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Faculty of Veterinary Medicine and Animal Science

Presence of Hypergonadotropic Hypogonadism in Dogs with Primary Adrenocortical Insufficiency

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SUMMARY

Primary adrenocortical insufficiency (PAI) is a complex endocrine dysfunction that can occur in both dogs and humans. The three layers of the adrenal cortex are destroyed, which is most often an autoimmune process. There is evidence of other autoimmune endocrinopathies, such as hypothyroidism, occurring concurrently with PAI in dogs. This is also known in humans with PAI, as part of autoimmune polyendocrine syndromes that are genetically linked. Sometimes these syndromes can include dysfunction of the gonads, and a consequential hypergonadotropic hypogonadism (HH).

The aim of this study was to investigate whether there is a connection between PAI and HH in dogs. Twenty intact Swedish dogs with PAI were selected from the patient records of the University of Agricultural Sciences animal hospital to be included in the study. Serum specimen were acquired and analysed for luteinizing hormone using a semi-quantitative rapid immune-chromatographic essay. A positive test was interpreted as confirmed HH and a negative result as non-HH. A third category, suspected HH, was used for the cases when a clear test band was discernible of lower intensity than the control band. Animal hospital journals were utilized to acquire additional information about the dogs, such as for example whether they were diagnosed with other endocrinopathies.

The female to male proportion of the dogs was 60% to 40% and the most common breed was Standard Poodle. The age of the dogs ranged from 2 to 10 years, with a median of 7 years. Phenotypically, the study group was comparable to what previously has been reported in studies on PAI. Out of the 20 dogs in this study, none had a clear positive result on the rapid essay. However, three dogs (15%), one female and two male dogs, were classified as having suspected HH. Interestingly two of these three dogs had concurrent hypothyroidism.

The present study shows that HH may be present in dogs with PAI, and that suspected HH may occur in dogs with concurrent polyendocrine diseases. More studies are needed, including larger sample size and quantitative hormone analysis, before presence of HH can be confirmed in dogs with PAI.

CONTENT

ABBREVIATIONS

ACTH = Adrenocorticotropic hormone

ACTH-RH = Adrenocorticotropic hormone -releasing hormone (also known as corticotropin-releasing hormone, CRH)

AIRE = Autoimmune Regulator Gene

APS = Autoimmune Polyendocrine Syndromes

CAR = Cortisol to ACTH ratio

DHEA = Dehydroepiandrosterone

FSH = Follicle Stimulating Hormone

GnRH = Gonadotropin-releasing hormone

HH = Hypergonadotropic Hypogonadism

IMHA = Immune Mediated Haemolytic Anaemia

LH = Luteinizing Hormone

NOAI = Naturally Occurring Adrenocortical Insufficiency

PAI = Primary Adrenocortical Insufficiency

POF = Primary Ovarian Failure

SEAbs = Steroidogenic Enzyme Antibodies

TSH = Thyroid Stimulating Hormone

TT4 = Total Thyroxine

INTRODUCTION

Canine primary adrenocortical insufficiency (PAI) can affect any dog, but overall, young-middle aged females are predisposed (Peterson *et al.,* 1996; Hanson *et al.,* 2016). The disease is characterized by a destruction of the adrenal cortex, a process which is most commonly immune-mediated (Hadlow, 1953; Friedenberg *et al.,* 2018). In PAI all three layers of the adrenal cortex are most often destroyed resulting in deficiency of the hormones that are normally produced by the cortex. The most important of these hormones are cortisol and aldosterone. A deficiency in these hormones can result in a variety of unspecific clinical signs including gastrointestinal disturbances, lethargy, weight loss and tremor. Aldosterone deficiency and the subsequent electrolyte disturbances puts the patient in risk of Addisonian crisis, which is a life-threatening condition that require urgent diagnosis and treatment (Peterson *et al.,* 1996; Sjaastad *et al.,* 2010). Concurrent with PAI, the patient may have other diseases, for example other autoimmune endocrinopathies, including hypothyroidism and diabetes mellitus (Peterson *et al.,* 1996; Blois *et al.,* 2011).

Primary adrenocortical insufficiency in dogs is similar to the disease observed in humans. The presence of multiple autoimmune endocrinopathies is described as Autoimmune Polyendocrine Syndromes (APS) where APS-1 and APS-2 include PAI (Neufeld *et al.,* 1981; Bruserud *et al.,* 2016). These are genetically linked conditions that can sometimes include hypergonadotropic hypogonadism (HH) due to premature ovarian failure (POF) in women. There is also evidence that hypogonadism can occur in males with PAI (Ross *et al.,* 2014).

Hypogonadism in dogs with PAI is scantly researched. To investigate this a large enough number of intact dogs with PAI is needed. There is a wide variation between countries in the approach to neutering dogs. For example, in Sweden neutering dogs for non-medical reasons was not allowed until 1989. Nowadays, Swedish veterinarians do preform castration routinely, yet only 22.3% of Swedish dogs were castrated in 2012 according to a nationwide survey by the government agency Statistics Sweden. This can be compared to a survey provided by The People's Dispensary for Sick Animals (PDSA) in 2019 taken by 5036 United Kingdom dog owners which concluded that 74% of dogs were castrated. This means that the ease with which a large enough study group of intact dogs differs between countries.

Since there is a relatively large population of intact dogs in Sweden, it is an appropriate country for acquiring a study group to investigate the presence of HH in dogs with PAI, which is the purpose of this study.

LITERATURE REVIEW

Canine primary adrenocortical insufficiency

Primary adrenocortical insufficiency (PAI), also commonly called primary hypoadrenocorticism or Addison's disease is an important, but complex endocrine dysfunction. It can appear in all dog breeds, but some breeds have a predisposition for the disease, for example Nova Scotia Duck Tolling Retriever (Hughes *et al.,* 2011), Standard Poodle and Bearded Collie (Hanson *et al.,* 2016). Genetic studies of the disease suggest an autosomal recessive mode of inheritance in Standard Poodle (Friedenberg *et al.,* 2017) and Portuguese Water Dogs (Oberbauer *et al.,* 2006). There is also evidence that young to middle-aged dogs are predisposed but the disease can be diagnosed in dogs of all ages (Peterson *et al.,* 1996). Sex proportion varies by breed, for example, there is an equal distribution between sexes in Standard Poodle dogs whereas in the Golden Retriever breed there is a predominance of female dogs having adrenocortical insufficiency (Hanson *et al.,* 2016).

Primary adrenocortical insufficiency is characterized by a destruction of the adrenal cortex resulting in a deficiency of the hormones it produces. This can occur as a result of for example neoplasia, infarction, amyloidosis, trauma or iatrogenic during treatment of hyperadrenocorticism. However, the most common reason for PAI in both humans and dogs is an immune-mediated process causing destruction of all three layers of the adrenal cortex (Hadlow, 1953; Friedenberg *et al.,* 2018). The precise pathogenesis is yet to be established in canine PAI. A lymphocytic-plasmacytic inflammation with primarily a T-cell mediated response has been found (Friedenberg *et al.,* 2018). In humans with Addison's disease specific autoantibodies against vital steroid producing enzymes have been discovered, which will be reviewed later.

Physiology of the adrenal cortex

To understand the consequences of the disease there is a need for understanding the physiology of the adrenal cortex which consists of three layers. The outer zona glomerulosa (in dogs, called the zona arcuata because of its histological morphology) produces mineralo-corticoids of which aldosterone is the most active (Sjaastad *et al.,* 2010). Aldosterone activity increase the number of Na^+-K^+ pumps in the distal tubules and collecting ducts of the kidneys, leading to increased resorption of Na^+ and increased excretion of K^+ to the urine. Since water will follow in the direction of Na^+, aldosterone plays an important role in maintaining blood pressure. Aldosterone secretion is mainly regulated by the renin-angiotensin system and the extracellular concentration of K^+ (Himathongkam *et al.,* 1975). Secretion of the hormone is increased with increased activity of the renin-angiotensin system, or with increased concentration of extracellular K^+. Adrenocorticotropic hormone (ACTH) which is released from the anterior pituitary gland can also increase secretion of aldosterone, however this is believed to be of minor importance in regulating the hormone and is not a prerequisite for hormone secretion in the adult dog (Klein, 2012).

The middle layer of the adrenal cortex is called zona fasciculata. It produces glucocorticoids, of which cortisol is the most important. Cortisol is regulated through the hypothalamic-pituitary-adrenal axis. Adrenocorticotropic hormone -releasing hormone (ACTH-RH, also known as corticotropin-releasing hormone, CRH) is produced by the hypothalamus and

transported to the anterior pituitary gland where it stimulates release of ACTH into the circulation. In the adrenal cortex ACTH promotes secretion of cortisol. A loop of negative feedback by cortisol inhibits both secretion of ACTH-RH and ACTH. Secretion of cortisol from the adrenal cortex is episodic throughout the day (Feldman & Weidenfeld, 1995). In humans the highest peak occurs in the morning whereas in dogs this diurnal difference seems to be less pronounced, though cortisol concentrations do alter throughout the day in dogs as well (Kolevská *et al.,* 2003; Kalsbeek *et al.,* 2012). Together with other glucocorticoid hormones cortisol has multiple essential impacts on the body. For example, it is required for normal effects on the metabolism, immune system, function of catecholamines and glucagon as well as in coping with stress (Sjaastad *et al.,* 2010).

Zona reticularis is the inner layer of the adrenal cortex and produces androgen hormones by influence of ACTH. Two hormones it produces are dehydroepiandrosterone (DHEA) and androstenedione, which both need to be converted to testosterone or oestrogen in the target organs to be effective (Guyton & Hall, 2000; Sjaastad *et al.,* 2010). Small quantities of oestrogens and progesterone are also produced in the zona reticularis. The androgens produced by the zona reticularis are believed to be of minor importance to adults since they do not seem to answer to gonadotropins and the quantities produced are significantly smaller than those produced by the gonads. However, they are probably of larger importance in pubertal development as well as in geriatric females (Guyton & Hall, 2000).

It is important to differentiate between primary and secondary hypoadrenocorticism since the aetiology differs. Secondary hypoadrenocorticism is the result of a deficient stimulation from the pituitary gland due to a lack of ACTH. This means that the zona glomerulosa remains intact and there is no deficiency of aldosterone (Peterson *et al.,* 1996).

Clinical signs

For clinical signs to appear from hormone deficiency, in a non-stressful situation, it is estimated that 85-90% of the adrenal function needs to be reduced. Increased cortisol secretion is needed to adequately cope with stress, therefore, initial clinical signs are often seen in stressful situations. Since the adrenal cortex is so diverse in function, PAI leads to a variety of consequences for the patient. Clinical signs of disease are often nonspecific, and can mimic those of many other diseases, including gastrointestinal disturbances, lethargy, poor appetite, weight loss, tremor, dehydration, and polyuria/polydipsia (Peterson *et al.,* 1996). Severe electrolyte disturbances due to aldosterone deficiency can lead to hypovolemic shock, prerenal azotaemia and arrythmias. This so called Addisonian crisis is a life-threatening condition that needs immediate attention.

Diagnosis and treatment

Standard laboratory results can give an indication of the disease in patients presented with hyperkalaemia, hyponatremia, azotaemia and/or hypoglycaemia (Peterson *et al.,* 1996). The electrolyte disturbances are due to aldosterone deficiency since the ability for the kidney to excrete potassium and conserve sodium is lost. Consequently, electrolyte disturbances can be an important step in differentiating primary hypoadrenocorticism from secondary hypoadrenocorticism (where aldosterone is not affected). However, hyperkalaemia and hyponatremia can

be seen in other conditions than PAI, thus important differential diagnoses as for example kidney failure need to be considered. Patients with PAI may also present with normal plasma electrolyte concentrations (Nelson & Couto, 2013).

Diagnosis is most commonly performed by demonstrating hypocortisolism and reduced adrenocortical response to ACTH in the so-called ACTH stimulation test (Lathan *et al.*, 2008*)*. Further classification of hypocortisolism as primary or secondary is performed by measurement of plasma or serum electrolyte concentration or direct measurement of ACTH-concentrations in plasma.

Lately, the availability of synthetic ACTH analogues used for ACTH-stimulation has varied. Therefore, an alternative method of evaluating the pituitary-adrenal axis was developed by using the ratio of baseline cortisol and plasma ACTH concentrations (Boretti *et al.*, 2015). Cortisol to ACTH ratio (CAR) is generally significantly lower in dogs with PAI than in dogs without the disease since cortisol is low, and due to the loss of negative feedback ACTH is high.

The emergency treatment for a patient with Addisonian crisis focuses on correcting hypovolemia, electrolyte disturbances and providing a glucocorticoid supplementation (Kintzer & Peterson, 1997). After stabilization chronic treatment for the disease is initiated consisting of continuous supplementation of mineralocorticoids and glucocorticoids. When PAI is diagnosed and the dog responds well to initial treatment the prognosis is very good, but frequent monitoring of the patient to is necessary. This is important when treatment requires adjusting, but also to see signs of complications such as other concurrent diseases that can complicate adequate treatment.

Canine PAI is a complex disease and several factors regarding for example the pathogenesis are still unknown. Interestingly, the canine disease seems in many ways comparable to human Addison´s disease and therefore a lot of information can be acquired by learning about the human counterpart.

Addison's disease in humans

Human Addison's disease has a lot of similarities to canine Addison's disease. Women are predisposed to developing the disease and some forms of the disease are inherited, caused by one or more genetic defect (Neufeld *et al.*, 1981; Bruserud *et al.*, 2016). Clinical signs and symptoms in an affected patient are often non-specific but can include nausea, vomiting and weight loss (Ross & Levitt, 2013).

The most common underlying aetiology for Addison's disease in humans of the western world is an immune-mediated destructive process. Histologically there is evidence of a T cell-mediated response since lymphocytic infiltration is present, which would indicate a similar pathogenesis as for example autoimmune thyroiditis (Irvine *et al.*, 1967; Ajjan & Weetman 2015). In a study by Bratland *et al.* (2009) it was shown that in many patients with Addison's disease the T-cell response is directed to an enzyme specifically found in the adrenal cortex (cytochrome P450 21-hydroxylase; 21OH) and needed for steroid hormone synthesis. Autoantibodies against 21OH is often detected in patient sera, which is used as an additional diagnostic test in patients with suspected adrenal hypofunction (Colls *et al.*, 1995).

There are guidelines set up by the Endocrine Society in the Unites States for how to diagnose and treat Addison's disease in humans (Bornstein *et al.,* 2016). It is recommended to perform an ACTH stimulation test in combination with measurements of plasma ACTH concentrations on patients with suspected Addison's disease. For treatment, the primary recommended glucocorticoid supplement is hydrocortisone. In patients with confirmed low levels of plasma aldosterone concentrations fludrocortisone is recommended. The importance of screening for other autoimmune diseases such as thyroid disease, diabetes mellitus, premature ovarian failure and celiac disease is stressed in the recommendations, due to a known increased prevalence of such diseases in patients with Addison's disease.

Autoimmune Polyendocrine Syndromes

Human patients with Addison's disease sometimes present with other endocrinopathies due to loss of immune tolerance. Autoimmune Polyendocrine Syndromes is a term for a heterogenous group of syndromes characterized by circulating autoantibodies and lymphocytic infiltration of the affected organs (Husebye *et al.,* 2018*).* The syndromes are genetic but have different mechanisms of inheritance and can include autoimmune reactions in endocrine organs as well as in non-endocrine organs. Autoimmune Polyendocrine Syndromes that can include Addison's disease is commonly divided in type 1 and type 2:

- Autoimmune Polyendocrine Syndrome type 1 (APS-1): patient presented with chronic mucocutaneous candidiasis, hypoparathyroidism and PAI, or at least two of the conditions. Other components can be for example, enamel hypoplasia, enteropathy and premature ovarian failure (POF) but presentation of the syndrome varies greatly which complicates diagnosis (Bruserud *et al.,* 2016). Autoimmune Polyendocrine Syndrome type 1 is monogenic i.e., the expression is determined by the alleles of one single gene called the autoimmune regulator gene (AIRE). In patients with APS-1 AIRE is not functioning normally due to a mutation, which leads to a failure in the deletion process of T cells that may initiate an autoimmune response (Aaltonen *et al.,* 1997).

- Autoimmune Polyendocrine Syndrome type 2 (APS-2): patient presented with type 1 diabetes, autoimmune thyroid disease, and PAI, or at least two of the conditions. Many patients, especially the ones with PAI, also develop other conditions for example celiac disease, alopecia and POF. Autoimmune Polyendocrine Syndrome type 2 is more common than APS-1 and is usually presented at a later age (Neufeld *et al.,* 1981; Husebye *et al.,* 2018*).* Unlike APS-1, APS-2 is polygenic i.e., caused by effects of many genes. Research has shown that these affected genes code for important regulatory proteins in the immune system and thus dysfunction causes autoimmune diseases in several organs. For example, variants in major histocompatibility complex HLA DR3-DQ2 and DR4-DQ8 can be seen in patients with APS-2 in risk of celiac disease (Sollid *et al.,* 1989) as well as type 1 diabetes (Noble *et al., 1996*) and Addison's disease (Erichsen *et al.,* 2009).

Hypogonadism

Premature ovarian failure is defined as absence of menstruation for four months or more in women younger than 40 years in combination with primary hypogonadism and no previous

medical history of anything that can have influenced ovarian function negatively (Lawrence & Nelson, 2009). The underlying cause for POF is often an autoimmune process that may be related to other autoimmune diseases. The prevalence of POF is about 1% in the general population but may be as high as 20% in women with autoimmune Addison's disease (Reato *et al.* 2011). When dividing the women based on APS types, POF was discovered in 40.8% of women with APS-1 and 16% of women with APS-2. None of the women with only Addison's disease had POF in this study.

Women with a combination of Addison's disease and POF have specific steroidogenic enzyme antibodies (SEAbs) that can be used to indicate and predict POF. In men SEAbs appears to have no diagnostic value for testicular function (Dalla *et al.,* 2014). They occur in a high frequency in men with Addison's disease, but no correlation with testicular function has been found. Nevertheless, hypogonadism occurs in men with Addison's disease. This was proved in a study by Ross *et al.* (2014) where they researched hypogonadism in both men and women with Addison´s disease. The overall prevalence of hypogonadism in the men, measured by serum testosterone, was 33% (14/42). Most of these men had secondary hypogonadism, but two men with autoimmune Addison's disease had primary hypogonadism. In the same study 11% of women were documented to have POF.

Autoimmune polyendocrine syndromes and hypogonadism in dogs

There is limited information on concurrent autoimmune endocrine diseases in dogs with PAI. In a study by Peterson *et al.* from 1996, 225 dogs with naturally occurring adrenocortical insufficiency (NOAI) were included. Nine (4%) of these had concurrent hypothyroidism and one (ca. 0.4%) had insulin-dependent diabetes mellitus. Another patient had a combination of hypothyroidism, hypoparathyroidism and diabetes mellitus, as well as PAI. In a study of 13 512 dogs examined at the Ontario Veterinary College Teaching Hospital, 35 dogs (0.3% of the total hospital population) were identified with two or more endocrinopathies (Blois *et al.*, 2011). The second most common combination was hypoadrenocorticism and hypothyroidism, which was seen in 22.9% (8/35) of the dogs. In addition, one dog had a combination of diabetes mellitus, hypothyroidism and hypoadrenocorticism. There are also multiple case reports describing dogs with PAI and other concurrent autoimmune endocrinopathies, mainly primary hypothyroidism (Smallwood & Barsanti, 1995; McGonigle *et al.*, 2013; Cartwright *et al.*, 2016; Vanmal *et al.*, 2016,).

As with APS in humans, concurrent autoimmune endocrine diseases in dog are suspected to have a genetic component. One recent study found three risk haplotypes in Bearded Collies with PAI (Gershony *et al.,* 2019). These haplotypes are, similarly to what has been have found in humans, major histocompatibility complex (DLA, dog leukocyte antigen) class II haplotypes. The same haplotypes have also been found in association with hypothyroidism and diabetes mellitus in different dog breeds and might therefore be an indication for susceptibility to autoimmune diseases in multiple organs.

Regarding PAI and hypogonadism in dogs, information is scarce. A reason for this may be high number of castrated dogs in many counties limiting the population-size of dogs possible to study.

Canine reproductive endocrinology

The reproductive cycle in the female dog and the spermatogenesis of the male dog are elaborately controlled by various hormones. The main regulatory centre is the hypothalamus. It secretes GnRH (gonadotropin-releasing hormone) which stimulates the anterior pituitary gland to release the gonadotropins; FSH (follicle stimulating hormone) and LH (Luteinizing hormone), in a pulsatile fashion. The gonadotropins work together to stimulate gametogenesis and secretion of gonadal hormones (Sjaastad *et al.,* 2010).

In the domestic female dog hormone secretion varies in a cyclic fashion, with the cycle being described as monoestrous. This means that the spontaneous ovulation is followed by a luteal phase (of approximately 75 days) which in turn is followed by anoestrus that lasts for 2 to10 months (Concannon, 2011). At the end of anoestrus there is an increase in pulses of GnRH that stimulates gonadotropin secretion. In the ovaries, LH acts on the theca cells stimulating production of androstenedione that is then converted to oestradiol and secreted by the granulosa cells. Pituitary secretion of FSH stimulates follicle development and secretion of inhibin by the granulosa cells. Pro-oestrus lasts 5-20 days, when a serosanguineous vaginal discharge can be seen in the dog. During this phase, FSH concentrations decline through inhibin feed-back inhibition and oestradiol gradually increases to a peak that is followed by a short peak in LH secretion within two days after the oestradiol peak. This marks the end of pro-oestrus and the beginning of oestrus. This next phase lasts about 5-15 days and includes ovulation at approximately 48-60 h after the LH peak. During oestrus oestradiol concentration in serum continue to decline, while progesterone concentration increases due to preovulatory luteinisation. Oestrus is followed by 50-80 days of metestrus. In the beginning of this phase progesterone peaks and then slowly stars to decrease over 4-8 weeks, reflecting luteal regression due to luteolytic mechanisms performed by the uterus. There is no clear definition when metestrus progresses into anoestrus, but one way commonly used to define it is when progesterone concentrations decline to below 1 or 2ng/ml. Anoestrus is defined as absence of ovarian activity. During this phase basal-LH is low (<1-2 ng/ml) but do have pulses of higher values at intervals of 7-18 h or longer (Concannon, 2011).

In the domestic male dog FSH acts on the Sertoli cells to stimulate spermatogenesis while LH acts on the Leydig cells to stimulate production of testosterone. This is the most important male androgen and has multiple effects on the body, including development of reproductive organs (Ramaswamy & Weinbauer, 2014). Testosterone together with inhibin, which is a hormone secreted by the Sertoli cell in response to FSH, have a negative feedback effect on the anterior pituitary gland and the hypothalamus and thus keep secretion of gonadotropins at balance.

Primary hypogonadism is defined as a condition with malfunction in the gonads causing a depletion in the hormones they produce (Sterling *et al.,* 2015*).* When this happens the negative feedback from gonadal hormones on the anterior pituitary gland is diminished, which results in an increased release of gonadotropins – hypergonadotropic hypogonadism (HH). This should be separated from hypogonadotropic hypogonadism, or secondary hypogonadism, which is the result of a pathology of the pituitary gland causing a decrease in gonadotropins and thus a decrease in gonadal hormones. To differentiate these conditions, and to diagnose HH, analyses

of various hormones are used. Low levels of oestrogen or testosterone in combination of high levels of LH and FSH indicates hypergonadotropic hypogonadism.

If there is a suspicion of a destructive process in the gonads, as with hypogonadism in association with autoimmune disease, there is an indication for investigation for HH. The aim of this present study was to investigate whether uncastrated dogs with PAI also had concurrent HH. Analysis of the gonadotropin LH in serum of uncastrated dogs with PAI was performed, using an easy-available semi-quantitative rapid immune migration assay, that can be used to determine the presence of the pre-ovulatory LH peak or whether a dog has been castrated or not (Alm & Holst, 2018).

MATERIAL AND METHODS

Dogs

The study population was selected from the hospital population of the University Animal Hospital (UDS), Swedish University of Agricultural Sciences, Uppsala, diagnosed with PAI and visiting UDS between 2017 and 2019.

Dogs were only included if diagnosis of PAI fulfilled the diagnostic criteria of 1) confirmed hypocortisolism on ATCH-stimulation test in combination with measurements of either increased basal ATCH concentration and/or presence of hyperkalaemia and hyponatraemia. 2) Decreased basal cortisol concentration in combination with increased ACTH concentrations in plasma (CAR).

The goal was to include serum specimen from 20 non-neutered dogs, and five neutered dogs as positive control dogs. Apart from in the small control group, both surgically and medically castrated dogs were excluded from the study. To avoid peri-estrus related increases in LH concentrations, only intact female dogs that were sampled in anoestrus were included in the study. Anoestrus was defined as not being in heat or suspected heat for at least two months. Information about the reproduction status was retrieved from clinical files and owner interviews. Clinical files were also used to find out whether the dog was diagnosed with any additional diseases.

Sample collection

Venous blood specimens were drawn from the cephalic vein and collected in 4 ml Greiner Bio-One vacuette ® or BD vacutainer ® (BD Plymouth) tubes used for serum biochemistry. The tubes were kept in room temperature for at least 30 minutes to allow clotting, and were then centrifuged. Serum was aliquoted into 0.5 ml protein low binding tubes (Sarstedt AG & co. Nümbrecht, Germany) The tubes were stored in -80 °C until time of analysis. Serum specimen with haemolysis measured at above 0.5 g/L were excluded from the study as instructed by the manufacturer of the test performed (see appendix 1).

Analysis of LH

Serum LH was analysed using a semi-quantitative rapid immunochromatographic assay (Witness® LH, Synbiotics Corporation, San Diego, CA, USA). In the test, gold-conjugated antibodies react with LH which is visualized as a line in the test area and compared in intensity to a control line at the end of the test area. A test line of similar or of greater intensity than the control line indicates a serum LH concentration exceeding 1 ng/ml (see appendix 1). The tests were performed and read by the author and the results presented in this report were not blinded. A photograph was taken from each test-device for documentation and to enable re-evaluation.

Serum from castrated dogs with PAI was used as positive controls. Serum from one specific dog (no. 7) was included as positive control at every occasion of analysis to facilitate interpretation between occasions.

Samples from dogs determined as having suspected HH after the initial analysis were re-analysed at an additional occasion for further confirmation of the result. This second occasion

was one month after the first and was performed on another aliquot collected at the same time as the first tested serum sample, using the same positive control and technique for analysis.

Statistical analysis

Descriptive data was tested for normality using the histogram function in Excel 2016. Normally distributed data is presented as mean and SD. Non-normally distributed data is presented as median and range. The data from the analyses of LH were interpreted using OpenEpi.com where prevalence, P-values (using Fisher's exact test) and Odds Ratio were analysed. Power calculations and sample size were performed with the package ´pwr´version 1.2-2 in the open resource software R version 3.3.2 (2016-10-31).

Ethics

This study was approved by the Uppsala Ethical Committee of Animal Experimentation (Dnr: C12/15). All owners gave written consent for sampled serum to be used for research.

RESULTS

Dogs

Serum from 20 intact dogs were included in the study. There were 4 Standard Poodles, 3 mixed breed dogs, 2 Bearded Collies and 2 Samoyed dogs. The rest of the breeds included, represented by one dog each, were Cocker Spaniel, Nova Scotia Duck Tolling Retriever, Pumi, Siberian Husky, Puli, Swedish Elkhound, Shetland Sheepdog, Rottweiler and Pomeranian.

The median age was 7 years (range 2 to 10 years).

Of the 20 dogs there were 12 (60%) female dogs and 8 (40%) male dogs. Out of the 12 female dogs 8 were reported to have normal reproductive cycles. The remaining 4 dogs all had clinical history that could indicate abnormal reproduction, for example scarce or no visible bleeding during suspected heat. All 4 dogs had irregular cycles, 2 of which were reported to have periods of longer than a year without being in confirmed or suspected heat.

Of the male dogs, 3/8 were described to pay interest to female dogs in heat, one of these even had an issue with ejaculating in his sleep. Two of the dogs had small and soft testicles reported in the clinical examination form, without further information about sexual behaviour. Information of reproductive behaviour was lacking for the remaining 3 male dogs.

The group of castrated dogs that were used as a positive control consisted of 3 female dogs and 2 male dogs. Dog breeds represented in this group were Finnish Lap Dog, Labrador Retriever, Briard, Miniature Poodle and Mixed breed. The median age was 10 years (range, 8-10 years).

Autoimmune polyendocrine syndromes

In the test group, 5 of the 20 dogs were diagnosed as having concurrent hypothyroidism by analysis of serum concentrations of thyroid stimulating hormone (TSH) and total thyroxine (TT4). One of these dogs also had concurrent diabetes mellitus. Of the remaining 15 dogs, 4 dogs had tested negative for hypothyroidism and 11 dogs were not tested for the disease.

One additional dog in the test group were previously diagnosed with immune mediated haemolytic anaemia (IMHA).

None of the castrated dogs in the positive control group had been diagnosed with hypothyroidism, diabetes mellitus or IMHA.

No other autoimmune diseases were diagnosed in any of the dogs included in this study.

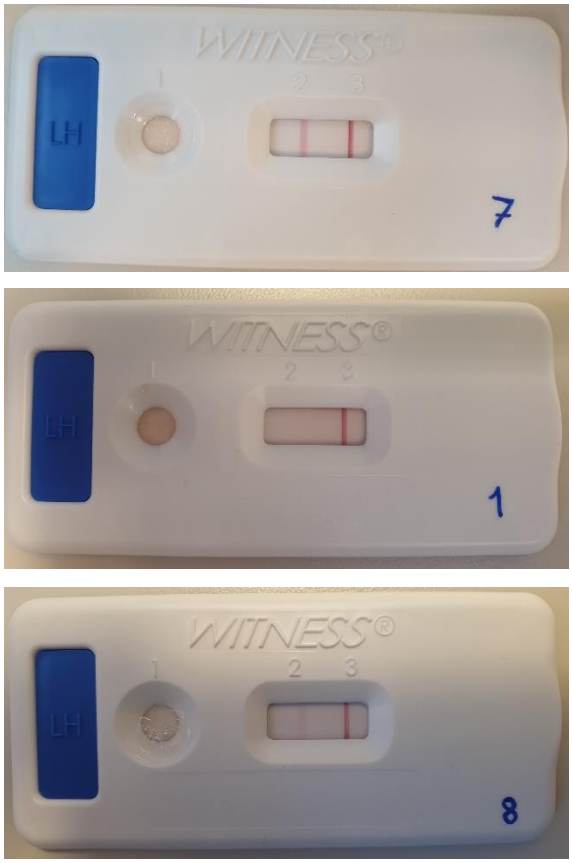
Results from LH-analysis

Positive control dogs

All five dogs in the positive control group had similar intensity of test lines compared to the control lines (figure 1A). This corresponds to a positive result, and an LH level > 1 ng/ml. The control sample that was analysed at every occasion of analysis (dog no. 7) showed a consistent positive result in each analysis.

Test group

Of the 20 intact dogs included in this study, no dogs had a positive result according to the instructions of the semi-quantitative rapid immunochromatographic essay. Seventeen dogs had no visible line in the test area and were labelled as negative. Three dogs (dogs no. 5, 8 and 20) had a visible line on the test area of lower intensity than the control-lines (figure 1C) and were classified as suspected HH. These serum-samples were tested at one additional occasion with the same outcome.



A

B

C

Figure 1. *Rapid immunochromatographic essay for LH (luteinizing hormone) concentrations showing A) positive result of a castrated dog in the control group, LH concentration >1 ng/ml. B) negative result of an intact dog in the sample group, LH concentration <1 ng/ml. C) negative result of an intact dog in the sample group, but with a visible test line. In this study labelled as suspected HH (hypergonadotropic hypogonadism).*

Prevalence of confirmed HH in this sample was 0/20, 0% (with confidence levels 0.0-20.0%). Prevalence of suspected HH was 3/20, 15% (with confidence levels 4.0-39%) (table 1).

Table 1. *Prevalence of hypergonadotropic hypogonadism* (*HH) in total sample, based on gender and in hypothyroid dogs*

Group	Number of dogs (n)	Prevalence (%)	With confidence limits (%)
Total prevalence of HH in sample			
Total confirmed HH	0	0.0	0.0-20
Total suspected HH	3	15	4.0-39
Prevalence of HH based on gender			
Suspected HH in males	2	25	4.5-64
Suspected HH in females	1	8.3	0.4-40
Prevalence of HH in hypothyroid dogs			
Suspected HH in hypothyroid dogs	2	40	7.3-83
Suspected HH in non-hypothyroid dogs	1	6.7	0.4-34

The male prevalence of suspected HH in this sample was 2/8, 25% (with a continuity correction of 4.5-64%) whereas the female prevalence of suspected HH was 1/12, 8.3% (with a continuity correction of 0.44-40%) (table 1). Using a two by two contingency table (table 2) this calculates to an Odds Ratio of 3.7 (with confidence limits 0.27-49). A probability of random association was computed using Fishers exact test which gave a two tailed p-value of 0.69.

Table 2. *Contingency table of association between gender and suspected hypergonadotropic hypogonadism (HH) in the sample*

		Suspected HH		
		YES	**NO**	
Gender	**Male**	2	6	8
	Female	1	11	12
	TOTAL	3	17	20

Amongst the five dogs in this sample with diagnosed hypothyroidism two also had suspected HH which calculates to a prevalence of 40% (with a continuity correction of 7.3-83%). Meanwhile, one of the 15 dogs without diagnosed hypothyroidism had suspected HH which calculates to a prevalence of 6.7% (with a continuity correction of 0.35-34%) (table 1). Using a two by two contingency table (table 3) an Odds Ratio of 9.3 (with confidence limits 0.62-140)

was computed. A probability of random association was computed using Fishers exact test which gave a two tailed p-value of 0.28.

Table 3. *Contingency table of association between diagnosed hypothyroidism and suspected hypergonadotropic hypogonadism (HH)*

		Suspected HH		
		YES	**NO**	
Diagnosed Hypothyroidism	**YES**	2	3	5
	NO	1	14	15
		3	17	20

Power

The probability of finding at least one true positive case (confirmed HH) with significance level of 0.05 in this sample of 20 dogs was calculated to 30%. This calculation is based on an estimated prevalence of HH in the general population of 1%, which is a rough estimate based on studies on prevalence of HH in the general human population.

Power for the study comparing the association between hypothyroidism and suspected HH was calculated based on normal approximation to 60%.

Power for the study comparing the association between gender and suspected HH was calculated based on normal approximation to 3.0%.

Sample size

The sample size needed for 80% power and a significance level of 0.05 was calculated to n=32. This calculation is based on an expected prevalence of HH at 10%, which is based on studies on prevalence of HH in humans with PAI.

DISCUSSION

This study, first of its kind, shows that hypergonadotropic hypogonadism (HH) may occur in dogs with primary adrenocortical insufficiency (PAI), especially in dogs diagnosed with concurrent hypothyroidism.

In the sample studied the most common breeds were Standard Poodle, followed by Bearded Collie and Samoyed dog. Standard Poodle and Bearded Collie are well-known breeds that are predisposed for the development of naturally occurring adrenocortical insufficiency (NOAI) (Peterson *et al.,* 1996; Hanson *et al.,* 2016). The two Samoyed dogs in this study were related, which may be caused by a genetic predisposition (Oberbauer *et al.,* 2006; Friedenberg *et al.,* 2017) or an incidental finding in this small material. In addition, 3 dogs in this study were mixed breed dogs. Mixed breed dogs were also commonly represented in a study of NOAI but without increased relative risk of developing the disease when related to the total number of mixed breed dogs in the study (Hanson *et al.,* 2016).

There was a wide range of ages in the included dogs, which corresponds to what has been seen in in previous studies (Peterson *et al.,* 1996). The percentage of female and male dogs in this study was 60% and 40% respectively, which is a similar ratio to the overall findings in a large epidemiological study on NOAI (Hanson *et al.,* 2016) where 64% were female and 36% were male dogs. In humans gender ratios of similar magnitude have been noted, for example in a study by Ross & Levitt (2013) in which 62% were women and 38% men. Thus the present study population, although small, corresponds to what previously has been published regarding breed and gender proportions, although there was a selection bias for non-castrated dogs.

The most common concurring endocrinopathy in this study was hypothyroidism which was found in 25% of the included dogs. Hypothyroidism has indeed been shown to be the most commonly concurring concurring autoimmune disease in dogs with PAI. However, in one study of 225 dogs only 4% had concurrent hypothyroidism (Peterson *et al.,* 2016). The discrepancy can most likely be explained by the population of referral patients from which the dogs in the present study were selected. One of the included dogs had concurrent hypothyroidism and diabetes mellitus of suspected autoimmune origin. This combination of three endocrinopathies has previously been noted, for example in a study of 35 dogs where one dog had this combination (Blois *et al.,* 2011). In other words, the prevalence of concurring autoimmune diseases was relatively high in this small sample compared to previous epidemiological studies on PAI.

In this study, a semi-quantitative rapid immunochromatographic assay was used to determine concentrations of LH in serum of dogs. This assay utilizes a control line and a test line, where a result is labelled as positive if the test line has similar or greater intensity compared to the control line. One issue with this type of test is the subjectivity when the analyser decides whether a test line is of lower, similar or greater intensity than the control line. In this study, none of the dogs in the positive control group had a test line determined as being of greater intensity than the control line. In fact, they all had test lines defined as being of slightly lower intensity, but still very similar to the control line. When comparing the positive controls with the intact dogs in the sample, most of the sample dogs had clearly no test line visible and thus

were negative. The problem of interpretation arises with the three dogs in the sample group who had test lines of lower intensity than the control line, and of lower intensity than the positive control group, but clearly visible. Acknowledging the semiquantitative nature of the test, dogs with clearly visible test bands of low intensity were classified as suspected HH, in which the serum LH-concentrations may be inappropriately above basal levels, even though they did not reach the cut-off concentration of 1 ng/ml.

Studying hypogonadism in dogs with PAI has not been done previously. In women with PAI, the prevalence of hypogonadism was 11% in one study (Ross *et al.,* 2014) and 20% in another study (Reato *et al.*, 2011). In men, one study reported a prevalence of hypogonadism of 33% (Ross *et al.,* 2014). In the present pilot study of 20 dogs none of the dogs had confirmed HH. However, three of the dogs were classified as suspected HH corresponding to a prevalence of 15% which, in case it is true, is in agreement with the previously reported prevalences in humans. Those studies, however, also included hypogonadotropic hypogonadism in addition to HH (Reato *et al.*, 2011; Ross *et al.*, 2014).

Interestingly, two of the three dogs with suspected HH were previously diagnosed with hypothyroidism. Although, there was no statistical difference between the groups in the present study, it has been shown that the prevalence of POF (and thus HH) was higher in women with APS than those only diagnosed with PAI. In fact, no women with PAI only were found to have HH while the prevalence in women with APS type 1 was 40.8% (Reato *et al.,* 2011). Dogs may be affected with multiple autoimmune syndromes, similar to APS in humans, and HH may be part of this.

The reproductive history of 6 of the 20 dogs included signs of abnormal reproduction such as irregular cycles in female dogs and small, soft testicles in male dogs. However, 2 of the dogs with suspected HH (one female dog no. 5; one male dog no. 8) had no comments indicating abnormal reproduction. Both of these dogs had concurrent hypothyroidism. It is therefore a risk that the test line of weak intensity in these dogs corresponds to normal activity of the pituitary gland. The third dog (male, dog no. 20) had soft and small testicles noted in the record.

One limitation in the present study is the relatively small sample-size of 20 dogs. The power to find at least one confirmed HH in this study (with a significance level of 0.05) was 30%. Ideally, the power should be over 80%, which had required the inclusion of 32 dogs (with a significance level of 0.05). Odds Ratio in the study connecting suspected HH to hypothyroidism had confidence limits including 1, which means that this study cannot prove that hypothyroidism (in combination with PAI) is a risk factor for suspected HH. When relating gender to the risk of HH the confidence limits for Odds Ratio were even wider. Accordingly, no significant difference between the groups was seen on Fisher's exact test.

Another study limitation is the chosen test for serum LH concentration. For this pilot study it was chosen to use an easily available test that was routinely run at the UDS Clinical Pathology lab for identifying female dogs with suspected remnant ovarian tissue after castration or unknown castration status (see appendix 1) (Alm & Holst, 2018). Since it is a semi-quantitative test there is a subjective component in interpretation of the intensity of the reaction bands seen in the test area. To enable reassessment of the interpretations, all test areas were documented

with digital photographs. The test used for analysing LH in this study can also be used to pinpoint the pre-ovulatory LH-peak in female dogs. To avoid LH-fluctuations associated with pro-estrus, oestrus and dioestrus, only samples that were taken during expected anoestrus were included in the study. Further studies may include the use of a quantitative test for LH, follicle stimulating hormone (FSH) and oestrogen for female dogs and testosterone for male dogs. This would also enable differentiation into hypergonadotropic and hypogonadotropic hypogonadism. These extensive hormonal analyses were not performed in the present study due to limited resources regarding time and economy.

In conclusion, the results of this study indicate that HH may exist in dogs with PAI as part of polyautoimmune syndromes corresponding to APS in humans. Additional studies are needed including larger sample sizes and more quantitative hormone analyses before it can be concluded whether dogs with PAI may have concurrent HH.

POPULAR SCIENCE SUMMARY

Primary adrenocortical insufficiency (PAI), also called Addison's disease, is a dysfunction that affects the cortex of the adrenal glands. This is most commonly caused by an autoimmune process in both humans and dogs. The cortex of the adrenal gland produces important hormones, for example cortisol and aldosterone, that are essential for the maintenance of normal body function including coping with stress and the maintenance of blood pressure. A deficiency in these hormones can result in multiple clinical signs such as gastrointestinal disturbances, weakness and weight-loss, that may be seen in many other diseases. Therefore, PAI has been nicknamed "The Great Pretender". Some patients with PAI have additional autoimmune-related dysfunctions of other hormone-producing organs. In dogs with PAI, dysfunction of the thyroid gland, so called hypothyroidism, is most often seen. In humans with dysfunction in more than one hormone-producing organ due to autoimmunity, so called autoimmune polyendocrine syndrome (APS), there is often an underlying genetic predisposition. These syndromes also include dysfunction of the gonads (ovaries in women and testicles in men), that produce sex hormones. The body response in both men and women to low concentrations of oestrogen and testosterone is to increase the secretion of the stimulating hormone LH from a small gland under the brain, the pituitary gland. The combination of low sex hormones and high LH is called hypergonadotropic hypogonadism (HH)

This study, first of its kind, aimed to investigate whether HH is present in dogs with PAI, by measuring LH with a ready to use rapid test commonly used for determining castration status in female dogs. After applying serum (derived from blood) to the device, the test result will be in form of a visible coloured test-line, the intensity of which is compared to a control line to decide whether a result is positive (high LH concentrations) or negative (low LH concentrations). In healthy non -castrated dogs, the serum concentration of LH is low. In castrated dogs, and those with a dysfunction in the ovaries or testicles making them produce less hormones, the serum concentration of LH is high.

The study group consisted of 20 intact dogs with PAI from the hospital population of the University Animal Hospital (UDS), Swedish University of Agricultural Sciences. There were 12 (60%) female dogs and 8 (40%) male dogs included, which is similar ratios as those seen in previous studies in dogs and humans with PAI. The age range at sampling was 2-10 years, with a median age of 7 years. The study included 4 Standard Poodle and 2 Bearded Collie dogs, two breeds that are known to be at higher risk of the development of PAI. None of the included dogs had a clearly positive test result on the rapid essay. Thus, HH could not be confirmed. However, in the test of three (15%) dogs, one female and two males, there was a visible test bend of lesser intensity than the control band that may represent increased LH secretion and hypergonadotropic hypogonadism. Interestingly, two of these dogs also had hypothyroidism.

Since a group of 20 dogs is a relatively small study group, the power of the study was low, which means that there may exist a true occurrence of HH in dogs with PAI, and differences between groups that the present study was unable to show. It was calculated that a group of at least 32 dogs would be needed to find at least one dog with HH. Therefore, the results of the present study need to be interpreted with caution.

In conclusion, the results of the present study indicate that HH may occur in some dogs with PAI, potentially as part of a polyautoimmune syndrome corresponding to APS in humans. However, additional studies are needed to provide evidence on whether HH occur in dogs with PAI.

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APPENDIX 1

WITNESS® LH

For the Detection of
Serum Luteinizing Hormone
DIRECTION INSERT

I. INTRODUCTION

Test Description

The **WITNESS® LH** test provides an accurate, semi-quantitative measurement of canine and feline luteinizing hormone (LH). The test is simple to perform and rapidly provides information for the veterinary clinician. This assay, when used in conjunction with progesterone testing, identifies the pre-ovulatory LH surge, and thus, the time of ovulation. **WITNESS® LH** is also used to distinguish ovariectomized from sexually intact bitches or queens.

Indications

Identification of the LH surge provides the most accurate means of canine ovulation timing. It may be used for routine breeding situations and is especially recommended in those instances where there are factors present that could adversely affect conception rates. These include:

- chilled/extended semen breedings
- frozen semen breedings
- bitches with a history of infertility
- breedings with stud dogs with low semen quality
- limited access to the stud dog

Determining bitches and queens that have had their ovaries removed is possible through the detection of luteinizing hormone. Serum concentrations of LH increase after gonadectomy in dogs and cats. Because LH concentrations in serum are < 1ng/mL in sexually intact bitches and queens, except during LH peak at estrus, it may be assumed that high values in bitches and queens not in estrus are indicative that the ovaries have been removed.

Ovulation Timing in the Bitch

Since the LH surge may occur within a 24 hour period, it is crucial that daily serum samples are tested. Blood should be drawn every day at approximately the same time beginning the 4th or 5th day of proestrus, or when vaginal cytology approaches 50% cornification (Cornification is defined as cells with a roughened, angulated border with or without a nucleus). A progesterone test should also be performed on the first day of testing to confirm a baseline progesterone level. If testing is started after the onset of estrus, it is likely that the LH surge will have already occurred and cannot be identified in retrospect.

Once an increase in LH is identified, Synbiotics recommends that daily serum samples should be tested for progesterone levels. If the detected increase in LH was indeed the actual pre-ovulatory surge, progesterone levels should rise, usually reaching a value above 2 ng/ml within 3 days, and then stay elevated. If progesterone remains low, it indicates that a proestrus fluctuation in LH was identified. Continue daily LH testing until the true LH surge occurs. *Note: the* **WITNESS® LH** *test and test interpretation is referenced with the OVUCHEK® Premate® progesterone assay. Use of other progesterone tests may require different interpretations to explain test results.*

II. TEST PRINCIPLES

WITNESS® LH is a luteinizing hormone assay that provides a convenient semiquantitative measurement of luteinizing hormone levels without special equipment. The test is an immunochromatographic assay that uses gold-conjugated antibodies to give a visual line in the presence of luteinizing hormone. One test device is used for each serum sample to be tested. A control line is included on each test device. Color development of the control line is indicative of proper testing technique. The WITNESS® LH luteinizing hormone assay allows identification of the LH surge. A positive result occurs when a line appears in the test area which is of *similar or greater intensity than the control line*. When this occurs, the LH level in the serum sample is greater than 1 nanogram per milliliter. Based on field observations, the test line may be distinct but of less intensity than the control line in some intact females, shortly before or after the LH surge, as well as in some ovariectomized females.

III. REPRODUCTIVE PHYSIOLOGY IN THE BITCH

Unsuccessful breedings result more often from improper ovulation timing than any other cause. While the estrous cycle of the bitch typically lasts for several weeks, the true fertile period is short (48-72 hours) and is difficult to identify without the use of hormonal assays.

The common indicators of estrus and breeding time, such as vaginal cytology and receptive behavior, are primarily controlled by changes in the hormone estrogen. Unfortunately, these changes are only an approximation of ovulation. Using only these parameters, ovulation could be "missed" by more than a week. Precise identification of the fertile period is made by measurement of the LH surge which actually triggers ovulation. This surge, which consists of a rapid increase in LH level, occurs in many cases within a 24 hour period. Ovulation occurs 2 days after the LH surge; the oocytes then require an additional 2-3 days to mature, and will live for about 48-72 hours. Thus the fertile period of the bitch falls between days 4-7 after the LH surge with the most fertile days being on days 5 and 6 post-LH surge.

Progesterone assays are useful for ovulation timing. Before the LH surge, serum progesterone remains low, generally between 0 and 1.0 ng/ml. At about the time of the LH surge, progesterone levels will begin to rise, usually changing from a baseline of 0-1.0 ng/ml into a range of 1.5-2.0 ng/ml. Progesterone will then continue to rise as the cycle progresses and will remain elevated for 2-3 months in non-pregnant as well as pregnant bitches. It is important to appreciate that the absolute progesterone values discussed above may vary by individual. The key event progesterone assays seek to identify is the initial rise of progesterone above the particular individual's baseline level. Once identified, this initial rise in progesterone may be used as an estimate of the LH surge. However, this first rise in progesterone may vary from the day of the actual LH surge in many bitches. While progesterone is elevated during the fertile period, the rate of rise varies from bitch to bitch, as does the absolute value of progesterone which coincides with the LH surge or with the optimal time to breed. As a result, progesterone measurements alone are, at best, only an approximation of the LH surge. Given the fact that good breeding management often plans for multiple breedings over a period of several days relying on the information provided by progesterone assays alone may be sufficient. Some breedings, however, will benefit from a more exact identification of the bitch's fertile period. Identification of the LH surge itself allows the most precise ovulation timing.

During proestrus, small, pulsatile fluctuations in LH may occur, while progesterone remains at low, baseline levels. Progesterone rises, however, after the pre-ovulatory surge in LH. By the third day post-LH peak, the majority of bitches will evidence a rise in progesterone levels above the 2 ng/ml level. Daily progesterone testing after a positive **WITNESS® LH** test result allows simple differentiation between small fluctuating increases in LH during proestrus and the true, pre-ovulatory LH surge. If there is no confirmation of progesterone rise within 3 days of a positive **WITNESS® LH** test, it may be assumed that the positive test result was due to a baseline LH fluctuation.

IV. WHEN TO CONDUCT INSEMINATIONS IN THE BITCH

The positive **WITNESS® LH** test identifies the day of the LH surge. This day is designated as day 0. Count forward to determine the fertile period. Days 4-7 post-LH surge encompass the true fertile period, with peak fertility on days 5 and 6. Properly planned breedings or inseminations coinciding with this window of fertility will optimize the probability of success.

Natural Breeding or Fresh Artificial Insemination

Fresh semen of a normal healthy stud may live up to 5 or more days in the bitch. Therefore, semen inseminated a day or two before the fertile period should be viable at the time of peak fertility. Synbiotics recommends breeding on days 2, 4 and 6 post-LH surge to completely cover the fertile period, and to maximize viable sperm numbers on the bitch's most fertile days. If only two breedings are being performed, they should be accomplished between days 3 and 6 post-LH surge.

Natural Breeding with Compromised Semen Quality

At times, stud dogs produce semen of compromised quality due to age, stress or disease. Breeding during the true fertile period will increase the likelihood of limited sperm numbers encountering mature eggs and will increase the chance of conception. Synbiotics recommends breeding on days 4, 5, 6, and 7 post-LH surge.

Surgical Insemination with Fresh Semen

Occasionally, it is desirable to perform surgical insemination with fresh semen due to low fertility of either the stud dog or the bitch. Synbiotics recommends that this procedure be performed on either days 5 or 6 post-LH surge.

Chilled Extended Semen Breeding

Chilled extended semen will usually live for 2 to 4 days after collection. One day of this time is typically used in shipping. This reduced survival time requires the breeding to be conducted closer to the short fertile period of the bitch. Synbiotics recommends that 2 inseminations be conducted on days 4 and 6 or 3 and 5 post-LH surge. If a surgical insemination is desired, it should be performed on day 5 or 6 post-LH surge.

Frozen Semen Breeding

Frozen semen survives less than 24 hours after thawing, so timing is crucial. Inseminations must be performed during the true fertile period. If one surgical insemination is planned, it should be performed on day 5 or 6 post-LH surge. If multiple vaginal or trans-cervical inseminations are to be conducted, they are recommended on days 4, 5 and 6.

V. DISTINGUISHING BETWEEN OVARIECTOMIZED AND SEXUALLY INACT BITCHES OR QUEENS

A negative test result (no test line, valid control line) indicates that the bitch or queen is intact.

A positive test result (test line similar to or greater intensity than a valid control line) indicates the ovaries have been removed from the bitch or queen. *To rule out that this positive result is due to brief episodic LH pulses in a sexually intact female, the test should be repeated in 2 hours with a fresh sample. If estrus is suspected, a positive result should be confirmed by repeating the test in 24 hours or more with a fresh sample. Subsequent test results from ovariectomized animals should remain positive.*

When a test line is present which is distinct but of less intensity than a valid control line, the animal may be intact; however, field observations have shown that some of these animals are ovariectomized.

VI. SAMPLE INFORMATION

WITNESS® LH requires only four drops of serum to run each test. Collect the blood sample in a plain (red top) vacutainer or serum separator tube. Allow the blood to clot and separate the serum by centrifugation. The sample should not be hemolyzed or lipemic. Testing should be performed the same day as sample collection. If this is not possible, refrigerate the serum for up to 24 hours. Serum may be frozen for prolonged storage. Do not thaw and refreeze.

VII. STORAGE AND STABILITY

Store kit at 2° to 25°C or 35° to 77°F. Do Not Freeze. The foil pouch containing the test device and pipette should not be opened prior to running the test. **WITNESS® LH** will remain stable until the expiration date provided that the kit has been stored properly.

FOR TECHNICAL ASSISTANCE CALL
888.ZOETIS1 (888.963.8471)

SYNBIOTICS

zoetis

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A Wholly-Owned Subsidiary of Zoetis Inc.
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Kalamazoo, MI 49007

30376000

Luteinizing Hormone Test

WITNESS® LH

For the Detection of Serum Luteinizing Hormone

DIRECTION INSERT

WITNESS® LH Test Procedure

NOTE: Only use SERUM as a sample.

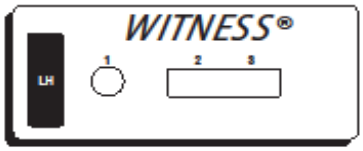
Prior to use, allow SAMPLE to come to room temperature (20° to 25°C; 70° to 77°F)

SAMPLE COLLECTION AND PREPARATION

- Only use SERUM as a sample.
- Collect the blood sample into a plain (red top) vacutainer or serum separator tube.
- Allow to clot and centrifuge sample.
- Transfer serum to a clean glass or polypropylene tube. Serum must be free of red cells, clots and visible debris.
- *DO NOT USE SEVERELY HEMOLYZED OR GROSSLY LIPEMIC SAMPLES.* When in doubt, collect a better quality sample.
- If serum sample will not be tested immediately, store in refrigerator for up to 24 hours or freeze for longer storage. *REMEMBER: Sample must be at room temperature prior to testing.*

TEST PROCEDURE

The **WITNESS® LH** Test includes a pipet and a test device. The device has a Serum Well "1" and a window with a Test Area "2" and a Control Area "3".



1. Remove test device from foil pouch by tearing at the notch. Place the device on a flat level surface at room temperature.
2. Draw some serum up into the provided pipet. Hold the pipet perpendicular to the device and add **4 drops** of serum to the Serum Well "1".
3. Allow the test device to sit undisturbed for **20 minutes.**
4. Read results at **20 minutes.** *Reading results prior to or beyond the 20 minute test interval will invalidate results.*

GOOD TECHNIQUE = ACCURATE RESULTS!

Lipemic or grossly hemolyzed samples may interfere with the rate at which the sample flows in the device or create a background color which will make interpretation of the results difficult.

If samples cannot be tested the same day, refrigerate for up to 24 hours or freeze for longer storage. Do not thaw and refreeze.

Do not use the kit beyond its expiration date.

Do not open the foil pouch until ready to perform the test.

Do not touch any of the surfaces in the windows of the device.

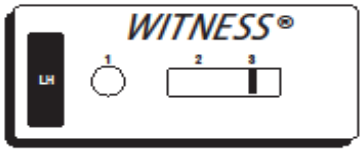
Keep device flat during testing. Do not disturb while test is being conducted.

INTERPRETING RESULTS

At the end of the test, a pink line should always appear in the area marked "3". This line assures that the test is complete.

FOR OVULATION TIMING

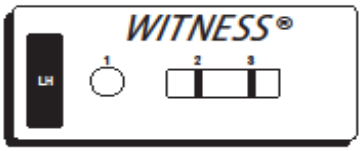
NEGATIVE RESULT:
If no line appears in the area marked "2", the LH value is less than 1 nanogram per milliliter. Continue LH testing on a daily basis to determine the optimal time to breed.



POSITIVE RESULT:
Field observations have shown if a line appears in the area marked "2" which is of similar or greater intensity than the control line "3", the LH value is greater than 1 nanogram per milliliter.

Field observations have shown if a distinct line appears in the are marked "2" of less intensity than the control line "3", the sample is likely to contain slightly less than 1 nanogram per milliliter and have been drawn before or after the LH surge (the shoulders of the LH peak). If the daily tests for the LH surge do not reveal a stronger test line, field observations have shown the weaker intensity line should be considered as indicative of the LH surge. Progesterone values may help confirm.

For ovulation timing purposes, in the bitch, the first time a positive result is observed is the day of the LH peak. This day is counted as Day 0. Follow the instructions under Section IV. WHEN TO CONDUCT INSEMINATIONS (reverse side of page). Note: It is recommended that you perform a progesterone test in 3 to 4 days to confirm a rise in progesterone.



FOR DISTINGUISHING OVARIECTOMIZED BITCHES/QUEENS

NEGATIVE RESULT:
If no line appears in the area marked "2", the LH value is less than 1 nanogram per milliliter. This indicates that the bitch or queen is intact.

POSITIVE RESULT:
If a line appears at the area marked "2" which is similar to or greater intensity than control line "3", the positive result indicates the ovaries have been removed from the bitch or queen. *To rule out that this positive result is due to brief episodic LH pulses in a sexually intact female, the test should be repeated in 2 hours with a fresh sample. If estrus is suspected, a positive result should be confirmed by repeating the test in 24 hours or more with a fresh sample. Subsequent test results from ovariectomized animals should remain positive.*

When a line is present in the area marked "2" which is distinct but of less intensity than the control line "3", the animal may be intact; however, field observations have shown that some of these animals are ovariectomized.

COMMONLY ASKED QUESTIONS REGARDING OVULATION TIMING

Q: How do I use **WITNESS® LH** with OVU-CHECK® Premate®?
A: Utilizing both LH and progesterone assays improves ovulation timing accuracy. Perform an initial progesterone test to establish a baseline level during the first 5 days of the bitch's season. When vaginal cytology indicates 50 percent cornification, begin daily **WITNESS® LH** testing. When LH testing indicates a positive result, make preparations to breed. Synbiotics recommends an additional progesterone test be run 72 hours post-LH surge to confirm a rise in progesterone.

Q: Why do I need a "confirming" progesterone test after a positive **WITNESS® LH** test result?
A: Due to the pulsatile nature of LH activity, there is a small chance that a positive **WITNESS® LH** test result can be obtained due to identification of a baseline fluctuation rather than the true LH surge. Confirming a rise in progesterone before actually performing the insemination will easily identify those few instances where a positive **WITNESS® LH** test result is due to identification of LH baseline fluctuation.

Q: When should I start LH testing?
A: Blood should be drawn the fourth or fifth day of proestrus or when vaginal cytology approaches 50 percent cornification. Sampling should be performed daily until the LH surge is detected. If sampling begins after the onset of estrus, the LH surge may have been missed. Remember to confirm a baseline progesterone level on the first day of testing.

Q: How long will I have to test?
A: Testing on 200 dogs has shown that, with daily testing beginning as recommended, the LH surge will be detected in most dogs within 6 days. There are six tests to a kit. Because the bitch may require additional testing, you may wish to have another kit on hand to avoid missing a day of testing.

Q: What if I miss a day of testing?
A: The duration of the LH peak varies from dog to dog and lasts only 1 day in approximately 40 percent of bitches. Samples should be drawn and tested on a daily basis. Failure to do so may result in improper timing. If an LH test day is missed, continue to test but supplement LH testing with progesterone assays in the event the LH peak occurred on the missed day.

Perform a progesterone assay if it is suspected that the LH surge was missed. If progesterone is low (below 2 ng/ml), continue testing. If progesterone is high (above 2 ng/ml), call Synbiotics for technical assistance.

FOR TECHNICAL ASSISTANCE CALL 888.ZOETIS1 (888.963.8471)

SYNBIOTICS
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