al, in leptin-treated spermatozoa of swine. Equine spermatozoa on the other hand presented different results. Upon leptin-treatment the motility parameters were decreased together with increased acrosome reaction rate (Lange-Consiglio et al., 2014).

Spermatozoa are able to produce leptin itself, which has been demonstrated in several species. Being able to do that, it is independent from systemic leptin and capable to balance its own metabolism based on its energy needs needed for gamete fertilizing processes (Wabitsch, 2001; Aquila et al., 2005; Aquila et al., 2008).

Leptin deficient mice are associated with impaired spermatogenesis and increased germ cell apoptosis. ob/ob male mice show reduced seminiferous tubule area, decreased pachytene spermatocytes, and decreased tubules with elongated spermatids and mature spermatozoa, during examination of cross section of testes. Inadequate gonadotropin support is most likely the reason for abnormal spermatogenesis and infertility in these mice and an accelerated germ cell death by apoptosis is seen. In leptin deficient mice, the impaired spermatogenesis may explain the reduced sperm quality as a result of obesity and leptin resistance. Reduced leptin signals leads to reduced GnRH neuronal activity. And with obesity there is an increased leptin resistance, which results in changed concentrations of reproductive hormones. Together, this may explain the association between BMI, altered semen parameters and infertility (Landry et al., 2013).

Studies exploring leptin and leptin receptors role in canine male reproductive function have not previously been done. Although it is though that their character is likely to be in an endocrine and auto-/paracrine aspect of the testicular, epididymal function.

The objective of this work was to map and characterize the presence and localization of leptin and leptin receptors in the male gonads of adult dogs. We wanted to identify whether leptin is expressed in epididymal or ejaculated spermatozoa, so we can be able to find out whether the hormone alter the fertlity ability in the future.

Materials and methods

Methods used in the previously performed pilot study

Immunohistochemical analysis of testis and epididymis

Sample collection

Upon performing routinely neutering of 3 sexually mature (n=3), healthy dogs without any testicular pathology present, testes and parts of the epididymis (caput, corpus and cauda) were collected simultaneously.

Sample preparation for IHC

8% neutral phosphate-buffered formalin was used to fixate the tissue for 24 hours. Fixation is done to prevent autolysis, preserve cellular components, stabilize cellular material and facilitate expected staining and immunostaining. Daily for one week the fixed tissues were washed in phosphate buffered saline, followed by dehydration in a graded ethanol series and finally embedded in paraffin.

Immunohistochemistry; IHC

IHC is a technique where an antibody is used to find antigen that is present in a cell. The fundamental concept is a demonstration of antigens, the biomarker, within tissue section by means of specific, known antibodies, being an immunological asset. Present on the immunoglobulin molecule, the antibody, there is binding sites for antigens and other antibodies (figure 4). A coloured histochemical reaction can be seen by light or fluorescent microscopy when the antibody-antigen complex (Ag-Ab) is formed (Ramos-Vara and Miller, 2013). Immunohistochemistry is an in-situ detection of antigens in whole tissue sections using monoclonal or polyclonal antibodies. If we want to detect a specific antigen, and only this, monoclonal antibodies can be used. They will only interact with one single epitope of antigens, whilst polyclonal antibodies interact with several antigenic epitopes at one time. The antigen-antibody complexes are visualized through microscope. Different types of IHC can be used, the direct or indirect method. The direct IHC is when the primary antibody directly interacts with the antigen and the enzyme or fluorescent tag creates a colour reaction. The indirect IHC is when the primary antibody is targeted with a secondary antibody, and the latter antibody is the one interacting with an enzyme and substrate or fluorescent tag (Ramos-

Vara and Miller, 2013).

With an indirect immunoperoxidase method our immunohistochemistry was performed. A rabbit polyclonal affinity purified antibody (Aviva Systems Biology) diluted 1:200 was used for the detection of leptin. For leptin receptors a goat polyclonal affinity purified antibody (Santa Cruz Biotechnology) diluted 1:50 were used. Pre-immune rabbit for leptin and goat IgG for leptin receptors were used as isotype controls. As positive controls for Leptin and leptin receptors murine ovaries with corpus luteum (CL) and canine CL were used. The expression of the proteins in the study was evaluated by the indirect method of the Streptavidin biotin-peroxidase, using the solution 3,3'-diaminobenzidine tetrahidrocloret (DAB) as the chromogenic agent. Evaluation of the slides with immunostaining for leptin and leptin receptors were done under 100x magnification.

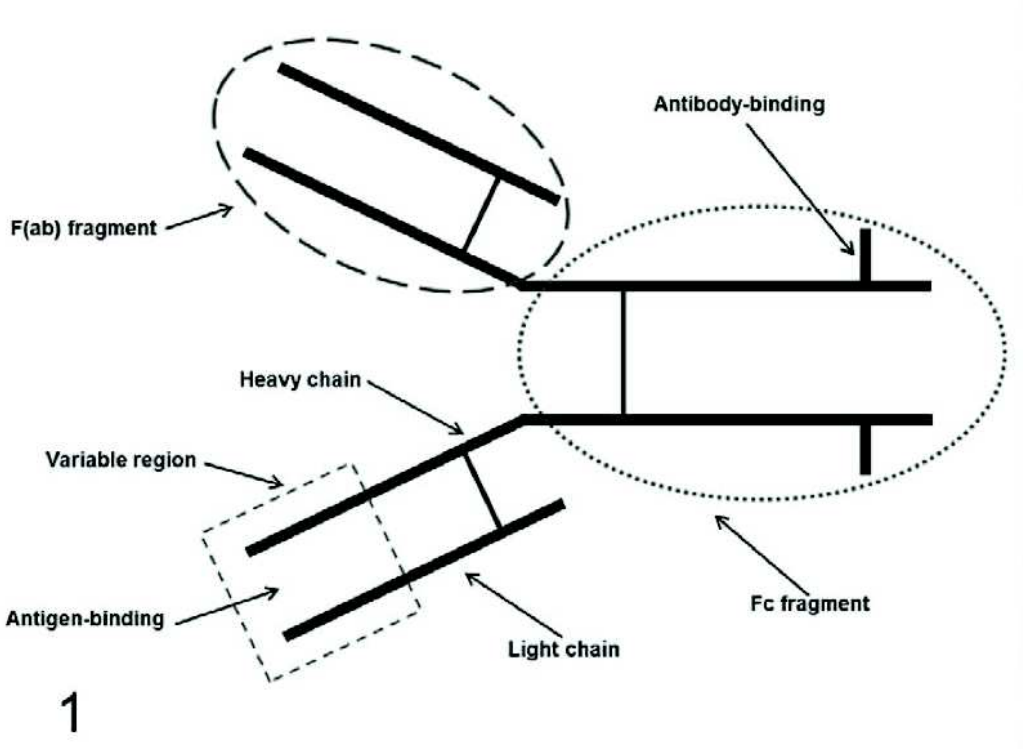

Figure 4. Immunoglobulin structure. Fc fragment is shown with an oval dotted line, F(ab) fragment with an oval dashed line (Ramos-Vara and Miller, 2013).

Immunocytochemical analysis of ejaculated spermatozoa

Sample collection

From three other sexually mature, healthy dogs, semen (n=3) samples were collected through manual stimulation prior to the surgery. All the samples were full sperm-rich portions.

Sample preparation

Immediately after collection, the semen samples were purified using the swim-up technique. The swim-up technique selects sperm on their motility and their capability to swim out of the semen. It makes it possible to separate the spermatozoa from the seminal fluid (García-López et al., 1996). 100 µl of freshly collected dog semen was placed under a layer of PBS (equal volume). The samples were incubated for 40 min at 38.5°C. After this time, the upper fraction containing the motile spermatozoa was collected.

3 of the semen samples were collected, purified and processed, submitting them to a smear procedure in silane coated slides (3-Aminopropyltriethoxysilane, Sigma ®), according to the conventional methodology and fixated in alcohol at 95%.

Immunocytochemistry; ICC

Immunocytochemistry focuses on finding the antigen present in the cell by using antibodies, but without the extracellular components like in IHC. This is used more for an experimental than diagnostic perspective level. The immunocytochemical expression of the antibodies was obtained through the same indirect immunoperoxidase method.

Results

By completion of conducting immunohistochemistry, our results indicated that leptin immunoreactivity was recognized in all spermatogenic cells in the seminiferous tubules. The signals were in fact found to have the strongest affinity in primary spermatocytes and spermatids. Spermatocytes and spermatogonia presented a weak leptin receptor stain in contrast to spermatids, which displayed an intense stain. The leptin signals in Leydig cells were weak.

The ductual epithelium of the epididymis presented a positive reaction for both proteins, with a demonstration of a stronger signal towards cauda of epididymis. Weak sporadic leptin and leptin receptor staining in blood vessels of testis and epididymis was additional findings.

Testis

In the samples taken from testis, leptin immunoreactivity was detected in all spermatogenic cells in the seminiferous tubules. The most evident signal was established in primary spermatocytes and spermatids. Leydig cells within the testis however, demonstrated a more weakened leptin signal, but was still present (Figure 5).

Regarding spermatids the leptin receptor staining was intense, whilst the spermatocytes and spermatogonia had a more weakened stain. A sporadically, weakened positive figure was seen in Leydig cells (Figure 6).

Epididymis

In the samples from epididymis, the ductual epithelium was positive for leptin (Figure 7 left column) and leptin receptors (Figure 7 right column). The closer to cauda of epididymis the samples were taken, the stronger signal was developed in case of both proteins.

Additionally, a weak sporadic leptin and leptin Receptor staining could be recognized in blood vessels of the testis and epididymis.

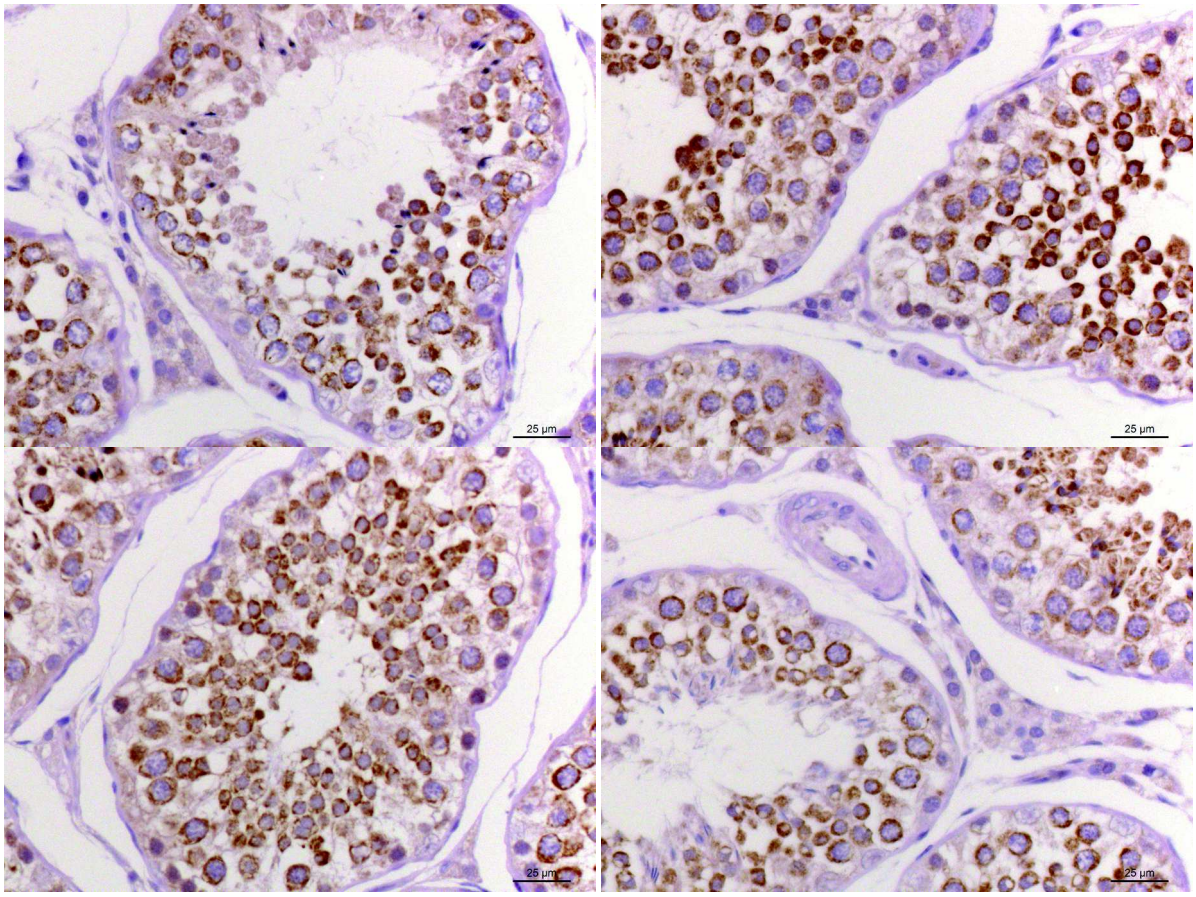

Figure 5. Leptin signals in the testis are strongest in primary spermatocytes and spermatids among all spermatogenic cells. Leydig cells stain weakly positive. Inset shows the isotype control. Bar 25 µM.

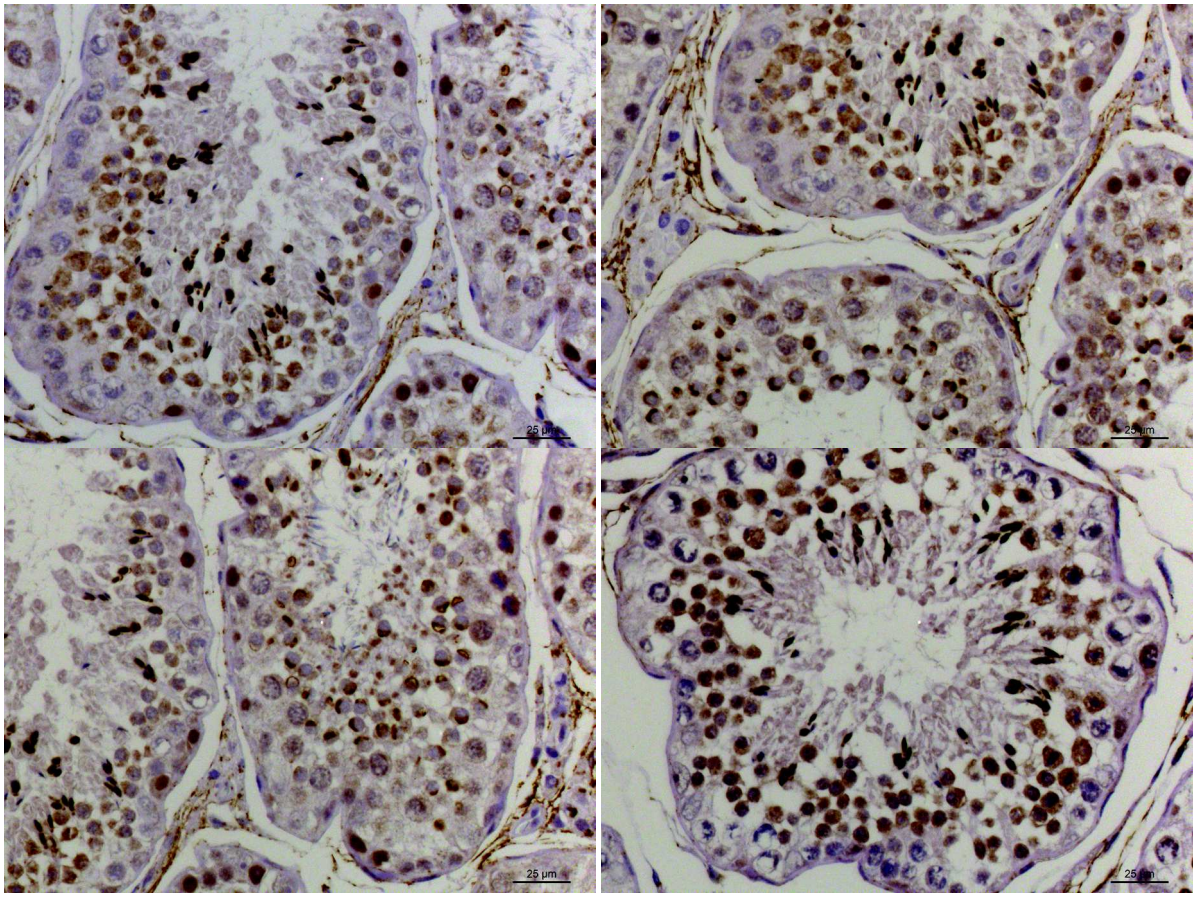

Figure 6. Leptin receptor immunoreactivity in the testis is present in germ cells with strongest signals in spermatides. Leydig cells stain sporadically and weakly. Inset shows the isotype control. Bar 25 µM

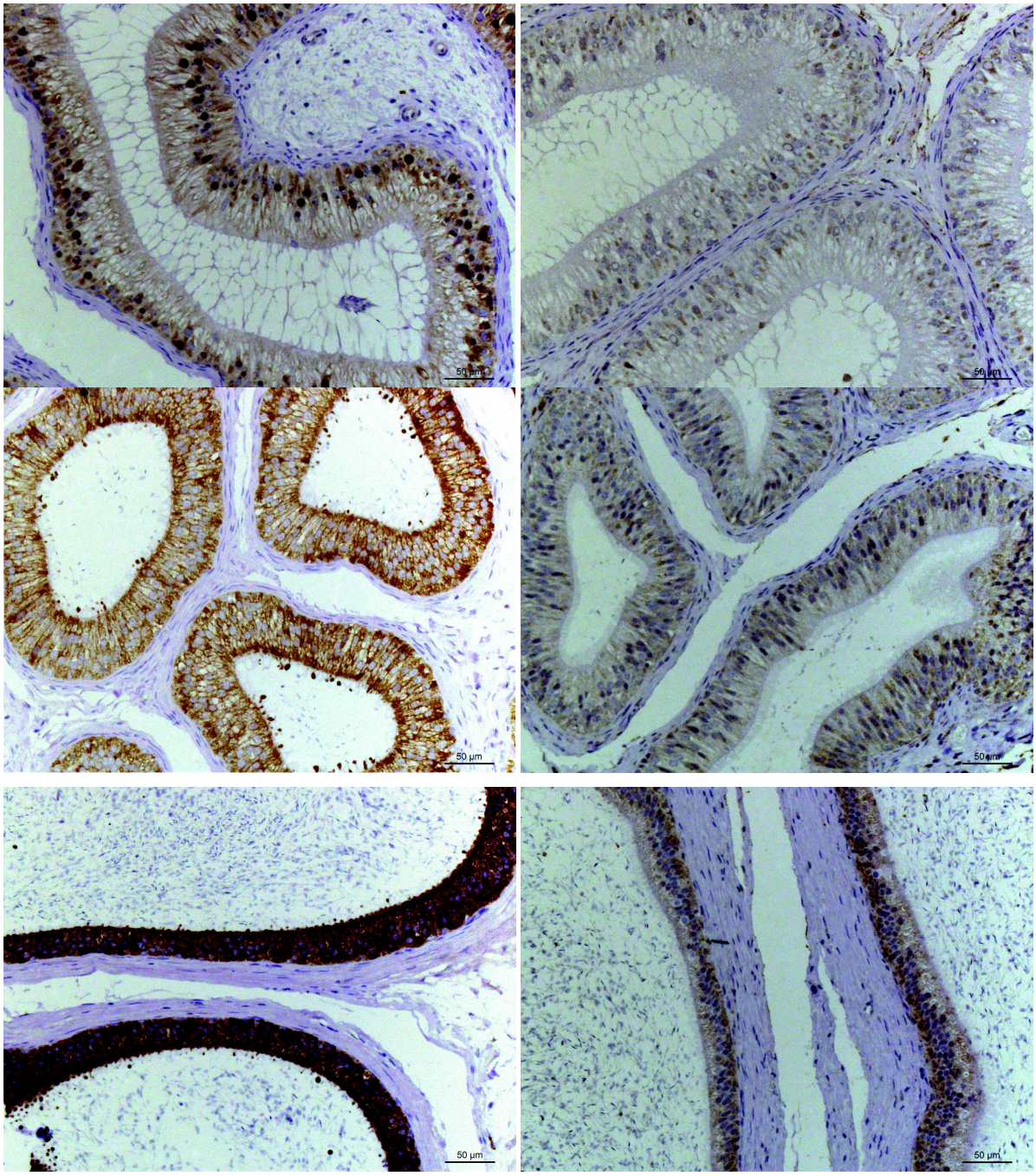

Figure 7. Leptin immunoreactivity (left column) is detected in the ductal epithelium of the epididymis, which becomes gradually stronger from the caput towards the cauda. Leptin receptor (right column) is present in the epithelial cells lining the epididymal ducts with strongest signals in the caput. Bar is 50 µM.

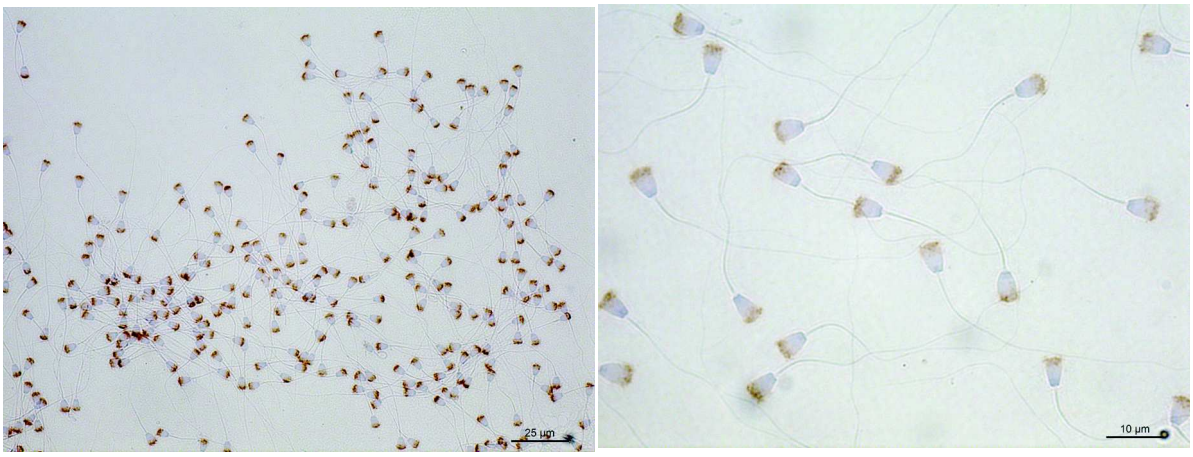

Figure 8. Leptin immunoreactivity is detected in ejaculated semen.
On the left, the presence of positive immunolabeling was visible for the large majority of cells present (Scale bar = 25 µm). In the right, in higher magnifications it was clearly visible that the immonoreaction was limited to the acrosome area, whilst the flagellum remained negative for this molecule (Scale bar = 10 µm).

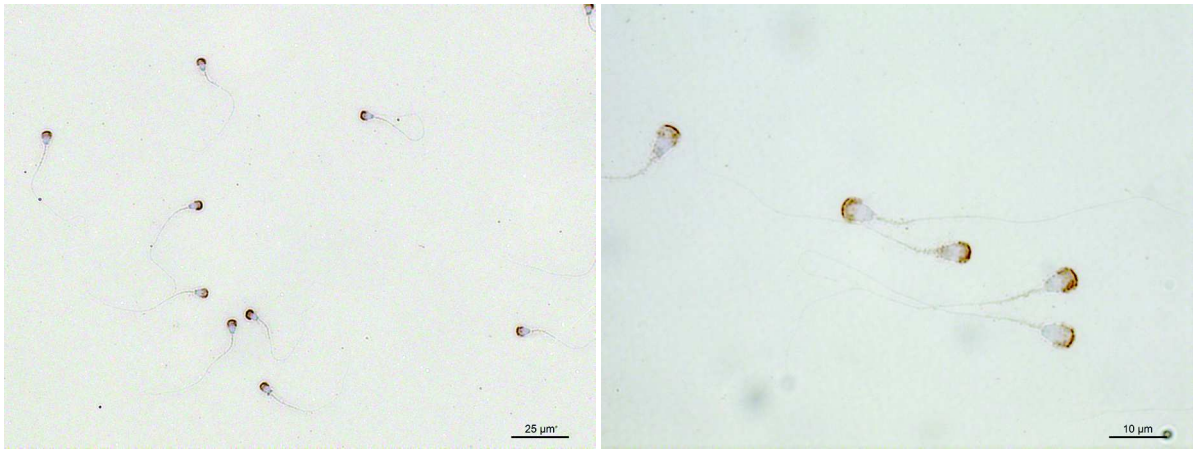

Figure 9. Leptin receptor immunoreactivity is detected in ejaculated semen.

Discussion

A growing interest has been directed towards the presence of leptin and leptin receptors in gonadal tissue in different mammalian species. Several research studies have revealed the occurrence of the two proteins in tissues beyond the hypothalamic-pituitary axis. In an attempt to clarify leptins role in the male reproductive system, different research has been conducted specifically concerning this field. They have verified that leptin and its receptors are not only present in reproductive tissues, but that it additionally obtains diverse physiological traits in different animals (El-Hefnawy et al., 2000; Tena-Sempere and Barreiro, 2002). Thus a noteworthy influence caused by leptin on reproduction is recognized, both with a deficient amount or an excessive amount present. The fact that leptin shows a direct effect on the gonads contributes to the increasing popularity in research of this topic (Yu et al., 1997).

If a specific relationship between leptin and reproduction in additional species can be established, it can play an important role in understanding and perhaps coping at a new level with the largest nutritional problem found in the small animal practice today, the obesity. In this case, it can further be investigated what consequences e.g. obesity brings in terms of reproduction.

Some animals have gone through more research than others, rodents being one of them. Rat and mouse testis have encountered numerous analyses and several times proven to possess a significant amount of both leptin and leptin receptors in several parts throughout their male reproductive tract. Through several examinations, different cells in the spermatocytogenesis together with interstitial tissue in the testis were positive for leptin receptors. Leptin and leptin receptors have been found in Leydig cells, Sertoli cells and in the seminiferous tubules in rat. Both mouse and rat expressed leptin receptors in their spermatogonia and spermatocytes. Mice did not have any receptors on its Leydig or Sertoli cells, but they were present on spermatocytes at the stage of clearance. In young rats they were mostly found on the spermatogonia whilst in adults on the spermatocytes (Caprio et al., 1999; El-Hefnawy et al., 2000; Caprio and Fabbrini, 2003). Now, other species have additionally been investigated since the rodents.

As we know, Rago et al., identified leptin and leptin receptors in the testis and epididymis of young and adult swine in their study performed with immunohistochemical analysis in 2009. Immature swine expressed leptin and leptin receptors in interstitial compartments of the testis. Leptin was not found in their epididymis, whereas leptin receptors were. In mature swine testis however, both leptin and leptin receptors were present in Leydig cells, seminiferous tubules and in the epididymal tissue. Ob-Rb was additionally identified on spermatozoa, more specifically on the acrosome. Other species evaluated in this matter is humans and research has been done with them as well. The presence of leptin in seminal plasma with its receptors located on spermatozoa has been confirmed. On the spermatozoa it was found more specifically on their tail (Jope et al., 2003). Bulls together with swine had leptin and its receptors confirmed in ejaculated spermatozoa. In sheep it has been located in epididymal spermatozoa and in Leydig cells (Mehr et al., 2012; Mehr et al., 2013; Gil et al., 2014).
An overview on the different species and the presence of leptin and leptin receptors in their tissues is put together. See tables 2.1 and 2.2 for the expression of leptin, whereas table 3.1 and 3.2 demonstrate the leptin receptor expression in the various mammals.

In our study, which was performed on 3+3 individual dogs, leptin immunoreactivity was discovered in spermatogenic cells within the seminiferous tubule. The strongest leptin reaction was found in primary spermatocytes and spermatids and leptin receptors were found most prominent in spermatids. Epididymis was positive for both proteins with the strongest signals towards the caudal part of epididymis. We may certainly conclude, based on our results, that leptin is present in the canine testis, epididymis and spermatozoa of the animals investigated in our study.

Unfortunately studies conducted in canines are poor, and because of that there is an absence of data on leptin and its receptors and what their effects in dogs are. The article Iwase et al published in 2000 presented findings of leptin receptor in adipose tissue in canines. What they published demonstrated a correlation found between the leptin molecules in canines compared to other species. Their data verified that the molecular structure of canine leptin and leptin receptors were close to identical to those of other mammals. The findings in this article can therefore be set as an indicator for leptins possible role in canine reproduction. Given that other animals have identical molecules and that an effect on reproduction is seen in them, we have the reason to believe it may exert a physiological effect towards spermatogenesis and

steroidogenesis in the canine testis as well. It might also be possible that it has a functional role during epididymal sperm transport and storage seen that it serves this effect in other species having leptin receptors present in the same tissues to what we found in the canines. As seen in rats with receptors in Leydig cells, leptin proved to have a negative effect on the testosterone production of the testis thus reducing the testicular function (Caprio and Fabbrini, 2003). Contrary to this, in mice it didn't show a negative effect towards testosterone, since there weren't any receptors present in the interstitial tissue of the testis. These studies imply leptin as primarily inhibitory to gonadal function.

Another interesting factor that appears to be relevant is that leptin receptors may induce molecular changes associated with sperm capacitation and survival. Leptin, through leptin receptors, has had an important effect towards sperm function and male fertility. An interaction between leptin and insulin in regulating glycogen synthesis in mature spermatozoa effects sperm motility. This has been demonstrated in humans and is also believed to have the same effect in sheep, pigs and bulls (Aquila et al., 2008; Nikbakht et al., 2010; Mehr et al., 2012).

With the knowledge provided from Iwase et al., there is an additional reason for us to believe that leptin could exert an effect in canine male reproductive system. Especially now, that we have found a specific presence of leptin and leptin receptors in canine gonadal tissue.

Considering our study being a pilot study and that it was based on 3+3 animals, we cannot draw a complete conclusion. We can, however, surely say that the method is working and that the location of the protein and the receptors did not differ, even in this small sample count. Within the coming future, the plan is to repeat the study in a larger sample size to get a better verification on our results and to confirm a conclusion in the end.

Firstly, the plan is not only to examine the protein expression in canines, but additionally examine it on a RNA level with PCR. This way we will get more specific results on what kind of leptin receptors is present and not.

Table 2.1 Expression profiles of leptin in male gonads in different mammalian species

Species	Testis	Epididymis	Semen	References
Canine	All spermatogenic cells in seminiferous tubules: Spermatids (intense) Primary Spermatocytes (intense) Spermatogonia (weak) Leydig cells (weak, sporadically) Blood vessels (weak)	Cauda (strongest) Ductual epithelium Blood vessels (weak)	Ejaculated spermatozoa → acrosomal area	Our pilot study
Immature Porcine	Leydig cells	-	-	Rago et al., 2009 De Ambrogi et al., 2007 Aquila et al., 2008
Mature Porcine	Leydig cells Seminiferous tubule	Epididymal tissue	Spermatozoa → acrosomal area	Rago et al., 2009 De Ambrogi et al., 2007 Aquila et al., 2008
Sheep	-	Epididymal spermatozoa	-	Mehr et al., 2012 Mehr et al., 2013 Gil et al., 2014
General				Landry et al., 2013;

Table 2.2 Expression profiles of leptin in male gonads in different mammalian species

Species	Testis	Epididymis	Semen	References
Bovine	Yes	-	Ejaculated spermatozoa	Nikbakht et al., 2010
Rat	Leydig cells Sertoli cells Seminiferous lumen	-	-	Caprio et al., 1999 El-Hefnawy et al., 2000 Caprio & Fabbrini, 2003 Tena-Sempere, 2001 Tena-Sempere and Barreiro, 2002
Mouse	Spermatogonia (young) Spermatocytes at stage of clearance (adult)	-	-	Caprio et al., 1999 El-Hefnawy et al., 2000 Caprio & Fabbrini, 2003
Human	Seminiferous tubules Spermatocytes (adult) Leydig cells (adult)	-	Seminal plasma	Jope et al., 2003 Aquila et al., 2005
General				Landry et al., 2013

Table 3.1 Expression profiles of leptin- receptors (lep-R) in male gonads in different mammalian species

Species	Testis	Epididymis	Semen	References
Canine	Spermatids (intense expression) Spermatocytes (weak expression) Spermatogonia (weak expression) Leydig cells (weak, sporadically) Blood vessels	Cauda (strongest) Ductual epith. Blood Vessels	Ejaculated spermatozoa → acrosomal area	Our pilot study
Immature Porcine	Interstitial tissue	Epididymal tissue	-	Rago et al., 2009 De Ambrogi et al., 2007 Aquila et al., 2008
Mature Porcine	Leydig cells Seminiferous tubule	Epididymal tissue	Spermatozoa → acrosomal area, subequatonal area, whole tail or on the midpiece	Rago et al., 2009 De Ambrogi et al., 2007 Aquila et al., 2008
Sheep	Leydig cells	Epididymal spermatozoa → Ob-R & Ob-Rb	Ejaculated spermatozoa → Ob-R & Ob-Rb	Mehr et al., 2012 Mehr wt al., 2013 Gil et al., 2014
General				Landry et al., 2013

Table 3.2 Expression profiles of leptin- receptors (lepR) in male gonads in different mammalian species

Species	Testis	Epididymis	Semen	References
Bovine	Yes, at low levels w/highest expression of Ob-b, Ob-a, Ob-c respectively	-	Ejaculated spermatozoa	Nikbakht et al., 2010
Rat	Leydig cells (mature) → Ob-Rb & Ob-Ra Sertoli cells Seminiferous lumen Spermatogonia A (some B) → young Spermatocytes → older	-	-	Tena-Sempere, 2001 Tena-Sempere and Barreiro, 2002 El-Hefnawy et al., 2000 Caprio et al., 1999 Caprio & Fabbrini, 2003
Mouse	Spermatogonia (young) Spermatocytes at stage of clearance (adult)	-	-	Caprio et al., 1999 El-Hefnawy et al., 2000 Caprio & Fabbrini, 2003
Human	Seminiferous tubules Spermatocytes (adult) Leydig cells (adult)	-	Spermatozoa → tail	Jope et al., 2003 Aquila et al., 2005
General				Landry et al., 2013

Summary

Leptin, a 16-KD protein that originally was thought to exert its primary role at a nutritional level has now proven to be a pleiotropic mediator involved at neuroendocrine and reproductive levels as well. Through numerous studies the hormone has been located and demonstrated in the male reproductive tract in several species.

This pilot study was performed on 3 + 3 healthy and mature male dogs. We wanted to investigate whether there was any leptin and/or leptin receptor expression in the testis, epididymis or in the ejaculated sperm. Prior to surgery, a full sperm-rich fraction of semen was collected through manual stimulation from three suitable dogs. In another three dogs we collected tissues from the testis and epididymis through a standard neutering procedure. After the samples had been collected they were fixed and stored properly in formalin before they were processed into histological tissue samples. Two different immunoreactive procedures with an indicating chromogenic agent were used to identify the proteins. Immunohistochemistry identification was done on testis and epididymis samples, whilst immunocytochemistry was done on the ejaculated sperm to locate leptin and/or leptin receptors. The results were finally evaluated under 100x magnification.

The results revealed the presence of both leptin and leptin receptors in the male canine reproductive organs. The strongest expression of leptin was found in spermatids and primary spermatocytes, although it was present in all spermatogenic cells within the seminiferous tubule and in Leydig cells. In the epididymis the most intense immunoreaction was seen towards the cauda and ejaculated sperm had its leptin expression on the acrosomal area. The expression of leptin receptors was found in testis, epididymis and in sperm. In the testis, the signal was most intense in spermatids, and the expression in epididymis was stronger towards the cauda here as well.

With a conclusion based on our results, we can for certain say that leptin and leptin receptors were found in the gonads of those canines tested in this study. However with a low number of animal tested we are planning to conduct this project at another level where a larger number of animals are being evaluated.

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M.P. Kowalewski and I.M. Reichler contributed equally to the design of the experiment, data analysis and interpretation, manuscript revision and final approval, and made the project that enabled my work thank you.

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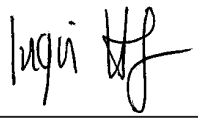

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