HYPERADRENOCORTICISM IN FERRETS

HYPERADRENOCORTICISME BIJ FRETTEN
(*met een samenvatting in het Nederlands*)

PROEFSCHRIFT

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Chapter 1

Aim and scope of the thesis

The adrenal cortex produces mineralocorticoids, glucocorticoids, and sex steroids. Thus in principle, three distinct syndromes may arise as a result of adrenocortical hyperfunction. Indeed, syndromes of glucocorticoid excess are well-known in mammalian species such as dogs and cats. The occurrence of mineralocorticoid excess has also been described recently in dogs and cats. So far, distinct syndromes due to an excessive secretion of sex steroids have been described only in humans, [1, 5] although recent reports indicate that such syndromes also occur in dogs and cats. [2, 10, 11] There is increasing evidence that sex steroids play a primary role in hyperadrenocorticism in ferrets. [6, 8, 12]

The first case report on hyperadrenocorticism in ferrets was published in 1987. [3] In a series of 50 cases, hyperadrenocorticism appeared to be a common disorder, primarily affecting neutered ferrets. [9] Some of the signs (vulvar swelling in neutered jills and recurrence of sexual behavior in neutered hobs) pointed towards an excessive production of sex steroids rather than to an excessive production of glucocorticoids. [9] Consistent with this, elevated plasma concentrations of androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone, and estradiol have been reported. [8] Only rarely do plasma cortisol concentrations exceed the upper limit of the reference range, [8] whereas the urinary excretion of cortisol is often increased. [4] The possible involvement of pituitary hormones in hyperadrenocorticism in ferrets has not been studied.

In hyperadrenocorticism in ferrets, the adrenocortical lesion may be a unilateral adenoma or carcinoma. These tumors are not associated with atrophy of the contralateral adrenal cortex. [9] On the contrary, the contralateral adrenal cortex may be hyperplastic as well as tumorous. Indeed, the disease may also be due to bilateral adrenocortical hyperplasia. Macroscopic changes of the pituitary gland have not been reported. [7] Resection of a unilateral tumor usually leads to improvement, albeit without substantial lowering of the plasma cortisol concentration. [9]

The studies reported in this thesis, were aimed at the further characterization of adrenocortical hyperfunction in ferrets, in an attempt to elucidate the pathogenesis of the condition and to clarify some of the diagnostic problems. To this end, the literature was first reviewed (**chapter 2**). Then, to improve diagnostic accuracy, the influence of anesthesia and manual restraint on variables of the pituitary-adrenocortical axis was studied (**chapter 3**). Subsequently, reference ranges for plasma concentrations of adrenocorticotropic hormone (ACTH) and α-melanocyte-stimulating hormone (α-MSH) were established for normal ferrets and these concentrations were compared to those of ferrets with hyperadrenocorticism (**Chapter 4**).

Urinary corticoid-creatinine ratios were measured in privately owned healthy ferrets and in ferrets with signs of hyperadrenocorticism to elucidate the contradiction between normal plasma cortisol concentrations and the increased urinary cortisol excretion. Possible seasonal variations in urinary corticoid excretion were studied in healthy ferrets and ferrets with hyperadrenocorticism for nineteen months (**Chapter 5**).

The possible pathogenetic role of the pituitary gland in hyperadrenocorticism was investigated by comparing the histology of the pituitary gland in ferrets with hyperadrenocorticism and healthy ferrets (**Chapter 6**). The involvement of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) was investigated in a preliminary study by asking the owners of (castrated) ferrets whether they currently had ferrets with

signs of hyperadrenocorticism, or if they had such ferrets in the past, to find out whether there was any correlation between the age at neutering and the age at which hyperadrenocorticism developed (**Chapter 7**).

The presence and functioning of LH and FSH receptors in adrenocortical tissue was examined by immunohistochemistry, RT-PCR, and *in vitro* and *in vivo* stimulation tests (**Chapter 8 and 9)**. Possible alternatives to surgical neutering are discussed, with a view to eliminate the possible role that this type of neutering has on the development of hyperadrenocorticism (**chapter 10**). Lastly, findings are put in a broader context in a summarizing discussion (**Chapter 11)**.

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Chapter 2

General introduction

Hyperadrenocorticism in ferrets is thought to be primarily of adrenal origin. This introduction will provide an overview of current knowledge on adrenocortical morphology, (regulation of) adrenocortical steroidogenesis, and adrenocortical diseases. Only the literature on hyperadrenocorticism in ferrets published before our first publication (2000) will be discussed.

1. Morphology of the adrenal cortex

In ferrets the adrenal glands are paired structures, embedded in retroperitoneal fat craniomedially to the kidneys. The left adrenal gland, 6 – 8 mm long, usually lies close to the aorta, just cranial to the origin of the cranial mesenteric artery. The adreno-lumbar vein runs across its ventral surface. The right adrenal gland is larger, 8 – 11 mm long, and is located more cranially than the left gland and is always related to the latero-dorsal surface of the caudal vena cava.[17]

The adrenal gland consists of two functionally distinct endocrine glands of different embryological origin. The medulla is of ectodermal origin and secretes epinephrine and norepinephrine, whereas the adrenal cortex is of mesodermal origin and contains three major zones.

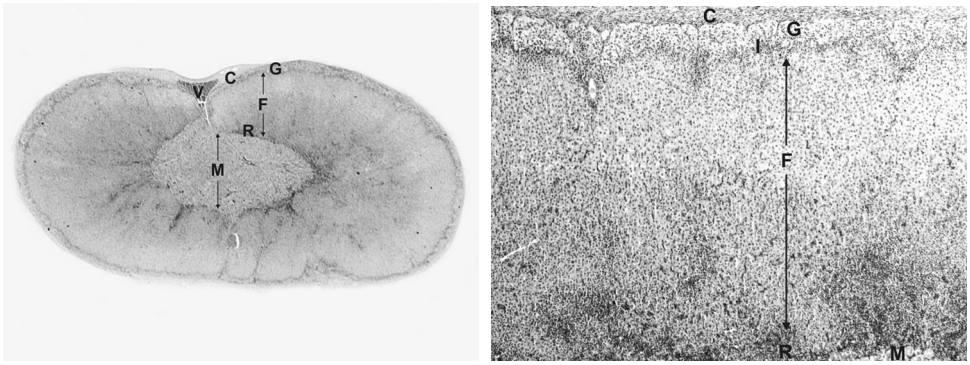

Figure 1. Histological sections of an adrenal gland of a healthy ferret: M = medulla, R = zona reticularis, F = zona fasciculata, I = zona intermedia, G = zona glomerulosa, C = capsule, V = adreno-lumbar vein.

The outermost zone of the adrenal cortex is the zona glomerulosa, which produces mineralocorticoids (primarily aldosterone). Further inward is the zona intermedia. The cells in this zone are smaller than those in either of the adjacent zones,[17] and are regarded as the progenitors of the cells of the adrenal cortex.[30,50] In rats, the cells of the zona intermedia lack the enzymes aldosterone synthase and 11β-hydroxylase, and thus mineralocorticoids and glucocorticoids are not produced in this zone. Yet these cells can be stimulated by ACTH to replicate and migrate toward the zona fasciculata, the second major zone (Fig 1). Activation of the renin-angiotensin system, by providing a Na-deficient diet to rats, results in cell replication in the innermost portion of the zona glomerulosa.[30]

The zona fasciculata consists of an outer and inner part, and produces glucocorticoids (cortisol and corticosterone) and androgens. The cells in both parts are usually arranged in columns. The cells in the outer part are large, have a centrally located nucleus, and abundant, spongy appearing cytoplasm. The cells of the inner part are smaller and stain more deeply than those of the outer part.[17]

The most interior zone is the zona reticularis, which is extremely variable in its prominence and its cellular composition. This zone contains the smallest cells of the adrenal cortex[17] and produces primarily androgens.[27] A notable feature of the ferret adrenal gland is that islands of cortical cells can be found among the cells of the medulla. These cells resemble those of the inner part of the zona fasciculata.[17]

One or more detached nodules of accessory adrenal tissue, consisting only of cortical cells, are sometimes seen, whereas intracapsular nodules containing cortical cells are more common.[17]

2. Adrenocortical steroidogenesis

Cholesterol is the precursor of adrenocortical steroidogenesis, which involves the concerted action of several enzymes, including a series of cytochrome P450 enzymes (Fig 2). After side-chain cleavage pregnenolone is formed, which in turn is converted to progesterone or 17α-hydroxypregnenolone. 17-Hydroxylation is a prerequisite for glucocorticoid synthesis. Cytochrome 17-hydroxylase also possesses 17,20-lyase activity, which results in the production of the C19 adrenal androgens, dehydroepiandrosterone (DHEA) and androstenedione. 21-Hydroxylation of either progesterone (zona glomerulosa) or 17α-hydroxyprogesterone (zona fasciculata) is accomplished by 21-hydroxylase, to yield deoxycorticosterone or 11-deoxycortisol, respectively. The zona glomerulosa does not express 17-hydroxylase. The final step in cortisol biosynthesis involves the conversion of 11-deoxycortisol to cortisol. In the zona glomerulosa, 11β-hydroxylase may also convert deoxycorticosterone to corticosterone. Aldosterone synthase converts corticosterone to aldosterone.[50] Of the glucocorticoids, corticosterone is the predominant glucocorticoid in rodents and birds, whereas cortisol is the most important glucocorticoid in ferrets.[47]

3. Regulation of adrenal steroidogenesis

3.1 Functional zonation

The localized expression of aldosterone synthase means that mineralocorticoids are only synthesized in the zona glomerulosa, whereas glucocorticoids are not synthesized in the zona glomerulosa because it lacks 17α-hydroxylase.[50] Cortisol is mainly secreted from the zona fasciculata, while androgens predominantly originate from the zona reticularis.[18]

3.2 The hypothalamus-pituitary-adrenocortical axis

Adrenocorticotropic hormone (ACTH) is the principal pituitary-derived hormone that stimulates adrenocortical glucocorticoid secretion. ACTH is synthesized as part of a much larger precursor, pro-opiomelanocortin (POMC), which is then cleaved in a tissue-specific fashion. Other products that may be formed are melanocyte-stimulating hormones (MSHs, α, β, γ), β-lipoprotein, β-endorphin, and joining peptide. The function of many of these products is unknown.[50] Corticotrophin-releasing hormone (CRH), which is synthesized in

neurons of the hypothalamus, is the main stimulant of ACTH secretion, although arginine-vasopressin (AVP) may potentiate CRH-mediated ACTH secretion.[50] Both ACTH and α-MSH are secreted in a pulsatile fashion, but in the dog more ACTH pulses than α-MSH pulses per 24h are seen.[22] Glucocorticoids exert a direct negative feedback control on their own secretion, by inhibiting POMC gene transcription in the pituitary and CRH and AVP synthesis in the hypothalamus (Fig 3).[50]

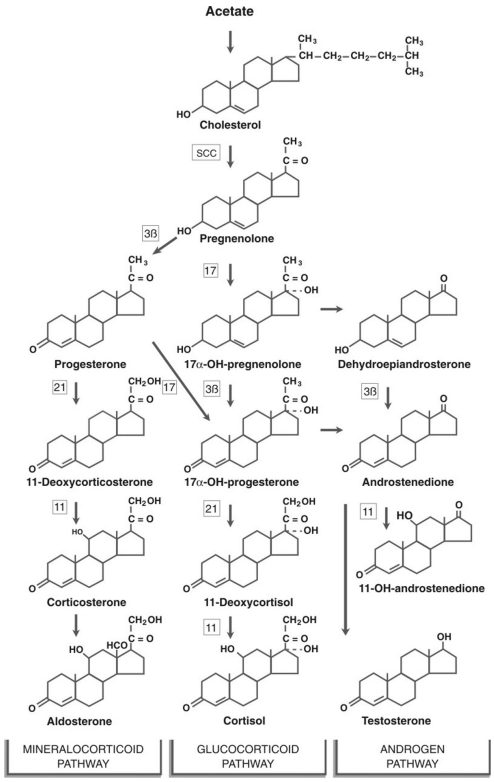

Figure 2.
Major pathways of adrenocortical steroid synthesis.
SCC = side chain cleavage
3β = 3β-hydroxysteroid dehydrogenase
11 = 11β-hydroxylase
17 = 17α-hydroxylase
21 = 21-hydroxylase
(Adapted from Rijnberk [41])

3.3 Adrenal androgen secretion

ACTH is also considered to be important in controlling the secretion of adrenal androgens.[39] However, differences in the secretion of adrenal androgens and glucocorticoids have led to the suggestion of the existence of an additional androgen-stimulating hormone.[50] For example, cytochrome b_5 has a profound effect on the activity of 17,20-lyase, the enzyme that converts 17α-hydroxypregnenolone into DHEA and 17α-hydroxyprogesterone into androstenedione.[19]

3.4 Mineralocorticoid secretion

Aldosterone is secreted from the zona glomerulosa under the control of three secretagogues, angiotensin II, potassium, and to a lesser extent ACTH. It is beyond the scope of this introduction to discuss these factors in detail

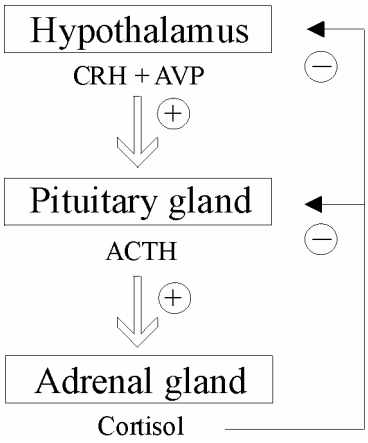

Figure 3.
Regulation of adrenal glucocorticoid secretion. Hypothalamic CRH and AVP stimulate ACTH secretion by the pituitary gland, which in turn stimulates the adrenal cortex to produce cortisol. Cortisol exerts a direct negative feedback on the hypothalamus and pituitary gland.

4. Adrenocortical disease

4.1 Adrenocortical insufficiency

Primary adrenocortical insufficiency, or Addison's disease, occurs either spontaneously after progressive destruction of the adrenal cortices by more than 90%,[41] after surgical removal of both adrenal glands, or after chemotherapeutic destruction with o,p'-DDD.[10] To date, spontaneous hypoadrenocorticism has not been diagnosed in ferrets. Subtotal bilateral adrenal surgery, however, may lead to this disease.[58]

The main features of this disease in dogs, i.e. lethargy and weakness, are predominantly due to a deficiency of mineralocorticoids. Lethargy may be related to hypotonic dehydration due to sodium loss, while hyperkalemia affects neuromuscular function, in particular leading to conduction disturbances in the heart.[41] The diagnosis is based on the results of the ACTH stimulation test with failure of low baseline plasma cortisol concentration to increase after ACTH administration being regarded as diagnostic.[41] Weiss et al. used a plasma sodium-to-potassium ratio to determine whether ferrets had a mineralocorticoid deficiency after subtotal bilateral adrenalectomy.[58] A ratio lower than 25:1 was considered indicative of adrenocortical insufficiency. An ACTH stimulation test, however, was not performed. Intramuscular injection of the mineralocorticoid deoxycorticosterone pivalate (DOCP; 2.2 mg/kg) provides adequate substitution for the deficiency in aldosterone.[58]

4.2 Hyperadrenocorticism

In line with the three main groups of hormones secreted by the adrenal cortex, i.e. glucocorticoids, mineralocorticoids, and androgens, three hyperfunction syndromes exist: (1) hypercortisolism (Cushing's syndrome), (2) hyperandrogenism, and (3) hyperaldosteronism (Conn's syndrome). Hyperandrogenism may lead to hyperestrogenism. This introduction concentrates on hypercortisolism and hyperandrogenism / hyperestrogenism.

4.2.1 Hypercortisolism

Hypercortisolism (Cushing's syndrome) is by far the most common of the three syndromes in humans, dogs, and cats. The clinical features in humans, which were first described by Harvey Cushing in 1932,[9] include weight gain, lethargy, weakness, menstrual irregularities, loss of libido, depression, hirsutism, acne, purplish skin striae, and hyperpigmentation.[37] Common symptoms in dogs with Cushing's syndrome are centripetal obesity, alopecia, lethargy, weakness, polyuria, and polydipsia.[41] In cats hyperadrenocorticism is almost without exception associated with concurrent insulin-resistant diabetes mellitus.[34] Cushing's syndrome may be ACTH-dependent or ACTH-independent. The most common form of this disease in humans (60% – 80% of cases),[5] dogs (approximately 85% of cases),[41] and cats (approximately 80% of cases),[34] termed Cushing's disease, is generally due to an ACTH producing pituitary microadenoma. In humans, ACTH-dependent Cushing's syndrome may also be caused by ectopic ACTH secretion by a non-pituitary tumor.[37]

Unilateral hyperfunctioning adrenocortical tumors account for approximately 90 – 98% of ACTH-independent forms of Cushing's syndrome in humans,[23] while other causes include ectopic CRH secretion,[6] or stimulation of the adrenal cortex by gastric inhibitory polypeptide (GIP), β-adrenergic agonists, human chorionic gonadotropin (hCG)/LH, vasopressin, serotonin (5-HT4), and possibly leptin.[23] Autonomous glucocorticoid-secreting unilateral adrenocortical tumors may be the sole cause of hyperadrenocorticism, but in dogs these tumors have also been observed to occur simultaneously with pituitary adenomas.[16,53,55]

The diagnosis of Cushing's syndrome is established by biochemical analysis of blood and urine, concentrating on the excessive secretion of endogenous cortisol and the loss of the normal feedback control of the hypothalamus-pituitary-adrenocortical axis. Abdominal ultrasound, pituitary computed tomography (CT), and magnetic resonance imaging (MRI) are used to further characterize and localize the lesion.

Measurement of plasma cortisol concentrations has little diagnostic value because the episodic secretion of ACTH results in variable plasma cortisol levels, which may at times be within the reference range.[41] Therefore (24-h) urinary cortisol is measured in humans,[37] dogs,[1] and cats.[14] To correct for urine dilution due to polyuria, the urinary cortisol concentration is expressed relative to the creatinine concentration.[8,14,51]

A high dose dexamethasone screening test can be used to differentiate between a tumor of pituitary and non-pituitary origin (ectopic ACTH-secreting tumor or adrenocortical tumor). After the oral administration of dexamethasone, plasma cortisol concentrations or the urinary corticoid-to-creatinine ratio is measured. Suppression of the plasma cortisol

concentration, or of the urinary corticoid-to-creatinine ratio, by more than 50% is an indication of pituitary-dependent Cushing's disease.[13,14,54] Another way to distinguish between ACTH-dependent and ACTH-independent Cushing's syndrome is to measure plasma concentrations of ACTH. Concentrations persistently below the detection limit of the assay are found in ACTH-independent Cushing's syndrome, which may be due to cortisol-producing adrenal tumors, autonomous bilateral adrenal hyperplasia, or Cushing's syndrome due to the administration of exogenous glucocorticoids.[37,41]

Pituitary surgery is the most widely accepted treatment for Cushing's disease in humans.[5] Transsphenoidal hypophysectomy is also an effective treatment of Cushing's disease in dogs and cats.[28,29] Another option is bilateral adrenalectomy. Both options require subsequent glucocorticoid and mineralocorticoid replacement therapy.[5,41] Unilateral adrenalectomy is performed for adrenal adenomas or carcinomas. Glucocorticoid-replacement therapy is necessary after surgery for a few months until the function of the hypothalamic-pituitary adrenal axis is restored.[5,41]

Many drugs have been used in the treatment of pituitary-dependent hyperadrenocorticism. The most commonly used inhibitors of steroid biosynthesis in clinical use are trilostane, metyrapone, aminoglutethimide, and ketoconazole. Side effects occur with each of these drugs.[5,35,40] In dogs, trilostane provides the most satisfactory results.[35] In addition, there is the option of selective or non-selective destruction of the adrenal cortices with o,p'-DDD. [10,20,42]

4.2.2 Hyperandrogenism / hyperestrogenism

In some very rare cases androgen-secreting adrenal tumors have been documented in women.[3,25] Hyperadrenocorticism due to unilateral adrenal tumors producing estradiol, progesterone, and 17α-hydroxyprogesterone has been described in two dogs. In one of these dogs plasma concentrations of androstenedione were also elevated.[52] Hyperadrenocorticism due to unilateral adrenal carcinomas producing progesterone has been described in two cats.[4,48] In contrast, the overproduction of sex steroids seems to be the rule, rather than the exception in ferrets with hyperadrenocorticism.[26,45,56]

5. Hyperadrenocorticism in ferrets

Although hyperadrenocorticism is now generally considered to be a common disease in ferrets,[43,57] the first case was only published in 1987.[12] The clinical features include symmetrical alopecia (Fig 4), vulvar swelling in neutered female ferrets (jills), recurrence of sexual behavior after neutering in male ferrets (hobs), and pruritus.[26,46,57] The alopecia usually begins in spring, which coincides with the start of the breeding season, and may disappear without treatment. The next year the alopecia usually returns but does not resolve spontaneously at the end of the breeding season.[46]

Other concurrent signs include urinary blockage in males, due to prostatic enlargement and cysts,[7,44] and occasionally mammary gland enlargement in jills.[31] The pathogenesis of this disease is unknown in ferrets, but it has been suggested that early neutering could play a role,[26,46] because in certain strains of mice nodular adrenocortical hyperplasia and adrenocortical tumors occur after neutering at an early age.[11,33,49]

Routine neutering of ferrets started in the early 1980s, following the publications of studies showing estrogen-induced bone marrow suppression in jills with prolonged estrus.[2,21] There is no medical reason to castrate hobs, but castration reduces aggression, so that hobs can be kept in groups, and decreases the intensity of the musky odor produced by the sebaceous glands.[32] The hormonal regulation of reproduction in intact ferrets, the consequences of neutering, and the hypothesis concerning its possible role in the development of hyperadrenocorticism are represented in figure 5.

Figure 4. Symmetrical alopecia in a ferret with hyperadrenocorticism

In approximately 85% of ferrets with hyperadrenocorticism, one adrenal gland is enlarged without atrophy of the contralateral adrenal gland, while in the other 15% of cases there is bilateral enlargement.[46,57] After unilateral adrenalectomy in the case of unilateral enlargement, the disease may recur due to enlargement of the contralateral adrenal gland.[57] The histological changes of the adrenals range from (nodular) hyperplasia to adenoma and adenocarcinoma.[46] No macroscopically visible changes have been found in the pituitary glands of ferrets with hyperadrenocorticism.[43]

The diagnosis of hyperadrenocorticism in ferrets is usually based on plasma concentrations of androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate, and estradiol;[45] urinary corticoid/creatinine ratios; [15] and ultrasonography of the adrenals.[43] Because plasma cortisol concentrations are rarely elevated in ferrets with hyperadrenocorticism,[45] the merit of measuring the urinary corticoid/creatinine ratio has been questioned.[43] Ultrasonography has been reported to be only accurate in 50 % of the cases,[43] while others reported much more positive on the detection of adrenal glands in ferrets.[36,38] One of the latter reports, however, stated that there was no statistical significant difference between the ultrasonographic or gross size of normal adrenal glands and those

with hyperplasia.[36] The results of ACTH stimulation tests[46,56] and dexamethasone suppression tests are not considered diagnostic in ferrets.[43] Pituitary hormones have not been measured in ferrets in relation to hyperadrenocorticism.

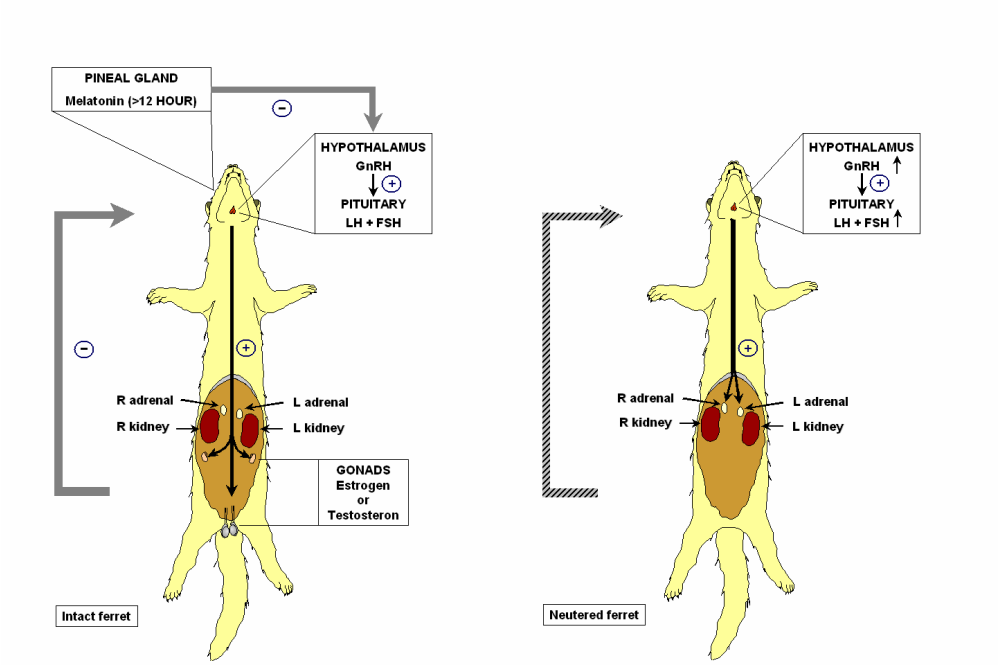

Figure 5. Scheme showing aspects of the regulation of reproduction in intact ferrets, the consequences of neutering, and its possible role in the development of hyperadrenocorticism in this species. High melatonin concentrations for more than 12 hours/day suppress the release of gonadotropin releasing hormone (GnRH). With increasing day length this suppression is lost and GnRH is released in a pulsatile fashion, resulting in the release of luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which in turn stimulate the release of estrogen and testosterone from the gonads. These hormones exert a negative feedback on the hypothalamus and pituitary gland. When ferrets are neutered, this negative feedback is lost, resulting in an increased release of gonadotropins, which may promote steroidogenesis and induce non-neoplastic and neoplastic adrenocortical enlargement.

The most common form of therapy is adrenalectomy,[24,46,56,57] which may include partial resection of the contralateral adrenal gland.[43,58] Glucocorticoid-replacement therapy is rarely necessary after surgery, because the contralateral adrenal gland is usually not atrophic.[43,57] Both o,p'-DDD and ketoconazole have been used for the medical treatment of ferrets with hyperadrenocorticism, but neither is considered to be very effective.[43]

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Chapter 3

Effects of Anaesthesia and Manual Restraint on the Plasma Concentrations of Pituitary and Adrenocortical Hormones in Ferrets

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Summary

Two experiments were carried out to investigate the effect of sampling techniques on the plasma concentrations of pituitary and adrenocortical hormones in ferrets (*Mustela putorius furo*). In the first experiment blood was collected on two occasions from 29 ferrets that were either manually restrained or anaesthetized with isoflurane. In the second experiment eight intact ferrets were fitted with jugular catheters and blood was collected on four occasions, just before and as soon as possible after they had been manually restrained or anaesthetized with medetomidine or isoflurane; blood was also collected 10 and 30 minutes after the induction of anesthesia. Medetomidine anesthesia had no effect on the plasma concentrations of pituitary and adrenocortical hormones. Isoflurane anesthesia resulted in a significant increase in the plasma concentration of alpha-melanocyte-stimulating hormone (α-MSH) directly after the induction of anesthesia. Manual restraint resulted in a significant increase in the plasma concentrations of cortisol and adrenocorticotropic hormone (ACTH) and a decrease in the plasma concentration of α-MSH.

Introduction

Hyperadrenocorticism is a common disease in pet ferrets. The disease differs from that in human beings and dogs in that there are increases in the basal plasma concentrations of the adrenal androgens 17α-hydroxyprogesterone, androstenedione, and to a smaller extent dehydroepiandrosterone, rather than in cortisol.[13] In some cases, the plasma concentration of oestradiol is also increased.[6,13,22] The signs of hyperadrenocorticism in ferrets, which initially occur only during the breeding season, are dominated by the effects of the excessive production of these steroids, for example, symmetrical alopecia, vulvar swelling in neutered jills and the recurrence of sexual behavior after neutering.[6,13,14,20,23]

To elucidate the pathophysiology of hyperadrenocorticism in ferrets, it is important to measure the plasma concentrations of cortisol, adrenocorticotropic hormone (ACTH), alpha-melanocyte-stimulating hormone (α-MSH), androstenedione and 17α-hydroxyprogesterone.[13,19] However, the influence of sampling technique on the plasma concentrations of these hormones has not been studied.

For the collection of blood from ferrets, four veins are commonly used: the jugular, cephalic, lateral saphenous and anterior caval veins.[10] The lateral saphenous and cephalic veins can be used to collect only small amounts of blood, but the other two can be used to collect the larger samples needed for hormone measurements. However, the collection of these larger samples requires the ferrets to be kept under firm manual restraint or to be anaesthetized, and these procedures are known to affect the results. Manual restraint causes significant increases in the plasma levels of cortisol, ACTH and α-MSH in cats,[24] and in ferrets hematological variables have been shown to decrease during isoflurane anaesthesia.[7] Different injectable anesthetics have also been found to either increase or decrease plasma androstenedione concentrations in cats.[1]

The objective of this study was to determine the effects of manual restraint and anesthesia on the plasma concentrations of pituitary and adrenocortical hormones in ferrets as they might occur during routine blood collection in a clinical setting.

Materials and Methods

Two experiments were carried out, both of which were approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University.

Experiment 1

Twenty-nine healthy ferrets, approximately three years old (14 intact and four neutered females, and six intact and five neutered males), were used. They had been purchased at six weeks of age, and were individually housed in outdoor suspended cages with a night box. No additional lighting was provided. Water and ferret pellets (FerRet; Hope Farms) were provided ad libitum.

The ferrets were randomly divided, by using the statistical computer program S-PLUS 2000 (MathSoft) into groups of 14 and 15. Blood samples were taken from all the ferrets on

two occasions, one week apart. For the first blood collection all the ferrets of group 1 were manually restrained, and the ferrets of group 2 were anaesthetized with 4 per cent isoflurane (IsoFlo; Schering-Plough Animal Health), inhaled through a mask in oxygen delivered at one liter per minute. As soon as the ferret could be placed in dorsal recumbency without restraint, the blood sample was collected, while anesthesia was maintained with 2 per cent isoflurane in oxygen delivered at 1 liter per minute. This protocol is similar to that commonly used for taking blood samples in a clinical setting.[18] No stimuli were applied to assess the depth of anesthesia, because they might have evoked an endocrine response.[21] The ferrets did not react to the blood collection, and after it had been completed they were allowed to wake up, which they usually did within five minutes. One week later the same protocol was followed, but in this case the groups were switched. In both groups the blood samples for the measurement of ACTH, α-MSH and cortisol were collected from the cranial vena cava.

Experiment 2

Eight healthy, intact three-year-old ferrets (three male and five female) were used during the reproductive season, so that 17α-hydroxyprogesterone could be measured together with cortisol, ACTH and α-MSH.

A jugular catheter was introduced, via vena section, under isoflurane anesthesia, into each of the ferrets. The catheters were tunneled subcutaneously to the base of the skull and connected to a 20-gauge cannula with an injection port (Vasofix Braunüle; Braun). The injection port was attached to the skin with polyglactin 910 (Vicryl; Ethicon) 2-0 USP sutures. The catheters (Silastic Medical Grade Tubing 602-155; Down Corning) had an internal diameter of 0.64 mm and an external diameter of 1.19 mm. To keep the catheter patent it was filled with a mixture of polyvinyl pyrrolidone (PVP) and heparin (60 g PVP / 54 ml 0.9 percent sodium chloride + 6 ml heparin [5000 IU/ml]).

Three days after the introduction of the catheters anesthesia was induced and maintained with isoflurane as in experiment 1. As soon as the animals could be placed in dorsal recumbency the isoflurane concentration was reduced to 2 per cent, and this concentration was maintained during the 30 minutes of anesthesia. Two days later anesthesia was induced by the administration of 100 μg/kg medetomidine (Domitor; Pfizer Animal Health) into the lateral muscles of the thigh.[18] This dose allowed the ferrets to be kept in dorsal recumbency for 30 minutes. Again, no stimuli were applied to assess the depth of anesthesia. On both occasions blood samples were collected through the jugular catheter before the ferrets were anaesthetized, immediately after induction, and 10 and 30 minutes later.

Three days later a blood sample was collected via the jugular catheter, followed as soon as possible, by another blood sample taken by venepuncture of the anterior vena cava while the ferret was restrained manually. The interval between the collection of the two samples differed, depending on how much the ferrets resisted the procedure.

In five of the ferrets the catheter became occluded between test days. In one of them a new catheter was introduced and all the blood samples were obtained; in three of them blood samples were obtained on two occasions; and in the other ferret only on one occasion blood samples could be collected.

Sample handling and hormone determination

After collection, the blood samples were placed immediately in chilled EDTA-coated tubes and centrifuged at 4ºC. Plasma samples were stored at – 20ºC until they were analyzed within three months.

Plasma ACTH concentrations were measured with a commercially available immunoradiometric assay (Nichols Institute Diagnostics). The interassay coefficient of variation was 7.8 per cent and the sensitivity was 1 ng/l. There was no cross reaction with α-MSH.

Plasma concentrations of α-MSH were measured by radioimmunoassay (RIA), using antiserum to synthetic human α-MSH as described previously.[12] This antiserum had full cross reactivity with desacetyl-α-MSH but less than 0.1 per cent cross reactivity with ACTH (1-39) and 4 per cent cross reactivity with ACTH (1-24). Synthetic human α-MSH was used as a standard. The limit of detection was 5 ng/l and the intra- and interassay coefficients of variation were 10 per cent and 23 per cent, respectively.

Both assays were validated for use in ferrets. Dilution curves of a ferret plasma sample were compared with standard curves for ACTH (Nichols Institute Diagnostics) and α-MSH (Bachem AG). In both cases the curves followed the standard curve.[18]
Plasma 17α-hydroxyprogesterone concentrations were measured after toluene extraction by using a competitive RIA and a polyclonal anti-17α-hydroxyprogesterone-antibody (UCB i903, UCB Bioproducts). 17α-Hydroxy[1,2,6,7-^{3}H]-progesterone (TRK 611, Amersham Pharmacia Biotech Benelux) was used as a tracer after chromatographic purification. The lower limit of detection was 0.2 nmol/l and interassay coefficients of variation were 9.0 per cent, 7.4 per cent and 9.9 percent at 0.89, 5.13 and 26.0 nmol/l, respectively.

Statistics

In experiment 1 the data were transformed logarithmically to obtain a normal distribution. The paired Student's *t* test was used for the statistical analysis. In experiment 2 the Friedman test (a non-parametric analysis of variance) was used, followed by a multiple comparison test (Dunnett: comparison with a control).[25] Statistical significance was assumed at $P < 0.05$.

Results

Experiment 1

The mean plasma ACTH concentration of the manually restrained ferrets and the ferrets anaesthetized with isoflurane did not differ significantly. However, the mean plasma α-MSH concentration of the manually restrained ferrets was significantly lower, and their mean cortisol concentration was higher than those of the anaesthetized ferrets (Table 1).

Table 1. Mean (SEM) concentrations and (ranges) of plasma cortisol, adrenocorticotropic hormone (ACTH), and α-melanocyte-stimulating hormone (α-MSH) in 29 ferrets immediately after induction of anesthesia with isoflurane and during manual restraint. Asterisk indicates significant difference ($P < 0.001$).

Variable	Manual restraint		Isoflurane anesthesia	
	Mean (SEM)	Range	Mean (SEM)	Range
ACTH (ng/l)	84 (9)	18 – 183	68 (6)	21 – 152
Alpha-MSH (pg/ml)	16 (2) *	4 – 44	50 (11)	4 – 283
Cortisol (nmol/l)	43 (8) *	3 – 201	13 (1)	3 – 27

Experiment 2

The mean (SEM) plasma ACTH concentration of the ferrets was increased significantly by manual restraint from 35 (4) to 113 (15) ng/l and their mean plasma concentration of cortisol increased from 8 (3) to 47 (25) ng/l. In contrast, their mean plasma α-MSH concentrations decreased significantly from 27 (4) to 11 (2) ng/l. The plasma concentration of 17α-hydroxyprogesterone was not affected by manual restraint (Fig 1).

Under isoflurane anesthesia the mean plasma α-MSH concentration of the ferrets increased significantly directly after induction, from 33 (4) to 103 (33) ng/l, but the plasma concentrations of ACTH, cortisol and 17α-hydroxyprogesterone remained unchanged. The plasma concentration of α-MSH was still significantly increased after 10 and 30 minutes of anesthesia (Fig 1).

Medetomidine anesthesia caused no significant changes in the concentrations of any of the hormones (Fig 1).

Discussion

Stress is known to increase the rate of release of ACTH from the pituitary gland,[8] and this in turn stimulates the secretion of adrenocortical hormones.[9,11] ACTH enhances the rate of conversion of cholesterol to pregnenolone, and within minutes after its release the concentrations of adrenocortical steroids in adrenal venous blood start to rise.[11] As would have been expected as a result of these physiological changes restraining the ferrets normally resulted in significant increases in their plasma concentrations of ACTH and cortisol; similar changes have been observed in rats and cats.[4,5,24]

The secretion of α-MSH from the pars intermedia adenohypophysis has been studied extensively in rats. In this species, the secretion of α-MSH is inhibited by dopamine and aminobutyric acid, whereas serotonin, β-adrenergic agents and corticotrophin releasing hormone (CRH) may stimulate it.[15] Tonic dopaminergic inhibition has also been recorded in dogs, cats, sheep and horses, but is absent in rabbits.[8] In dogs the administration of CRH and various forms of neurogenic stress do not stimulate the secretion of α-MSH.[3] In contrast, in cats a mild stress such as handling results in increased plasma α-MSH concentrations,[24] as it does in rats. In the ferrets the plasma concentrations of α-MSH decreased after they were restrained manually, a change which, to the authors' knowledge

has not been described in any other species. In rabbits glucocorticoids have an inhibitory effect on the release of α-MSH;[17] if this were also true for the ferret, it would explain why the plasma α-MSH concentrations decreased, because the manual restraint was associated with a clear-cut increase in plasma cortisol concentrations.

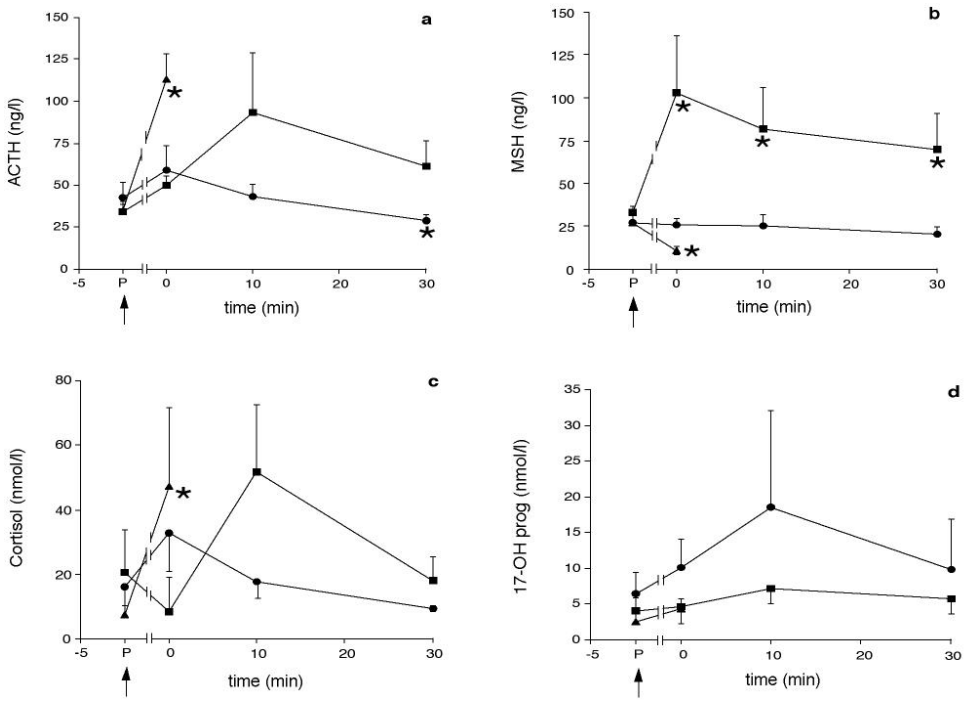

Figure 1. Mean (SEM) plasma concentrations of (a) adrenocorticotropic hormone (ACTH), (b) α-melanocyte-stimulating hormone (α-MSH), (c) cortisol and (d) 17α-hydroxyprogesterone (17-OH prog) in ferrets immediately before they were restrained manually or anaesthetized (P), during manual restraint (▲) [n = 6], and during anesthesia with isoflurane (■) [n = 7] or medetomidine (●) [n = 6]. Arrows indicate start of restraint or induction of anesthesia. An asterisk indicates significantly different from before restraint or anesthesia ($P < 0.05$)

Another unexpected finding with respect to α-MSH was the increase in plasma concentration during isoflurane anesthesia, but not during medetomidine anesthesia. Since manual restraint decreased rather than increased the concentration of α-MSH, it is not likely that the stress of mask induction was responsible for this increase, as suggested previously.[19] it is more likely that the increase was caused by an effect of isoflurane itself. One possible explanation might be that isoflurane has an antidopaminergic effect, because it has been shown that isoflurane can reduce the release of dopamine induced by either N-methyl-D-aspartate or nicotine.[2,16]

In both experiments no stimuli, other than dorsal recumbency and blood collection, were applied to assess the depth of anesthesia because such stimuli might have induced an endocrine response.[21] None of the anaesthetized ferrets reacted either to being placed in dorsal recumbency or having a blood sample taken. The differences between the hormone concentrations observed in the ferrets anaesthetized with isoflurane or medetomidine could theoretically have been due to the differences in the depth of anesthesia. However, from the uniform absence of any response to their placement or to the introduction of a needle, it is likely that the depth of anesthesia was very similar in both cases. It was the purpose of this study to determine whether two standardized anesthetic procedures, which are routinely used in practice, would affect the results of hormone measurements. In the absence of any evidence of a difference in the depth of anesthesia, the results suggest that different protocols used for blood collection may lead to different results.

In veterinary practice blood samples are usually collected immediately after the induction of anesthesia. A false increase in the plasma concentrations of ACTH, cortisol, and 17α-hydroxyprogesterone would therefore not be expected. Nevertheless, medetomidine anesthesia should be preferred to isoflurane anesthesia because the isoflurane anesthesia caused a significant increase in plasma α-MSH concentrations, which may affect the release of adrenocortical hormones. Medetomidine anesthesia did not affect the plasma concentrations of the pituitary and adrenocortical hormones.

Given that the levels of pituitary and adrenocortical hormones were not affected by medetomidine anesthesia, the question arises whether the reference ranges for the plasma concentrations of ACTH and α-MSH (13 to 98 ng/l and 16 to 74 ng/l respectively), which have been determined in blood samples collected during isoflurane anesthesia [19] are still valid. The mean (range) of plasma concentrations of ACTH and α-MSH measured in the present study were not significantly different from the values recorded in the previous study (68 [21 – 152] ng/l v 53 [4 – 145] ng/l for ACTH and 50 [4 – 283] ng/l v 56 [<5 – 238] ng/l for α-MSH) and it is therefore considered that these reference ranges can still be used, provided that the blood samples are collected under the same conditions.

Anesthesia is to be preferred to manual restraint when blood samples have to be collected from ferrets for the measurement of hormones of the pituitary-adrenocortical axis. Medetomidine anesthesia is to be preferred to isoflurane anesthesia because the latter causes a sharp rise in the concentrations of α-MSH.

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Chapter 4

Plasma Concentrations of Adrenocorticotropic Hormone and α-Melanocyte-Stimulating-Hormone in Ferrets (*Mustela putorius furo*) with Hyperadrenocorticism

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Summary

Objective – To determine plasma concentrations of adrenocorticotrophic hormone (ACTH) and α-melanocyte-stimulating-hormone (α-MSH) in healthy ferrets and ferrets with hyperadrenocorticism.

Animals –16 healthy, neutered privately owned ferrets, 28 healthy laboratory ferrets (21 sexually intact and 7 neutered), and 28 ferrets with hyperadrenocorticism.

Procedures – Healthy ferrets were used for determination of reference plasma concentrations of ACTH and α-MSH. Diagnosis of hyperadrenocorticism was made on the basis of history, clinical signs, urinary corticoid-to-creatinine ratios, ultrasonography of the adrenal glands, and macroscopic or microscopic evaluation of the adrenal glands. Blood samples were collected during isoflurane anesthesia. Plasma concentrations of ACTH and α-MSH were measured by radioimmunoassay.

Results – Plasma concentrations of ACTH in 23 healthy neutered ferrets during the breeding season ranged from 4 – 145 ng/l (median 50 ng/l). Plasma concentrations of α-MSH in 44 healthy neutered or sexually intact ferrets during the breeding season ranged from <5 – 617 ng/l (median 37 ng/l). Reference values (the central 95% of the values) for ACTH and α-MSH were 13 – 100 ng/l and 8 – 180 ng/l respectively. Plasma concentrations of ACTH and α-MSH in ferrets with hyperadrenocorticism ranged from 1 – 265 ng/l (median 45 ng/l) and 10 – 148 ng/l (median 46 ng/l) respectively. These values were not significantly different from those of healthy ferrets. Plasma ACTH concentrations of sexually intact female ferrets in estrus were significantly higher than those of neutered females.

Conclusions and Clinical Relevance –Ferrets with hyperadrenocorticism did not have detectable abnormalities in plasma concentrations of ACTH or α-MSH. The findings suggest that hyperadrenocorticism in ferrets is an ACTH and α-MSH independent condition.

Introduction

In humans, dogs, cats and horses the clinical signs of hyperadrenocorticism are primarily the result of excessive secretion of cortisol.[3,9,11,16] Hypercortisolism leads to muscle wasting, skin atrophy with alopecia, and centripetal obesity. In pet ferrets (*Mustela putorius furo)* the situation is different. The physical changes of hyperadrenocorticism are usually dominated by features of excessive production of sex steroid hormones, i.e., alopecia, vulvar swelling in neutered jills and recurrence of sexual behavior after neutering.[8,19,20,21,23,29] In agreement with these physical changes, plasma concentrations of androstenedione, 17α-hydroxyprogesterone, and dehydroepiandrosterone sulfate are usually increased whereas those of cortisol are not.[20] Besides these adrenal androgens, plasma concentrations of estradiol have also been reported to be increased in ferrets with hyperadrenocorticism.[8,20,28]

In ferrets, hyperadrenocorticism has been reported to be associated with adrenocortical hyperplasia and adrenocortical tumors.[21] In approximately 85% of ferrets with hyperadrenocorticism only 1 adrenal gland is enlarged, without atrophy of the contralateral adrenal gland.[21,29] After unilateral adrenalectomy there may be recurrence of the disease due to enlargement of the contralateral adrenal gland.[29] The presence of bilaterally affected adrenal glands in 15% of affected ferrets, and recurrence of the disease after unilateral adrenalectomy suggest that extra-adrenal tropic factors play a role in the pathogenesis of this disease.

In humans, dogs and cats the most common form of hyperadrenocorticism is pituitary-dependent, in which excessive adrenocorticotropic hormone (ACTH) is secreted by adenomas that may originate from corticotropic cells of the anterior lobe of the pituitary gland. In addition there is the possibility that neoplastic transformation of melanotropic cells of the pars intermedia, primarily producing the ACTH derivative α-melanocyte-stimulating hormone (α-MSH), causes hyperstimulation of the adrenal cortices.[10,16,22] This results primarily in hypersecretion of cortisol. The secretion of other adrenal steroids such as androstenedione, 17α-hydroxyprogesterone and dehydroepiandrosterone sulfate, however, may also be increased.[13]

In ferrets it is suggested that hyperadrenocorticism may be LH-dependent. First, there is the observation that hyperadrenocorticism is almost exclusively seen in castrated ferrets, with a significant correlation between the age at neutering and the age at diagnosis of hyperadrenocorticism.[23] Second, it has been reported that ferrets with hyperadrenocorticism can be treated successfully with depot GnRH-agonist leuprolide acetate.[27]

LH-dependent hyperadrenocorticism and LH-dependent adrenal androgen-secreting tumors have been described in humans.[6] In LH-dependent hyperadrenocorticism hypercortisolism is associated with suppressed concentrations of ACTH. In LH-dependent adrenal androgen-secreting tumors both cortisol and ACTH concentrations have been reported to be within the reference range.[7]

Thus there are several questions with regard to the factors involved in the development of hyperadrenocorticism in ferrets, such as the role of secretion of ACTH and α-MSH. In ACTH-dependent hyperadrenocorticism one would expect increased plasma concentrations of ACTH and possibly α-MSH. With hypercortisolism, the LH-dependent

form of hyperadrenocorticism would lead to suppressed plasma ACTH concentrations. To the authors' knowledge, there are no reports on plasma concentrations of ACTH or α-MSH in ferrets with hyperadrenocorticism.

The purpose of the study reported here was to determine plasma concentrations of ACTH and α-MSH in 28 ferrets with hyperadrenocorticism. The values are compared with those of 23 castrated and 21 sexually intact healthy ferrets.

Materials and Methods

Control ferrets

A group of 23 neutered control ferrets (13 female and 10 male) comprised 16 privately owned ferrets that were house mates of the ferrets with hyperadrenocorticism and 7 ferrets kept under laboratory conditions, individually housed in outdoor suspended cages with a night box. No additional lighting was provided. Water and ferret pellets (FerRet, Hope Farms, Woerden, The Netherlands) were provided ad libitum. The ages of the 23 ferrets ranged from 1.5 to 8 years (median, 3 years). Blood samples were collected between March 15 and September 29, 2000. None of the ferrets had any clinical signs of hyperadrenocorticism. All ferrets had urinary corticoid-to-creatinine ratio $< 1.7 \times 10^{-6}$. Urinary corticoid concentrations were measured by RIA for cortisol as described previously.[18] The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) by calculation of its quotient ($\times 10^{-6}$).[25]

In addition, blood was collected on April 4, 2000 from 21 sexually intact ferrets, aged 2 – 3 years (14 female and 7 male), kept under the same laboratory conditions as described.

Ferrets with hyperadrenocorticism

The 10 female and 18 male ferrets with hyperadrenocorticism were evaluated between April 1998 and September 2000. All ferrets had been neutered at 0.5 to 1 year of age. At the time of evaluation their ages ranged from 3 to 7 years (median, 5 years). In 22 ferrets alopecia was the main clinical sign. Of the 6 other ferrets, 5 were evaluated for swelling of the vulva, and in 1 ferret, a large adrenal mass was incidentally detected on the left side when abdominal ultrasonography was performed for other reasons. After 2 months this ferret died with metastases of an adrenocortical tumor.

The diagnosis of hyperadrenocorticism was made on the basis of history, physical signs, urinary corticoid-to-creatinine ratios [16,25] (range, $0.7 – 150 \times 10^{-6}$; in 26 of 28 ferrets these exceeded the upper limit of the reference range [4] [1.7×10^{-6}]), and ultrasonographic examination of the adrenal glands. Macroscopic appearance of the adrenal glands during surgery (n=23), histologic findings in the adrenal glands after surgery (n=19), or results of postmortem examination (n=9) provided additional support for the diagnosis.

In 21 ferrets there was unilateral enlargement of the adrenal gland, on the left side in 15 ferrets and on the right side in 6 ferrets. In all these ferrets the contralateral adrenal gland was of normal size (i.e. no atrophy). Seven ferrets had bilaterally enlarged adrenal

glands. In 24 of 26 ferrets ultrasonography had identified the alterations in the adrenal glands correctly.

Histologic examination of the adrenal glands of 26 ferrets (10 female and 16 male) revealed hyperplasia (n=9), adenoma (n=15), and metastasized adenocarcinoma (n=2). In 1 ferret the adrenal tissue was lost for histologic examination and in 1 ferret the adrenal tumor was left in place because of its growth into the caudal vena cava.

Blood collection

In affected ferrets and the control ferrets blood was collected between 2:30 and 4:00 p.m. from the anterior vena cava, by use of isoflurane anesthesia. Blood was immediately placed in chilled EDTA-coated tubes, and centrifuged at 4ºC. Plasma was stored at – 20ºC pending analysis. Samples were analyzed within 6 months after collection.

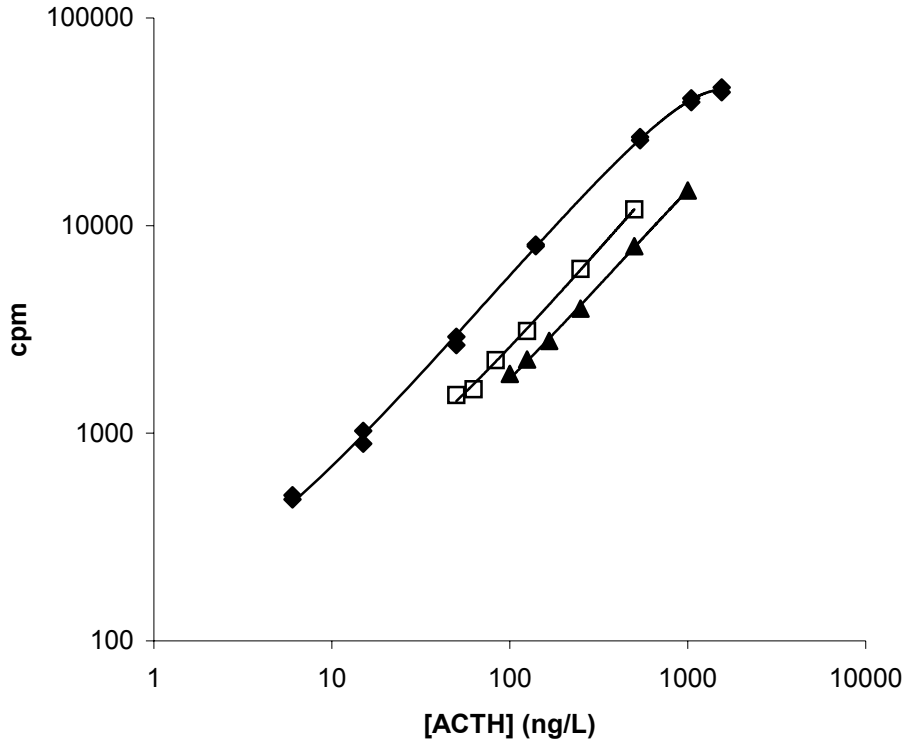

Figure 1. Dilution curves (concentration vs counts per minute [cpm]) of 2 ferret plasma samples (□,▲) and a human adrenocorticotropic hormone (ACTH) standard (♦) analyzed by use of a radioimmunoassay.

Hormone determination

Plasma ACTH concentrations were measured by use of a commercially available radioimmunoassay (RIA) as per manufacturer's instructions (Nichols Institute, Wijchen, The Netherlands). The interassay coefficient of variation was 7.8% and the sensitivity was 1 ng/l. No cross-reaction with α-MSH could be measured.

Plasma concentrations of α-MSH were measured by use of RIA with antiserum to synthetic human α-MSH, as described.[17] This antiserum had full cross-reactivity with desacetyl-α-MSH but < 0.1% cross-reactivity with $ACTH_{1-39}$ and 4% cross-reactivity with $ACTH_{1-24}$. Synthetic human α-MSH was used as a standard. The limit of detection was 5 ng/l and the intra- and interassay coefficients of variation were 10% and 23%, respectively.

Both assays were validated for use in ferrets. Dilution curves were made from a ferret plasma sample and compared with a standard for ACTH (Nichols Institute, Wijchen, The Netherlands), and for α-MSH (Bachem AG, Bubendorf, Switzerland). For both hormones, the curves followed the standard pattern (Fig 1 and 2).

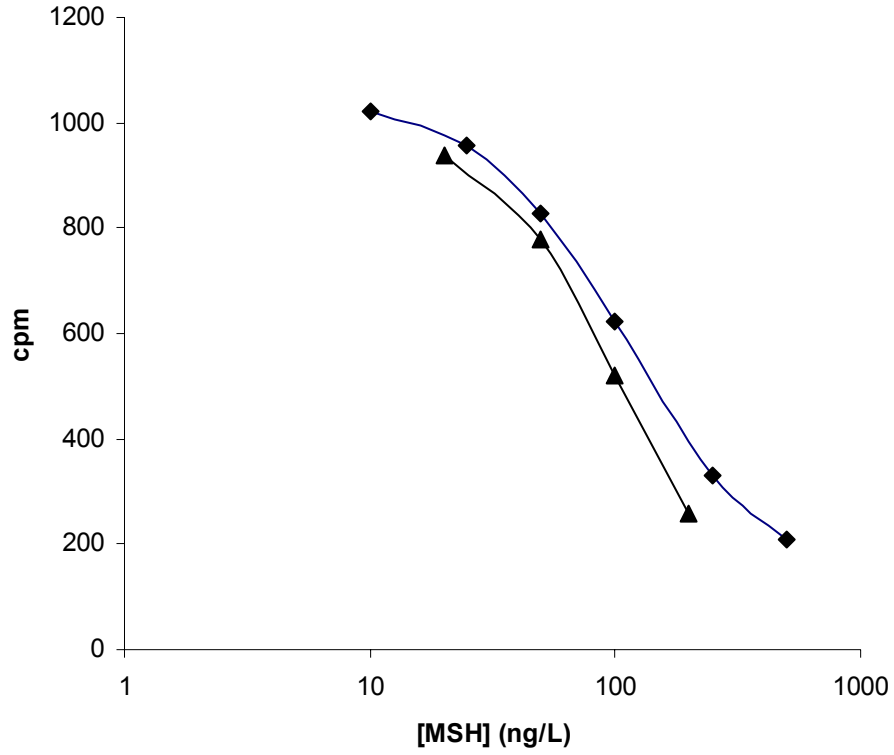

Figure 2. Dilution curves (concentration vs cpm) of a ferret plasma samples (▲) and a human melanocyte-stimulating hormone standard (♦) analyzed by use of a radioimmunoassay.

Statistical analyses

The percentile method was used to establish the reference ranges for plasma ACTH and α-MSH concentrations.[2] The percentiles $P_{2.5}$ and $P_{97.5}$ were determined with a probability of 95%. The Wilcoxon Rank Sum test was used for comparison of plasma ACTH and α-MSH concentrations of neutered and sexually intact ferrets.

Results were graphically represented in box-and-whisker plots. In these figures, the box represents the interquartile range from the 25th to 75th percentile, the horizontal bar through the box indicates the median, and the whiskers represent the main body of data, which in most instances is equal to the range.[1] For all comparisons, a value of $P < 0.05$ was considered significant.

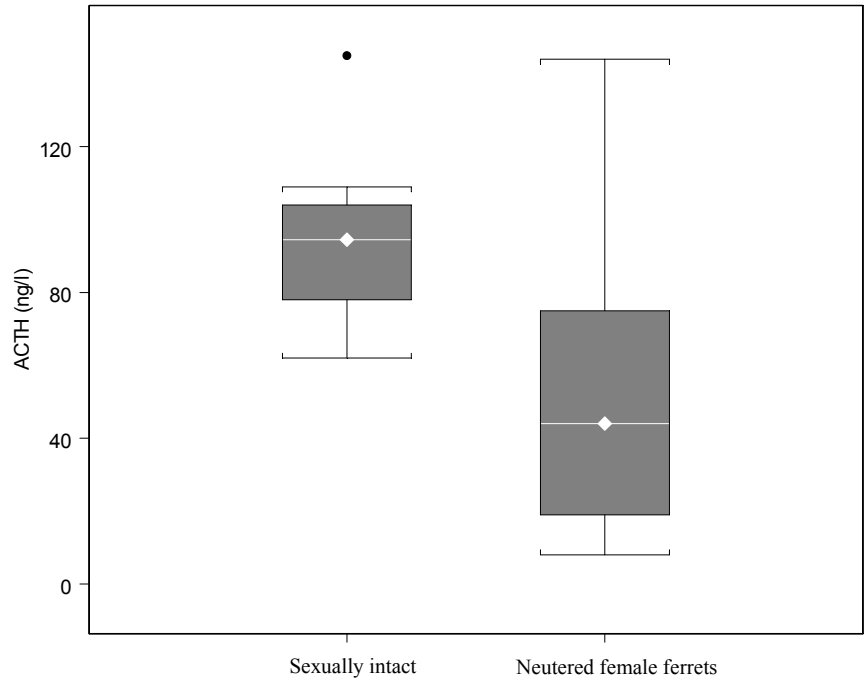

Figure 3. Boxplot of plasma ACTH concentrations in healthy sexually intact female ferrets (n=14) and healthy neutered female ferrets (n=13). Outlying data points are represented by dots.

Results

Healthy ferrets

Plasma ACTH concentrations in 23 neutered healthy ferrets ranged from 4 to 145 ng/l (median 50 ng/l; reference range, 13 – 100 ng/l). In the 21 sexually intact ferrets kept under laboratory conditions, plasma ACTH concentrations ranged from 38 to 144 ng/l (median 88

ng/l; reference range, 53 – 109 ng/l). The plasma ACTH concentrations in the sexually intact ferrets were significantly (P<0.01) higher than in the neutered ferrets.

Plasma ACTH concentrations in sexually intact female ferrets (n=14; range 62 – 144 ng/l; median 94.5 ng/l) were also significantly (P<0.01) higher than in neutered female ferrets (n=13; range 8 – 145 ng/l; median 44 ng/l) (Fig 3). The difference in plasma ACTH concentrations between sexually intact male ferrets (n=7; range 38 – 133 ng/l; median 68 ng/l) and neutered male ferrets (n=10; range 4 – 100 ng/l; median 66 ng/l) was not significant.

Plasma α-MSH concentrations of sexually intact (n=21) and neutered (n=23) ferrets did not differ significantly, nor was there a significant difference in the sexually intact ferrets between males and female; therefore, the data were combined to establish reference values. Plasma α-MSH concentrations in the 44 ferrets ranged from <5 – 617 ng/l (median 37 ng/l; reference range, 7 – 180 ng/l).

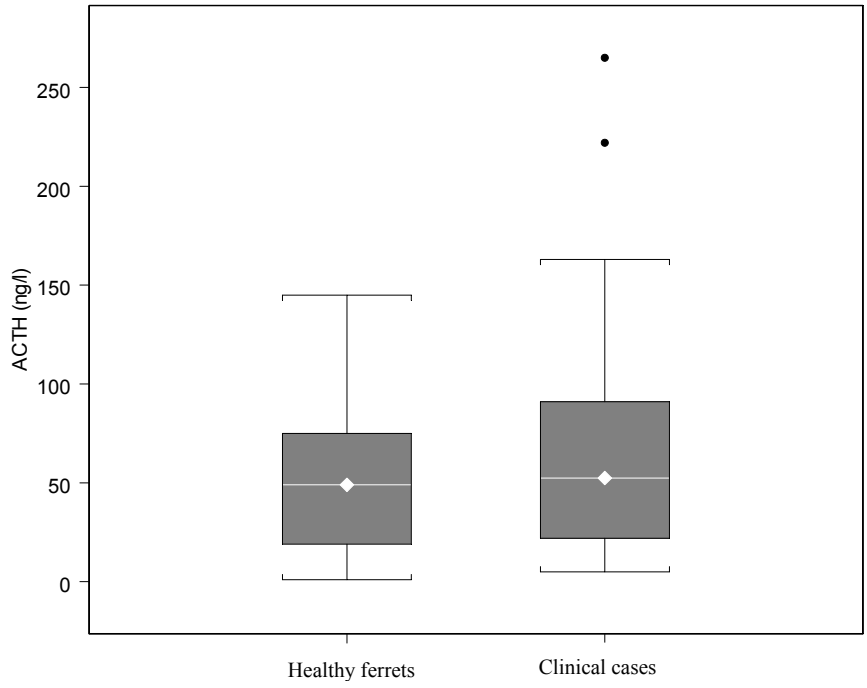

Figure 4. Boxplot of plasma ACTH concentrations in ferrets with hyperadrenocorticism (n=28) and healthy neutered ferrets (23). Outlying data points are represented by dots.

Ferrets with hyperadrenocorticism

Plasma ACTH concentrations in the 28 neutered ferrets with hyperadrenocorticism (range 1 to 265 ng/l; median 45 ng/l) were not significantly different from those in healthy neutered ferrets (Fig 4). In 6 hyperadrenocorticoid ferrets the plasma ACTH concentration exceeded the upper limit (100 ng/l) of the reference range of healthy ferrets.

The plasma α-MSH concentrations in ferrets with hyperadrenocorticism (n = 27, range 10 to 148 ng/l; median 46 ng/l) were not significantly different from those in healthy ferrets (Fig 5). The plasma α-MSH concentration did not exceed the upper limit (180 ng/l) of the reference range of healthy ferrets in any of the hyperadrenocorticoid ferrets.

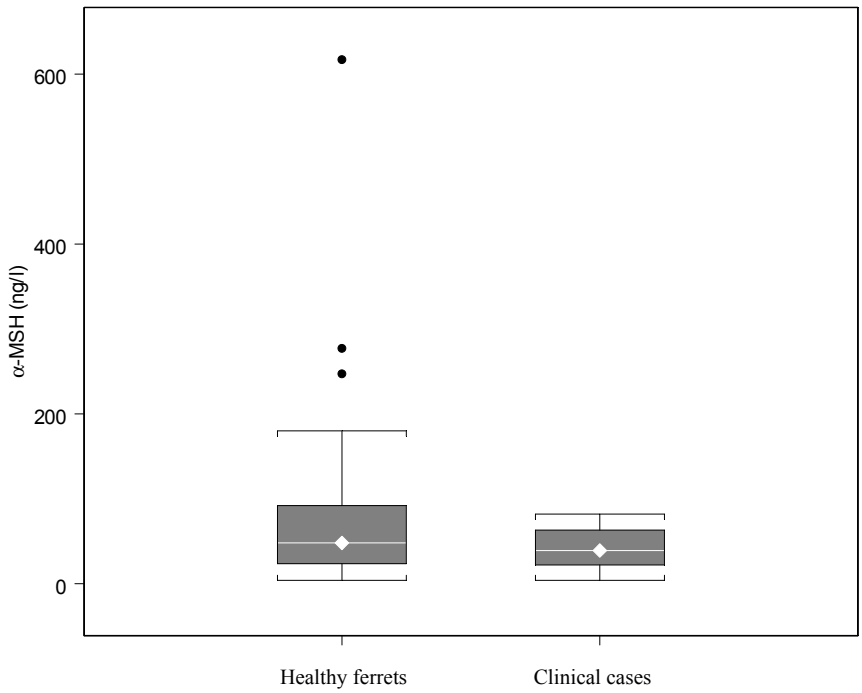

Figure 5. Boxplot of plasma α-MSH concentrations in ferrets with hyperadrenocorticism (n=27) and healthy ferrets (n=44). Outlying data points are represented by dots.

Discussion

In this study, plasma ACTH concentrations of healthy neutered ferrets ranged from 4 to 145 ng/l, which is similar to the ranges reported for dogs, cats and horses.[5,12,14,16] Plasma ACTH concentrations in sexually intact female ferrets were significantly higher than in neutered ferrets. This may be attributable to the fact that the blood samples were collected during the breeding season. In female frogs, secretory activity of ACTH cells increases during the breeding season.[15] The question remains whether this is due to the arousal associated with the breeding season or to a direct effect of the activated pituitary-ovarian axis on the corticotropic cells. The latter is the most likely explanation, because the plasma ACTH concentrations of sexually intact male ferrets were not significantly different from those of neutered male ferrets.

In our study basal plasma α-MSH concentrations in ferrets were similar to those in cats, which are somewhat higher than in dogs.[5,14] The values in ferrets may have been artificially increased by placement of a mask and induction of isoflurane anesthesia, because isoflurane has an unpleasant odor. It is known that, particularly in cats, handling may lead to increases in plasma concentrations of α-MSH,[30] and this may be true for ACTH concentrations as well. In addition, ACTH and α-MSH are released in a pulsatile manner in dogs.[5] Consequently, an occasional high value might be found with single measurements, although – particularly with α-MSH – the pulse frequency is low.[5] To our knowledge, there are no reports on the release patterns of ACTH and α-MSH in ferrets, but pulsatile release is likely because it has been found in all species studied. These considerations should be taken into account when individual values are interpreted. In our study, group values were evaluated instead of individual samples. We believe that the effects of possible pulsatile hormone release were outweighed by the numbers of animals per group.

Because we found that plasma concentrations of ACTH and α-MSH of ferrets with hyperadrenocorticism are essentially identical to those of healthy neutered ferrets, we believe that the adrenocortical changes and clinical signs cannot be ascribed to hypersecretion of ACTH. In addition, it is likely that there was no primary hypercortisolism either, because this finding should be associated with either decreased or increased plasma ACTH concentrations. Consequently, the increased corticoid-to-creatinine ratios might not be a reflection of hypercortisolism but rather the result of other urinary steroid hormones or steroid hormone metabolites cross-reacting in the cortisol assay. The antibody used in the assay for measurement of urinary cortisol is known to cross-react with other endogenous corticosteroids such as 21-deoxycortisol (62%), corticosterone (11%), cortisone (2%), 11-deoxycortisol (1.3%), deoxycorticosterone (1.3%) and 17α-hydroxyprogesterone (0.1%).[26]

Thus, hyperadrenocorticism in ferrets should probably be regarded as a normocortisolemic and corticotrophin-independent hypersecretion of androgens, primarily. Many different ectopic and abnormal hormone receptors have been found in the adrenal cortices of humans with hyperadrenocorticism.[6] In ferrets with adrenocortical hyperfunction, involvement of the LH receptor is likely. Recent preliminary immunohistochemical studies by Wagner et al[27] and our group[24] have revealed that adrenal tumors possess cells with LH-receptors.

Results of our study suggest that hyperadrenocorticism in ferrets may be an ACTH- and α-MSH-independent condition accompanied by unchanged plasma cortisol concentrations.

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Chapter 5

Urinary Corticoid/Creatinine Ratios in Healthy Ferrets and Ferrets with Hyperadrenocorticism

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Submitted

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Summary

Hyperadrenocorticism in ferrets is usually associated with unaltered plasma concentrations of cortisol and ACTH, although the urinary corticoid/creatinine ratio (UCCR) is usually elevated. In this study cortisol metabolism and urinary steroid profiles in healthy ferrets and in ferrets with hyperadrenocorticism were investigated, as well as possible seasonal fluctuations in the UCCR.

In healthy ferrets and in one ferret with hyperadrenocorticism, approximately 10% of plasma cortisol and its metabolites was excreted in the urine. High-performance liquid chromatography revealed one third of the urinary corticoids to be unconjugated cortisol; the other peaks mainly represented cortisol conjugates and metabolites.

In 21 healthy sexually intact ferrets, the UCCR started to increase by the end of March and declined to initial values halfway the breeding season (June). In healthy neutered ferrets there was no significant seasonal influence on the UCCR. In two neutered ferrets with hyperadrenocorticism the UCCR was raised, primarily during the breeding season.

In 27 of 31 privately owned ferrets with hyperadrenocorticism, the UCCR was higher than the upper limit of the reference range (2.1×10^{-6}). In 12 of 14 healthy neutered ferrets dexamethasone administration decreased the UCCR by more than 50%, whereas in only one of the 28 hyperadrenocorticoid ferrets did the UCCR decrease by more than 50%.

We conclude that the UCCR in ferrets primarily reflects cortisol excretion. In healthy sexually intact ferrets and in ferrets with hyperadrenocorticism the UCCR increases during the breeding season. The increased UCCR in hyperadrenocorticoid ferrets is resistant to suppression by dexamethasone, indicating ACTH-independent cortisol production.

Introduction

Hyperadrenocorticism leading to a glucocorticoid-induced catabolic state, characterized by muscle weakness, skin atrophy, and centripetal obesity, occurs in humans,[1] dogs,[5,16] cats,[13,15] and horses.[11] The glucocorticoid excess may be due to an adrenocortical tumor or over-stimulation of the adrenal cortex. In pet ferrets (*Mustela putorius furo)* the situation is different. In this species hyperadrenocorticism is characterized by signs of excessive production of steroids (androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate, and/or estradiol), leading to symmetrical alopecia, vulvar swelling in neutered female ferrets (jills), and recurrence of sexual behavior after neutering in male ferrets (hobs).[10,19,20,21,24,35] Plasma concentrations of cortisol are only increased in a minority of cases.[20]

In humans, dogs, cats, and horses, the assessment of basal adrenocortical function usually includes the measurement of urinary corticoids relative to the urinary creatinine concentration, [1,7,18,31] and is expressed as the urinary corticoid/creatinine ratio (UCCR). The main advantage of the UCCR over plasma cortisol concentrations is that urinary corticoid excretion reflects the free plasma cortisol concentration over a period of time, and is thus less influenced by the pulsatile secretion of this hormone than is the plasma cortisol level. A practical advantage of this measurement is that urine samples can be collected at home without the stress of a clinic visit and the blood sampling, thereby avoiding iatrogenic elevations.

In ferrets the UCCR has also been assessed for diagnosing hyperadrenocorticism, with a UCCR higher than 1.6×10^{-6} being regarded as diagnostic for hyperadrenocorticism.[8] However, other have questioned whether measurement of the UCCR is an appropriate way to diagnose hyperadrenocorticism in ferrets,[19] because hypercortisolism is not considered to play an important role in hyperadrenocorticism in ferrets.[20]

Recently, we reported that urinary corticoid levels are increased during the breeding season in ferrets with hyperadrenocorticsm.[25] This increase coincided with the reported increase in plasma luteinizing hormone (LH) concentrations.[9] It is not clear whether the increased UCCR associated with hyperadrenocorticism in ferrets is due to hypercortisolism or the result of cross-reaction of other urinary steroids in the cortisol assay.[25]

Here, we report on the results of studies of cortisol metabolism and urinary steroid profiles in healthy ferrets and ferrets with signs of hyperadrenocorticism. In addition, we present the results of UCCR measurements performed at 2-week intervals in intact and neutered ferrets for a period of 19 months.

Materials and Methods

HPLC and radioimmunoassay

Urine samples (10-15 ml) for high-performance liquid chromatography (HPLC) were extracted on a C18 SepPak cartridge (Millipore, Bedford, MA). Urine samples were passed

over the cartridge followed by a wash step with water. Steroids were eluted with 100% methanol, evaporated to dryness, and dissolved in mobile phase before chromatography.

Steroids were separated by HPLC (Pharmacia, Uppsala, Sweden) on a reversed phase C18 column (Merck, Darmstadt, Germany; particle size, 5 µm) using a mobile phase of HPLC-quality MeOH (Labscan Ltd, Dublin, Ireland) and double-distilled water (60:40 v/v) at a flow rate of 1 ml/min. Retention times of synthetic steroids (Sigma-Aldrich, St. Louis, USA) were determined using UV detection at 254 nm and were: aldosterone, 4.5 min; dehydrocorticosterone, 6 min; hydrocortisone (cortisol), 6.5 min; corticosterone, 9 min; 11-deoxycortisol, 9.5 min; androstenedione, 14 min; 17β-estradiol, 15.5 min; progesterone, 21.5 min and 17α-hydroxyprogesterone, 33 min. Fractions were collected every 30 sec for 45 min for the measurement of their immunoreactivity in the radioimmunoassay (RIA) for cortisol, as described previously.[18] The cortisol antiserum was raised in rabbits against a cortisol-21-hemisuccinate-bovine serum albumin conjugate. This antiserum is known to cross-react with other steroids such as 21-deoxycortisol (62%), corticosterone (11%), cortisone (2%), 11-deoxycortisol (1.3%), deoxycorticosterone (1.3%), and 17α-hydroxyprogesterone (0.1%).[30] The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) by calculation of its quotient (x 10^{-6}).[27]

Urinary excretion of corticoids

Animals

For this study, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, three male and three female 5-year-old ferrets were used. All ferrets had been neutered. One of the neutered hobs had hyperadrenocorticism diagnosed on the basis of increased plasma concentrations of androstenedione and 17α-hydroxyprogesterone, and ultrasonography of the adrenals. There was a (histologically confirmed) adenoma in the left adrenal gland.

All ferrets were individually housed in outdoor suspended cages with a night box. Water and ferret pellets (FerRet®, Hope Farms, Woerden, The Netherlands) were available ad libitum.

Experimental procedure

The ferrets were sedated with an intramuscular injection of medetomidine (100 µg/kg; Domitor®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands) for placement of a 24-gauge cannula with an injection port (Vasofix® Braunüle®, Braun, Melsungen, Germany) in the cephalic vein. Ferret plasma mixed with 5 µCi [1,2,6,7 ^{3}H]-cortisol (Amersham, Buckinghamshire, United Kingdom) was injected through the cannula which subsequently was flushed with 1 ml saline. Sedation was reversed with atipamazole (400 µg/kg; Antisedan®, Pfizer Animal Health BV, Capelle a/d IJssel, The Netherlands).

For the next 4 days urine was collected once daily by means of propylene litter boxes with macrolon plates placed underneath the cages. A 2-mm space between the plate and the wall of the litter box allowed urine to drain away from the feces.[14] Ten 1-ml aliquots of each urine sample were mixed with 4 ml scintillation liquid (Ultimagold; Packard Instrument company, Downers Grove, IL), and after thorough mixing radioactivity was

measured in a β-counter (Rackbeta liquid scintillation counter; LKB, Bromma, Sweden). The percentage of [^{3}H]cortisol excreted in the urine was calculated as [($\sum^3$H/10) (dpm/ml) x U volume (ml) / ^{3}H administered (dpm)] x 100.

Seasonal influence on urinary corticoid/creatinine ratios (UCCR)

The UCCRs of 21 healthy, intact ferrets (7 male, 14 female) and 9 gonadectomized ferrets (5 male, 4 female) were measured from February 2000 until September 2001. In February 2000 the ferrets were 2 to 3 years old. Eight ferrets had been gonadectomized at 6 weeks of age and one hob at 9 months of age. The ferrets were housed and fed as described above.

Although no signs of hyperadrenocorticism were present in any of the animals, two of the gonadectomized hobs appeared to have hyperadrenocorticism, based on increased plasma concentrations of androstenedione and 17α-hydroxyprogesterone and ultrasonographic findings. Later on, this was confirmed by histological examination of the adrenal glands. The UCCR of the 9 gonadectomized ferrets, recorded over a year, have been published previously.[25]

Overnight urine samples were collected in litter boxes (see above) which were underneath the cages from 17:00 – 8:00 hours. Urine was stored in tubes at 4ºC pending analysis, which was performed within 5 days of urine collection.

UCCRs with HDDST in healthy neutered ferrets

Between March 15 and September 29 2001, the owners of 17 (8 male, 9 female) neutered ferrets (median age 3.5 years, range 1.5 to 8 years) collected urine samples from their ferrets. These ferrets were housemates of ferrets that had been presented with signs of hyperadrenocorticism. On physical examination no abnormalities were found in these ferrets. In 12 of these ferrets plasma concentrations of androstenedione and 17α-hydroxyprogesterone were within the reference ranges.[25]

The owners were requested to collect the first morning urine produced on 3 consecutive days. The ferrets were allowed to urinate on a smooth floor from which the urine could easily be collected. After the second urine collection, the owners administered 0.1 mg dexamethasone orally, 3 times at 8-hour intervals (high-dose dexamethasone suppression test; HDDST). The next morning the third urine sample was collected. Urine samples were stored in the refrigerator and sent to the laboratory of the Department of Clinical Sciences of Companion Animals of Utrecht University for analysis within one week.

Two consecutive morning urine samples were also collected in seven 4-year-old neutered ferrets (2 male, 5 female) kept under laboratory conditions.

UCCRs with HDDST in privately owned ferrets with hyperadrenocorticism

From 1997 through 1999, hyperadrenocorticism was diagnosed in 31 neutered ferrets (25 male, 6 female; median age 5 years, range 2 to 8 years). The history included symmetrical alopecia (27 of 31 ferrets), an enlarged vulva (5 of 6 jills), and increased libido (6 of 25 hobs). An enlarged adrenal gland was palpated in 8 ferrets. In 22 of 28 ferrets in which ultrasonography was performed, the results were consistent with surgical findings.

Histological examination of the adrenal glands collected at surgery or at post mortem examination revealed hyperplasia (n=10), adenoma (n=16), and adenocarcinoma (n=5).

Urine samples were collected as described above for the healthy ferrets. A HDDST was performed in 28 of 31 ferrets.

Calculations and statistics

In the serial measurements of UCCRs, the values are expressed as mean ratios ± standard error of the mean (SEM). The paired Student's *t* test was used to compare the mean UCCRs of intact ferrets in April with those in December, and the serial UCCR measurements for the 2 ferrets with hyperadrenocorticism with those of the 7 healthy ferrets during both the breeding season (March until September) and the non-breeding season (September until March). Statistical significance was assumed at $P < 0.05$.

In the diagnostic assessment of the UCCR, the mean of the ratios of the samples collected on two consecutive days was used. The percentile method was used to establish the reference range for the UCCR in healthy ferrets.[4] The percentiles $P_{2.5}$ and $P_{97.5}$ were determined with a probability of 95%.

Results

Urinary excretion of corticoids

Within two days of intravenous administration of [1,2,6,7 ^{3}H]cortisol in the 5 healthy ferrets, 10.1% (range, 1.7% – 22.5%) of the administered cortisol was cleared in the urine. The urinary excretion of [^{3}H]cortisol in the ferret with hyperadrenocorticism was 10.4%. Urine samples collected on the two following days contained practically no radioactivity, indicating that all [^{3}H]cortisol had been excreted in two days.

The HPLC fraction eluting at the retention time of cortisol (t = 6.5 min) contained 33.2 ± 4.5% (mean ± SEM, n=10) of the total urinary corticoids (measured by RIA). The other two peaks with retention times of 3.5 and 5.5 min were probably conjugated or hydroxylated cortisol metabolites. No immunoreactivity eluted with retention times longer than 10 min, indicating that other steroids, such as androstenedione, 17β-estradiol, progesterone, or 17α-hydroxyprogesterone, did not interfere with the assessment of the UCCR.

Seasonal influence on urinary corticoid/creatinine ratios

Intact ferrets: - In both years of the study the UCCRs of the 21 intact healthy ferrets started to increase toward the end of March, reaching a peak in April. The ratios returned to initial values by the end of June (Figure 1). These UCCR peaks at the beginning of the breeding season were similar in both genders; at the end of April 2000 and 2001 the UCCR (± SEM) for 7 intact hobs was $5.9 \pm 0.7 \times 10^{-6}$ and $4.3 \pm 1.0 \times 10^{-6}$, respectively, and that for 14 intact jills was $6.0 \pm 0.7 \times 10^{-6}$ and $3.2 \pm 0.3 \times 10^{-6}$, respectively. At the end of April 2000 and 2001, the UCCR in all healthy ferrets ($6.0 \pm 0.5 \times 10^{-6}$ and $3.5 \pm 0.4 \times 10^{-6}$, respectively) was significantly higher than at the end of December 2000 ($1.4 \pm 0.1 \times 10^{-6}$).

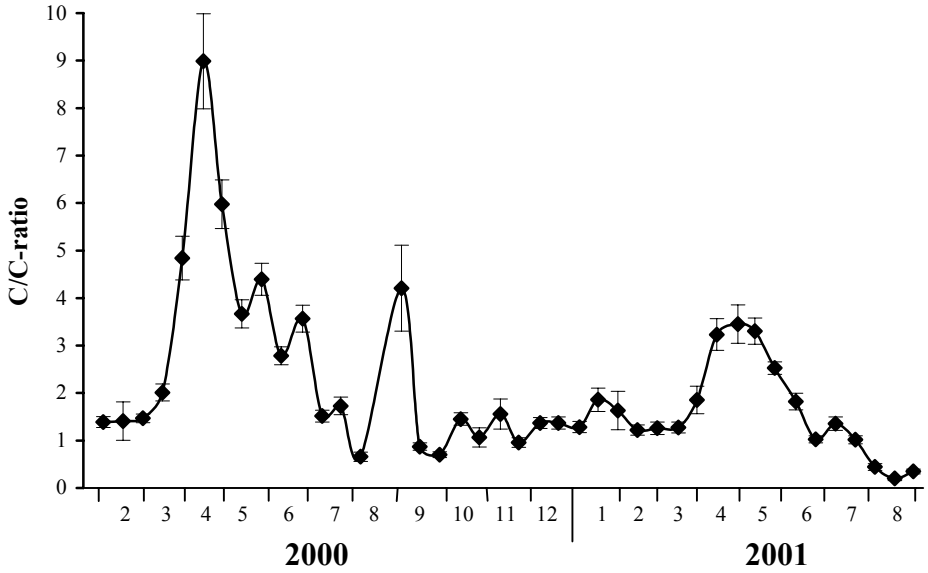

Figure 1. The mean (± SEM) urinary corticoid/creatinine ratio (x 10^{-6}) of 21 healthy intact ferrets from February 2000 until September 2001.

Neutered ferrets: - The UCCR was not affected by seasonal influences in healthy neutered ferrets. The mean value (1.3 ± 0.1 x 10^{-6}) was not significantly different from that of the intact ferrets in the non-breeding season (1.5 ± 0.2 x 10^{-6}). During the breeding seasons, the UCCR (± SEM) of the two hyperadrenocorticoid ferrets (3.5 ± 0.2 x 10^{-6} and 2.9 ± 0.2 x 10^{-6}) was significantly higher than that of the neutered control animals (1.0 ± 0.2 x 10^{-6}). Early in the non-breeding season, the UCCR of one of the hyperadrenocorticoid ferrets was still significantly higher (2.9 ± 0.3 x 10^{-6}) than that of the healthy control animals (1.6 ± 0.3 x 10^{-6}). In November and December, however, the UCCR of this ferret was no longer higher than that of the other animals (1.9 ± 0.4 x 10^{-6} versus 1.8 ± 0.2 x 10^{-6}) (Figure 2).

UCCRs and HDDST in healthy neutered ferrets

The mean UCCR of two consecutive morning urine samples in 24 healthy neutered ferrets ranged from 0.35 to 2.6 x 10^{-6} (median, 1.3 x 10^{-6}). The reference range (percentiles $P_{2.5}$ and $P_{97.5}$ with a probability of 95%) was 0.6 – 2.1 x 10^{-6}. Oral dexamethasone administration to 14 healthy neutered ferrets resulted in a more than 50% decrease in the UCCR in 12 ferrets, a 30% decrease in one ferret, and a 6-fold increase in another ferret (Figure 3).

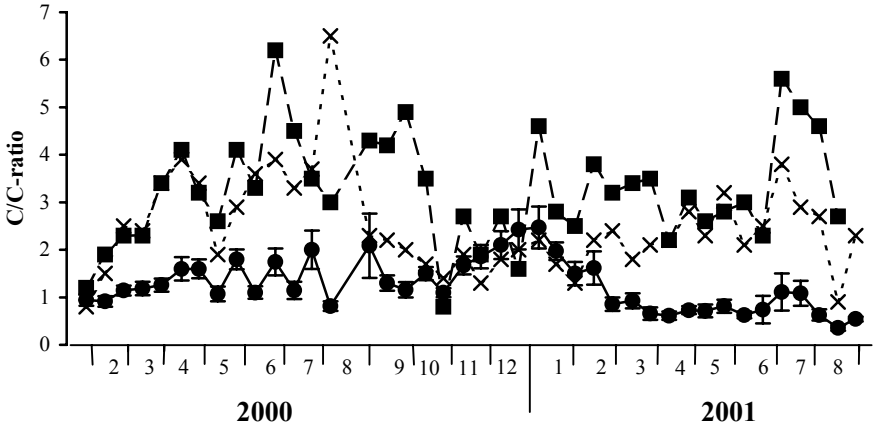

Figure 2. The mean (± SEM) urinary corticoid/creatinine ratio (UCCR) (x 10^{-6}) of 7 healthy neutered control ferrets (●), and the UCCR of 2 neutered ferrets (■, x) with hyperadrenocorticism from February 2000 until September 2001.
During both breeding seasons (March to August) the mean UCCR of both ferrets with hyperadrenocorticism was significantly higher than the mean UCCR of the 7 control ferrets ($P < 0.025$).

UCCRs and HDDST in privately owned ferrets with hyperadrenocorticism

The median UCCR in ferrets with hyperadrenocorticism was 6.8 x 10^{-6} (range, 1.5 – 96 x 10^{-6}). In 27 of 31 ferrets (87%) the UCCR was higher than the upper limit of the reference range (see above). Oral dexamethasone administration to 28 hyperadrenocorticoid ferrets resulted in an increase in the UCCR in 12 ferrets. In one ferret no change was seen while in 15 ferrets a decrease occurred. In only one of the latter ferrets was the decrease more than 50% (Figure 3).

Discussion

The present results indicate that in ferrets approximately 10% of cortisol and its metabolites is excreted in the urine, which is higher than the 2% found in cats,[7] but much lower than the 55% in dogs.[17] Apparently in ferrets, as in cats, the majority of cortisol is cleared by hepatobiliary excretion. Nevertheless, urinary cortisol concentrations can be measured by RIA.

Plasma cortisol concentrations in ferrets with hyperadrenocorticism are similar to those of healthy ferrets.[20] In contrast, UCCRs of hyperadrenocorticoid ferrets are significantly higher than those of healthy ferrets.[8] It has been hypothesized that urinary steroids or their metabolites, other than cortisol, cross-react in the cortisol assay.[25] For example, estrogens or androgens of adrenal origin and/or their precursors might cross-react in the urinary

cortisol assays. However, although the antibody used in the cortisol RIA cross-reacts with other steroids, no immunoreactivity was found in HPLC fractions corresponding with the elution of androstenedione, 17β-estradiol, progesterone, and 17α-hydroxyprogesterone. Thus the UCCR in ferrets mainly reflects urinary cortisol excretion. In our study, the upper limit of the reference range for the UCCR was 2.1 x 10^{-6}, which is slightly higher than the 1.6 x 10^{-6} reported previously.[8] Different methodology, e.g. the antibody used in the measurement of cortisol, might explain this difference.

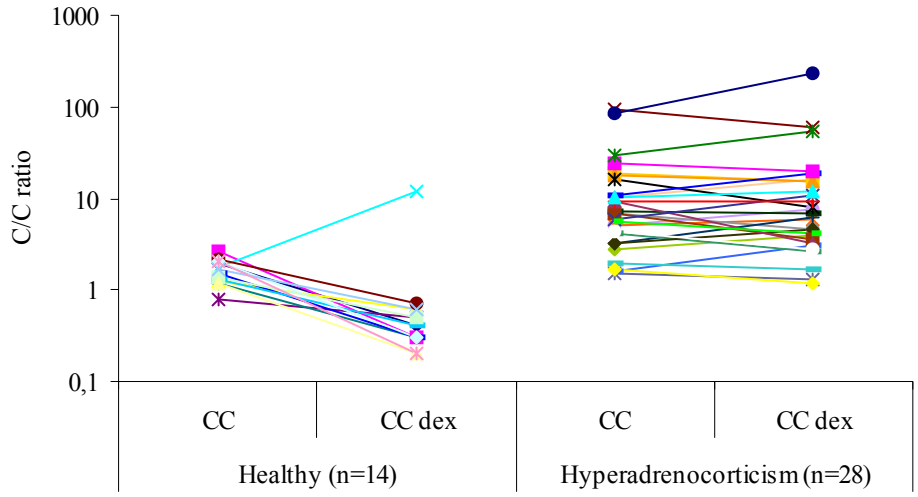

Figure 3. Logarithmic representation of the mean urinary corticoid/creatinine ratio (x 10^{-6}) measured in two consecutive morning urines (CC) from healthy ferrets and ferrets with hyperadrenocorticism. After the collection of these two urine samples the ferrets received three oral doses of dexamethasone (0.1 mg/kg) at 8-h intervals. The next morning a third urine sample was collected (CC dex). In ferrets with hyperadrenocorticism, the suppression of urinary corticoid excretion by dexamethasone was less than 50%, while in almost half of the cases a paradoxical rise occurred.

Interestingly, in healthy intact ferrets a distinct seasonal variation in the UCCR was observed. The ratio increased significantly in the middle of March, peaked at the beginning of April, and returned to basal values by the end of June. Similar variations in cortisol (metabolite) excretion have been found in non-human primates during periods of increased sexual activity.[12,29]

From a mechanistic point of view, there are several possible explanations for the increase in cortisol levels during the breeding season. First, there is the possibility that the elevated UCCR is due to activation of the pituitary-adrenocortical axis associated with the increased sexual activity. Our hobs and jills were housed in each other's vicinity without having the possibility to mate, and thus the hightend arousal may well have increased the UCCR. From studies of healthy pet dogs it is known that minor stresses, such as being briefly in a consultation room and hospitalization, can increase the UCCR.[32] In support of

this possibility is the fact that during the breeding season plasma adrenocorticotropic hormone (ACTH) concentrations are higher in intact jills than in neutered jills.[23] However, in intact hobs plasma ACTH concentrations are similar to those of neutered hobs.[23] This may be due to the effect of testosterone, which is known to inhibit the response of the hypothalamus-pituitary-adrenocortical axis to stress.[33] Since the peak UCCR at the end of April was similar in both genders, this rise cannot fully be ascribed to sexual arousal.

Another explanation for the elevated UCCR in the breeding season of ferrets concerns LH. The sexual maturation of ferrets, under the influence of stimulatory photoperiods, is associated with increased LH secretion.[22] Moreover, LH-receptors (LH-Rs) have been detected in the adrenal cortex of ferrets,[25] and *in vitro* stimulation tests have shown that LH stimulates adrenocortical cortisol secretion (Schoemaker *et al*., in preparation). One would expect, however, that the LH-R-mediated cortisol secretion would suppress endogenous plasma ACTH concentrations during the breeding season, which is not the case.[23] In addition, administration of a GnRH analogue to healthy (neutered) ferrets does not increase plasma concentrations of adrenocortical steroids.[25]

A third explanation for the increased cortisol excretion during the breeding season is that altered concentrations of sex steroids might influence of cortisol metabolism, for example, the capacity of cortisol-binding globulins (CBG) in plasma. In male sugar gliders, an inverse relationship has been found between testosterone concentrations and the CBG-binding capacity, resulting in increased plasma concentrations of free cortisol during the breeding season.[2] Changes in the concentration of carrier globulins may affect urinary cortisol excretion, as observed in a woman with clinical Cushing's disease and CBG deficiency. In this woman, total plasma cortisol concentrations were within the normal range, whereas plasma concentrations of free cortisol and the urinary corticoid excretion were elevated.[34]

In the healthy neutered ferrets there were no seasonal changes in the UCCR. The ratio remained constant at the same low level as in the intact ferrets during the non-breeding season. In contrast, two neutered hyperadrenocorticoid ferrets had an elevated UCCR during the breeding season. It is possible that under the influence of the castration-induced increased of gonadotropic hormones, the adrenocortical cells had become receptive to stimulation, e.g. by increased expression or a regain of functionality of LH-receptors.[26] As in sexually intact ferrets, plasma LH concentrations, which are influence by the photoperiods, [3] may have been low during the non-breeding season and high during the breeding season. The latter, in combination with adrenocortical responsiveness, may have led to an increase in the secretion of steroids. In turn, this may have led to an increase in the plasma free cortisol concentration and consequently to an increased UCCR. However, this explanation should be considered with some reservation. While LH-R-mediated cortisol production may contribute to the elevated UCCR in the breeding season, in the two hyperadrenocorticoid ferrets, hCG administration caused plasma concentrations of androstenedione and 17α-hydroxyprogesterone to rise without a concomitant increase in plasma cortisol concentrations.[25] However, sexual arousal and lowering of the CBG levels may have contributed to cortisol excretion, as described above for the sexually intact ferrets.

In 27 of the 31 privately owned ferrets with hyperadrenocorticism, the UCCR was higher than the upper limit of the reference range, confirming the previously reported increase in the UCCR in ferrets with hyperadrenocorticism.[8] In almost all of the healthy neutered ferrets, dexamethasone administration decreased the UCCR by more than 50%, whereas in the great majority of clinical cases there was resistance to suppression. In some cases, the UCCR even increased after dexamethasone administration. Such paradoxical increases, which have also been observed in humans [28,36] and dogs [6] with adrenocortical tumors, have been ascribed to a direct effect of dexamethasone on the adrenocortical tumor.[6,36]

Dexamethasone resistance is compatible with autonomy at adrenocortical level, although it may also be associated with some pituitary tumors. Autonomy at a pituitary level is very unlikely because hyperadrenocorticism is not associated with corticotropic adenomas in ferrets.[19, Schoemaker *et al*., submitted] Therefore ACTH-independent production of corticosteroids has to be assumed, for instance, LH-dependent production of adrenal steroids, including cortisol. The initially LH-dependent production of adrenal steroids may become LH-independent as the adrenal gland undergoes neoplastic transformation. Irrespective of which mechanism is operational, yet another mechanism has to be considered as explanation for the combination of an elevated UCCR, normocortisolemia, and unaltered plasma ACTH concentrations,[23] namely an increased production of steroids. These would affect the CBG concentration, leading to elevated free cortisol concentrations without an elevation of total cortisol levels. It is also possible that ACTH levels are not suppressed by the LH-dependent or autonomously hypersecreting adrenal lesions, because cortisol secretion was only marginally elevated.

In conclusion, our results indicate that the UCCR in ferrets primarily reflects cortisol excretion. In healthy ferrets and in ferrets with hyperadrenocorticism, the UCCR increases during the breeding season. The increased UCCR in hyperadrenocorticoid ferrets is resistant to suppression by dexamethasone, indicating ACTH-independent cortisol production.

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Chapter 6

Morphology of the Pituitary Gland in Ferrets (*Mustela putorius furo*) with Hyperadrenocorticism

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Summary

Pituitary tumors are the cause of hyperadrenocorticism in a variety of species, but the role of the pituitary gland in hyperadrenocorticism in ferrets is not known. In this species, the condition is mediated by the action of excess gonadotropins on the adrenal cortex and is characterized by an excessive secretion of sex steroids.

In this study, the pituitary gland of four healthy control ferrets, both intact and neutered, and ten neutered ferrets with hyperadrenocorticism was examined histologically following staining for adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), growth hormone (GH), thyroid-stimulating hormone (TSH), luteinizing hormone (LH), follicle-stimulating hormone (FSH) and prolactin. Immunohistochemistry revealed that somatotropes, thyrotropes and lactotropes are the most abundant cell types of the pars distalis of the pituitary gland in healthy ferrets. The distribution of corticotropes was similar to that in dogs and humans. In ferrets, similar to dogs, the melanotropic cell is almost the only cell type of the pars intermedia. Gonadotropes were found in the pars distalis of neutered individuals, but not intact, ferrets.

All the ferrets with hyperadrenocorticism had uni- or bilateral tumors of the adrenal gland. In addition, in the pituitary gland of two of these ferrets a tumor was detected. These tumors did not stain for any of the pituitary hormones, and had characteristics of clinically non-functional gonadotrope tumors seen in humans. In some of the other ferrets low pituitary immunoreactivity for gonadotropic hormones was detected, which might be due to the feedback of autonomous steroid secretion by the neoplastic transformation of the adrenal cortex.

It is concluded that the initially high concentrations of gonadotropins, as a result of castration, may initiate hyperactivity of the adrenal cortex. The low incidence of pituitary tumors and the low density of gonadotropin-positive cells in non-affected pituitary tissue in this study, suggest that persistent hyperadrenocorticism is not dependent on persistent gonadotropic stimulation.

Introduction

Hyperadrenocorticism occurs in several mammalian species, including humans,[25] dogs,[9,28] and cats.[18,26] In these species, the glucocorticoid excess leads to a catabolic state characterized by muscle weakness, skin atrophy, and centripetal obesity. The glucocorticoid excess may be due to an adrenocortical tumor or pituitary stimulation of the adrenal cortex. In humans, 60% to 80% of cases of hyperadrenocorticism are pituitary dependent,[5] while in dogs and cats, over 80% of the cases are secondary to a pituitary tumour.[9,24,28]

The situation is different in pet ferrets (*Mustela putorius furo)*. In these animals, the changes associated with hyperadrenocorticism are dominated by signs of excessive production of sex steroids, i.e. symmetrical alopecia, vulvar swelling in neutered jills, and recurrence of sexual behavior after neutering.[16,30,31,32,34,40] Plasma concentrations of androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate and/or oestradiol are increased, consistent with these signs.[31] In addition, elevated urinary corticoid/creatinine ratios have been reported in ferrets with hyperadrenocorticism.[10]

In most ferrets with hyperadrenocorticism (85%), only one adrenal gland is enlarged and there is no atrophy of the contralateral gland.[32,40] After unilateral adrenalectomy, the disease may recur in association with enlargement of the contralateral adrenal gland.[40] The histological changes of the adrenal glands range from (nodular) hyperplasia to adenoma and adenocarcinoma.[32]

The characteristics of hyperadrenocorticism in ferrets resemble those of some strains of mice in which nodular adrenocortical hyperplasia and adrenocortical tumors occur after neutering at an early age.[8,23,36] It has been suggested that castration also plays a role in the pathogenesis of hyperadrenocorticism in ferrets,[16,32] and recently the age at neutering was found to be significantly correlated with the age at onset of hyperadrenocorticism.[34] This suggestion, together with the observation that the signs of hyperadrenocorticism initially occur only during the breeding season,[30] that treatment with the gonadotropin-releasing hormone (GnRH)-agonist leuprolide acetate has beneficial effects,[39] and that functional luteinizing hormone (LH) receptors are present in the altered adrenal cortices of ferrets with hyperadrenocorticism [35] has strengthened the hypothesis that hyperadrenocorticism in ferrets is mediated by gonadotropic influence.

Given that the pituitary derived gonadotropic hormones play a role in the pathogenesis of hyperadrenocorticism in ferrets, it is of interest to know whether the disease in ferrets is associated with morphological changes in the pituitary. Previous reports indicate that there are no macroscopically visible changes in the pituitary glands of ferrets with hyperadrenocorticism,[30] or neoplastic diseases.[3,6,15] Here, we report on the pituitary histology of ten ferrets with hyperadrenocorticism and four control ferrets.

Materials and Methods

Healthy ferrets

Four healthy ferrets (two 1-year-old intact males, two 2-year-old neutered females) were euthanased during the breeding season, after which routine post-mortem examinations were performed within four hours of death. No histological abnormalities were found in the adrenal glands of these ferrets.

Table 1. Physical and histopathological data of ten neutered ferrets with hyperadrenocorticism

Ferret	sex	age (year)	alopecia	miscellaneous	surgery	histology		
						left adrenal	right adrenal	pituitary gland
1	m	6	yes	Increased libido	yes	hyperplasia	hyperplasia	NAF*
2	m	4	no			carcinoma	carcinoma	NAF
3	m	5	yes		yes	hyperplasia	carcinoma	adenoma
4	m	7	yes			carcinoma	carcinoma	NAF
5	m	5	yes			adenoma	hyperplasia	NAF
6	m	4	no		yes	carcinoma	carcinoma	NAF
7	f	5	yes		yes	adenoma	hyperplasia	NAF
8	m	5	no	enlarged prostate	yes	carcinoma	hyperplasia	NAF
9	m	6	yes		yes	hyperplasia	hyperplasia	NAF
10	m	5	no			hyperplasia	carcinoma	adenoma

* NAF = no abnormalities found

Clinical Cases

Ten (9 male, 1 female) neutered ferrets (age 4 – 7.5 years; median 5 years) were presented with signs of hyperadrenocorticism. At first presentation the following findings were observed. Six ferrets had alopecia. The female ferret had a slightly swollen vulva. One male ferret had an increased libido and another male had stranguria due to an enlarged prostate (Table 1). Mean urinary corticoid/creatinine (C/C) ratios (n=8) in two consecutive morning urines ranged from 3.2 to 86.5 x 10^{-6} (reference < 1.66 x 10^{-6})[10]. In two other ferrets only one urine sample was collected (Table 2); these C/C ratios also exceeded the reference range. Immediately after collection of the basal urine samples, a high-dose dexamethasone suppression test[29] was performed in six ferrets by oral administration of dexamethasone (0.1 mg/kg; 3 times at 8-hour intervals). The urinary C/C ratios after dexamethasone administration were compared to the mean urinary C/C ratios of the previous 2 days. The mean percentage change after dexamethasone administration in the six ferrets was 107.6% (range 11.5 – 276.3%).

Plasma concentrations of adrenocorticotropic hormone (ACTH), α-melanocyte-stimulating hormone (α-MSH), androstenedione and 17α-hydroxyprogesterone are

summarized in Table 2. Concentrations of androstenedione and 17α-hydroxyprogesterone exceeded the reference range in all cases (n=7) in which these hormones were measured.

Table 2. Laboratory data of ten neutered ferrets with hyperadrenocorticism

	Urine (C/C x 10^{-6})			Plasma			
Ferret	day 1*	day 2	day 3*	ACTH (pmol/l)	α-MSH (pmol/l)	a'dione* (nmol/l)	17-OH-prog* (nmol/l)
1	5.2	3.5	0.5	18.5	16.2		
2		4.6					
3		5.5				0.6	0.9
4	3.6	4.6	2.6	4.8	15.6	0.4	13.3
5	3.0	3.6		26.6	20.4	15.7	3.7
6	7.7	6.1	3.5	1.8	45.6	0.3	1.1
7	23	150	239	0.2	43.8	2.3	3.5
8	3.8	2.5	0.9	11.4	42.6	1.5	1.5
9	4.6	4.7	10	1.8	18.6		
10	2.9	3.8				4.2	6.8
reference	< 1.66 [10]	< 1.66		2.9 – 21.6 [33]	9.6 – 44.4 [33]	0.1 – 0.4 [35]	0.3 – 0.7 [35]

* C/C = corticoid/creatinine ratio; C/C day 3 = corticoid creatinine ratio after dexamethasone suppression; a'dione = androstenedione; 17-OH-prog = 17α-hydroxyprogesterone

Within four weeks after first presentation six ferrets underwent a laparotomy. Of these ferrets, one had left, three had right, and two had bilateral adrenal involvement. In three ferrets the adrenal glands were left in place because of either the extent of attachment of the gland to the wall of the vena cava or the presence of bilateral tumors. One ferret died immediately after surgery, and in two ferrets signs recurred after left adrenalectomy.

Post-mortem examinations were performed within 4 hours of death. Histology revealed adenoma (n=2) and adenocarcinoma (n=1) in the surgically removed adrenal glands, and hyperplasia (n=2) and adenoma (n=1) in the contralateral adrenal glands. The other seven ferrets all had bilateral adrenocortical involvement at post-mortem examination (Table 1).

Pituitary gland collection and tissue preparation

The entire head of one of the healthy female ferrets was fixed in 4% phosphate-buffered formalin. After 24 hours of fixation the skull was placed in a Na-EDTA solution (10%, pH = 7) for 2 weeks, to soften the bone before sectioning in a midsaggital plane. In this manner slides were obtained of the pituitary gland within the *fossa hypophysis* of the *os basisphenoidal*.

In the other three healthy ferrets and seven ferrets with hyperadrenocorticism the sphenoid bone (*os basisphenoidale*) was cut around the pituitary gland. The brain including the pituitary gland and sphenoid bone was removed, and the pituitary gland was carefully dissected from under the dorsum sellae and its posterior clinoid processes. Sections were cut in a midsaggital plane. Consecutive sections were cut of the pituitary gland of one of the healthy ferrets to investigate the distribution of corticotropes throughout the pituitary

gland. In the three remaining ferrets with hyperadrenocorticism the skull was opened dorsally and the brain was lifted so that the pituitary gland came out of the pituitary fossa. The pituitary glands in these ferrets were cut in a horizontal plane.

All tissues were fixed in 4% phosphate-buffered formalin for at least 24 hours and then embedded in paraffin wax. Sections (4 µm) were cut and stained with hematoxylin and eosin (HE). Multiple consecutive sections were stained with periodic acid-Schiff (PAS) or were used for immunohistochemistry of pituitary hormones.

Immunohistochemistry

Immunohistochemical staining of pituitary sections was performed with a monoclonal mouse antibody to synthetic $ACTH_{1-24}$,[17] a polyclonal rabbit antibody to synthetic α-MSH [PU060-UP, Biogenex Laboratories, San Remo, Ca, USA], a polyclonal rabbit antibody to porcine thyroid-stimulating hormone (TSH) [Biogenesis, Poole, England], a polyclonal rabbit antibody to porcine prolactin (PRL),[4] a polyclonal rabbit antibody to porcine growth hormone (GH),[37] a polyclonal rabbit antibody to human LH [DAKO, N 1543, ITK Diagnostics, Uithoorn, The Netherlands], and a polyclonal rabbit antibody to human follicle-stimulating hormone (FSH) [DAKO, N 1539, ITK Diagnostics, Uithoorn, The Netherlands]. Because of strong cross-reaction between the $ACTH_{1-24}$ and α-MSH antibodies, two additional ACTH antibodies were used to stain the pituitary glands of two ferrets (a polyclonal rabbit antibody to human $ACTH_{18-39}$ (hACTH) [Sigma-Aldrich Chemie bv., Zwijndrecht, The Netherlands] and a polyclonal rabbit antibody to synthetic carp $ACTH_{10-23}$ (cACTH) [KLH-cys-GKPVGRKRRPIKVY, Eurogentec, Seraing, Belgium]. One of these two ferrets, a 5-year-old neutered male, was euthanased during the breeding season because of an insulinoma. The adrenal glands of this ferret did not show any abnormalities on histological examination. The pituitary gland of the other ferret was from one of the clinical cases (ferret 10).

Specificity of the antibodies

The specificity of the $ACTH_{1-24}$, PRL, GH, and TSH antibody reactions was previously determined by staining of Western blots containing canine pituitary proteins.[17] The $ACTH_{1-24}$ antibody cross-reacted with α-MSH.[17] The GH antibody was highly specific, whereas the prolactin antibody showed considerable cross-reaction with other proteins. The TSH antibody reacted specifically with protein fragments with molecular weights of 23 and 27 kDa. The 27-kDa fragment corresponds with the molecular weight of TSH.[17] According to the manufacturer's information, the LH and FSH antibodies show 10% cross-reaction with each other.

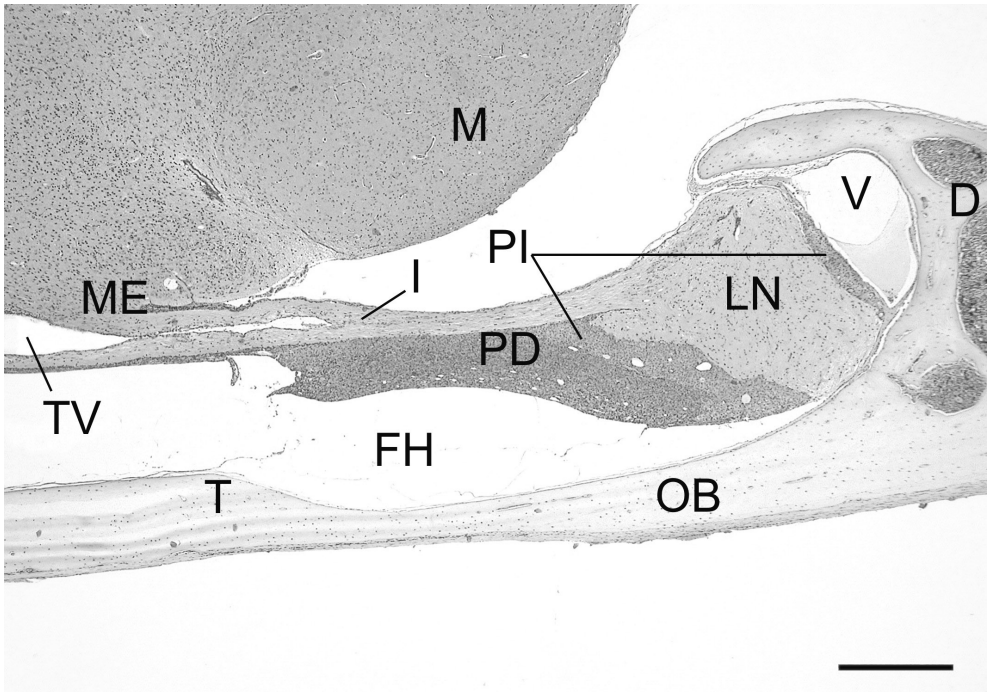

Figure 1. Midsagittal section of the pituitary gland of a 2-year-old neutered female ferret within the *fossa hypophysis* (FH) of the *os basisphenoidale* (OB). As an artifact the *pars distalis adenohypophysis* (PD) is pressed against the *pars intermedia adenohypophysis* (PI). Due to the artifact the *cavum hypophysis* (CH) is obscured (see figure 2). (D: dorsum sellae; I: *infundibulum neurohypophysis*; LN: *lobus nervosus neurohypophysis*; M: mammillary body; ME: median eminence; T: tuberculum sellae; TV: third ventricle; V: venous sinus connecting cavernous sinuses). Hematoxylin and Eosin. Bar is 0.5 mm.

Results

Healthy ferrets

By observing a saggital section of the entire hypothalamo-pituitary system of a ferret it is clear that the *dorsum sellae* extends dorsally over the *lobus nervosus neurohypophysis* of the pituitary gland (Fig 1), which makes it difficult to lift the pituitary gland out of its fossa without rupturing the pituitary stalk. There is almost no *pars tuberalis adenohypophysis* in ferrets. At most there are only a few cell layers. This thin layer did not show immunoreactivity against any of the pituitary hormones. When the pituitary gland was taken from the *fossa hypophysis*, the *infundibulum neurohypophysis* retracted, giving it a curved appearance (Fig 2).

Most of the $ACTH_{1-24}$ / α-MSH positive cells of the *pars distalis adenohypophysis* were in the anterior and mid-ventral portion of the pituitary gland. The number of $ACTH_{1-24}$

positive cells diminished with lateralization of consecutive sections. All of the cells in the *pars intermedia adenohypophysis* stained positive with these two antibodies (**Color section**; Fig 1). Cells in the *pars intermedia adenohypophysis* did not react with the hACTH antibody, whereas cells in the *pars distalis adenohypophysis* reacted with the hACTH (**Color section**; Fig 2) and cACTH antibodies (not shown). Only at high magnification a few cells staining with the cACTH antibody were seen in the *pars intermedia adenohypophysis* (not shown).

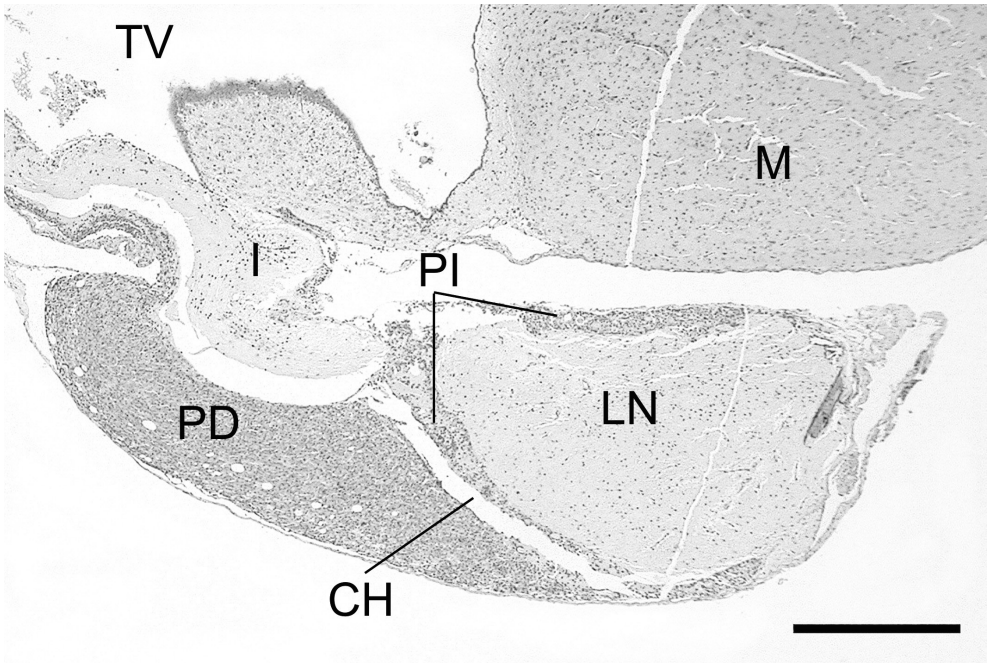

Figure 2. Midsagittal section of the pituitary gland of a 2-year-old neutered female ferret after removal from the pituitary fossa, illustrating retraction of the *infundibulum neurohypophysis*. For explanation of lettering see legend to Figure 1. Hematoxylin and Eosin. Bar is 0.5 mm.

By estimation, 30% to 40% of cells stained for PRL, another 30% to 40% of cells stained for TSH, and another 30% to 40% of cells stained for GH. The cells were diffusely scattered throughout the *pars distalis adenohypophysis* (**Color section**; Fig 1 and 3). At high magnification of the *pars intermedia adenohypophysis* a few cells were observed which stained positive for PRL and GH (not shown).

The number of cells staining positive for LH and FSH varied greatly. In the two healthy neutered ferrets approximately 15% to 20% of all cells were immunopositive (**Color section**; Fig 1), whereas in the other two (intact, 1-year-old, male) ferrets only a few cells were positive. In the *pars intermedia adenohypophysis* of the neutered jills, a small

number of cells stained positive for LH and FSH at the junction of the pituitary stalk and the *lobus nervosus neurohypophysis* (**Color section**; Fig 4).

Clinical cases

Two pituitary glands had evidence of neoplasia and 8 had no abnormalities. The pituitary neoplasias in the two 5-year-old male neutered ferrets (No 3 and 10) were round chromophobic, PAS-negative adenohypophyseal adenomas. These neoplasias were not surrounded by a capsule and contained mainly poorly differentiated cells with a low cytoplasm-to-nucleus ratio. Some normal pituitary cells were seen between the adenomatous cells. There was no necrosis and few mitotic figures were seen.

On immunohistochemical examination of the *pars distalis adenohypophysis*, in 5 of the 8 non-tumorous pituitaries only a few cells were positive for $ACTH_{1-24}$ (number 1, 6, 7, 8 and 9), while in the other three the number of cells positive for $ACTH_{1-24}$ was similar to that in the healthy ferrets. In three ferrets the number of cells positive for LH and FSH were similar to those in the neutered control ferrets. In five ferrets (numbers 1, 3, 4, 6 and 7) only a few cells were positive for these hormones. The distribution of the cells staining positive for other hormones did not differ from that in the healthy ferrets.

In the adenomas immunohistochemical staining for $ACTH_{1-24}$, α-MSH, TSH, GH, PRL, LH, and FSH was negative. Positive staining of some normal cells associated with the adenomas was observed. The cells of the non-affected part of the adenohypophysis stained positive for ACTH, α-MSH, TSH, GH, and PRL in ferret 3 (**Color section**; Fig 5) and positive for TSH, GH, LH, FSH, and PRL in ferret 10. In ferret 3 only a few LH- and FSH-positive cells were found while in ferret 10 very few ACTH- and α-MSH-positive cells were found in the surrounding tissue.

Discussion

As in other species, the cells of the ferret adenohypophysis are classified according to their specific secretory products: somatotropes (secreting GH), lactotropes (secreting PRL), thyrotropes (secreting TSH), gonadotropes (secreting LH and FSH), corticotropes (secreting ACTH) and melanotropes (secreting α-MSH).

Immunopositivity for ACTH was predominantly located in the anterior and ventral region of the *pars distalis adenohypophysis*, as has been described in humans and dogs.[7,17,38] Previous studies of ferrets did not describe the distribution of the cells in the pituitary gland.[20,21] The intense staining of the *pars intermedia adenohypophysis* for ACTH, as found with the antibody to $ACTH_{1-24}$, must be attributed to cross-reaction with α-MSH, as has previously been reported for dogs with the same antibody.[17] With the $cACTH_{10-23}$ antibody a few immuno-positive cells in the *pars intermedia adenohypophysis* were detected, but with the $hACTH_{18-39}$ antibody positive cells were not found in the *pars intermedia adenohypophysis*. Thus in ferrets, as in dogs,[11,27] the most abundant cell type of the *pars intermedia adenohypophysis* is the melanotrope. The staining with the $cACTH_{10-23}$ antibody and the observations of Mohanty *et al.* indicate that some corticotropic cells exist in the *pars intermedia adenohypophysis*.[20,21] Although Mohanty and co-workers attributed

this to cross-reaction with α-MSH, this is not possible with the $cACTH_{10-23}$ antibody, since these two hormone fragments do not have similar epitopes.

Mohanty *et al.* reported on the occurrence of corticotropes and gonadotropes in the *pars tuberalis adenohypophysis*.[20,21] However, comparison of their pictures with our sections of the pituitary gland within the sella turcica suggests that the area they described as *pars tuberalis adenohypophysis* may either be a transitional zone or the *pars intermedia adenohypophysis*. In this region, we not only found gonadotropes but also thyrotropes and somatotropes. By contrast, Mohanty *et al.* found somatotropes to be confined to the *pars distalis adenohypophysis*.[21]

In the present study, gonadotropes were well represented in the *pars distalis adenohypophysis* in the neutered ferrets whereas only a few gonadotropes were detected in the pituitary glands of the intact male ferrets. This is in agreement with observations in rats, in which castration leads to a 2-fold increase in the number of gonadotropes within 6 months due to loss of feedback by sex steroids.[12] Although Mohanty *et al.* detected gonadotropes in the *pars distalis adenohypophysis* of male ferrets, they did not specify whether the animals were castrated.[21]

In four of the neutered ferrets with hyperadrenocorticism the number of gonadotropes was similar to that found in the neutered healthy ferrets. In the other 6 ferrets only a few gonadotropes were found. A possible explanation for the low number of gonadotropes in some of these ferrets may be that hormones by adrenal tumors, such as oestradiol,[31] exert a negative feedback on the gonadotropes.

In the pituitary glands of 5 of 8 ferrets with hyperadrenocorticism, only a few corticotropes were found. In healthy humans corticotropes are mainly distributed in the anteriomedian part of the anterior lobe.[38] In the pituitary gland of a healthy neutered ferret, we examined multiple consecutive sections and found that the number of corticotropes diminished with lateralization of the sections. Thus sections with only low numbers of corticotropes may not have been taken from the median part of the pituitary gland. Another explanation, however, is that the adrenocortical neoplasia in some ferrets may have produced an excess of cortisol, which suppressed the ACTH synthesis in corticotrophs.[19] Although Rosenthal *et al.* have reported that hypercortisolism is unusual in hyperadrenocorticoid ferrets,[31,32] they also found elevated plasma cortisol concentrations in some cases.[31] The fact that hyperadrenocorticism in ferrets seems to be independent of ACTH and α-MSH [33] makes the first explanation the more plausible.

In the present study, blood samples for hormone measurements were collected when the animals were first presented. Since the time between first presentation and post-mortem examination ranged from several weeks to over 3 years, it is not possible to correlate the *in vivo* data with the number of corticotropes or gonadotropes found at post-mortem examination. Disease progression may have led to changes in the secretory pattern of adrenocortical hormones, which would affect the activity and distribution of pituitary gonadotropes and corticotropes.

In dogs, somatotropes account for 50% or more of the adenohypophyseal cells, with the other cell types each representing 5% to 15% of the cells.[27] The results of the present study confirm an earlier report that somatotropes are an abundant cell type in the ferret adenohypophysis.[21]

Up to 40% of the adenohypophyseal cells of healthy ferrets stained positively for prolactin. In cows, multihormonal cells have been found containing both GH and PRL,[2] but this has not been found in ferrets.[21] The cross-reaction of the PRL antibody with small protein fragments [17] might explain the high number of positive cells found in the present study. Cells staining positive for TSH also comprised up to 40% of the adenohypophyseal cells of ferrets. This percentage may also be an overestimation since the TSH antibody stained proteins of different molecular weights (23, 27 and 28 kDa).[17]

Among the ten ferrets with hyperadrenocorticism, there were two with a pituitary tumor, which are the first ever described in ferrets. This is in contrast to the situation in humans, dogs and cats, in which there is general agreement that pituitary-dependent hyperadrenocorticism is secondary to a tumor originating from corticotropes or melanotrophs.[5,9,24,28] The present pituitary tumors were immunonegative for all hormones tested.

The classification of pituitary tumors in man is based on morphology, immunohistochemistry, and ultrastructure.[14] Generally, tumors can be stained with an antibody raised against the hormone produced, i.e., the hormone of the cell of origin, the exception being the gonadotrope adenomas, which stain poorly or not at all for their respective hormones. These adenomas are usually chromophobic, have poorly developed cytoplasm, and are PAS negative.[13] The pituitary tumors described in this study meet this description and can be categorized as gonadotrope adenomas. In vitro studies have revealed that gonadotrope adenomas may secrete gonadotropic hormones or their alpha-subunits.[1] In the ferrets of this study, tumor development may have been initiated by the lack of negative gonadal feedback on hypothalamic GnRH as a result of neutering. This is in line with the hypothesis that persistent stimulation of the adrenal cortices by gonadotropic hormones plays a role in the pathogenesis of hyperadrenocorticism in ferrets.[34] Persistent stimulation may lead to adrenocortical hyperplasia and finally to autonomous hypersecretion due to tumor formation. The negative feedback action of adrenal androgens, produced by an autonomously hyperfunctioning adrenal tumor, might explain why at the time of necropsy very few LH- and FSH-positive cells were found in the non-tumorous part of the adenohypophysis in one of these ferrets.

Pituitary adenomas have been found at autopsy in approximately 11% (1.5 to 26.7%) of human beings not suspected of having pituitary disease while alive.[22] In the cases in which immunohistochemistry was performed for prolactin, approximately 40% of these tumors stained positively. No information was given on the immunohistochemistry of other hormones. The large number of pituitary tumors found in seemingly healthy humans suggests an alternative explanation for our findings, namely, that the pituitary adenomas found in the two ferrets reported here were not related to the adrenal disease and was an incidental finding.

In conclusion, our observations suggest that hyperadrenocorticism in ferrets is unlikely to be the result of persistent stimulation of the adrenal cortex as a result of a pituitary lesion. Instead, the decreased numbers of corticotropes and gonadotropes in the adenohypophysis seem to be a consequence, rather than a cause, of the adrenocortical hyperplasia and tumors. The non-staining pituitary adenomas found in two of the ten ferrets

with hyperadrenocorticism may be related to the disease, but may also be incidental findings unrelated to hyperadrenocorticism.

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Chapter 7

Correlation between age at neutering and age at onset of hyperadrenocorticism in ferrets

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Summary

Objective – To determine prevalence of hyperadrenocorticism in ferrets in The Netherlands and evaluate age, sex, and age at neutering in affected ferrets.

Design – Prevalence survey and retrospective study.

Animals – 50 ferrets with hyperadrenocorticism and 1,267 ferrets without hyperadrenocorticism.

Procedure – A questionnaire was sent to 1,400 members of a ferret-owners organization in The Netherlands; 492 (35%) owners returned the questionnaire, providing usable data on 1,274 ferrets. Seven of these ferrets developed hyperadrenocorticism during the survey period; medical records for these ferrets and 43 ferrets with confirmed hyperadrenocorticism were reviewed. Hyperadrenocorticism was confirmed by histologic examination of an excised adrenal gland (92% of ferrets) or clinical improvement after excision.

Results – Prevalence of hyperadrenocorticism in the survey population was 0.55%. Sex was not associated with prevalence of disease. Median time interval between neutering and diagnosis of hyperadrenocorticism was 3.5 years. A significant linear correlation between age at neutering and age at time of diagnosis was detected.

Conclusions and Clinical Relevance – Age at neutering may be associated with age at development of hyperadrenocorticism in ferrets.

Introduction

Hyperadrenocorticism is a common disease in pet ferrets.[3,6,7,8,10] A review of this disease has been published;[6] to our knowledge, reports have been based on observations made in the United States, where it is common practice to neuter ferrets at 6 weeks of age.[8] Because hyperadrenocorticism is most often detected in neutered ferrets, it has been suggested that early neutering may contribute to the high prevalence of hyperadrenocorticism in ferrets.[3,8] This theory is based on results of studies in certain strains of mice that develop nodular adrenocortical hyperplasia or adrenocortical tumors in 1 or both adrenal glands after neutering at an early age.[2,4,9]

Because many ferrets in the United States are neutered at an early age[8] and the population of sexually intact ferrets is relatively small, it is difficult to draw conclusions regarding a relation between hyperadrenocorticism and neutering at an early age. The objective of the study reported here was to determine the prevalence of hyperadrenocorticism in the Dutch ferret population, in which neutering at early age is not a common practice, and to determine influence of age, sex, and age at neutering for affected ferrets by review of medical records.

Materials and Methods

Owner survey

A short questionnaire was sent to 1,400 members of a Dutch ferret foundation (Stichting "De Fret") during April, May, and June 1997; the questionnaire solicited information on ferrets regarding age, sex, age at time of neutering, and whether members had ever owned a ferret with hyperadrenocorticism or presently owned a ferret with typical signs of hyperadrenocorticism, such as symmetric alopecia, swollen vulva (spayed females only), pruritus, and return of sexual behavior.

Retrospective study

Medical records of 50 ferrets with hyperadrenocorticism diagnosed at the Utrecht University Clinic for Companion Animals and several private veterinary practices in the Netherlands between January 1993 and September 1997 were reviewed; 7 of these ferrets were identified by results of the survey. Data regarding gross and microscopic pathologic findings, sex, age at neutering, age at time of diagnosis, and interval between neutering and time of diagnosis were recorded.

Statistical analyses

The Pearson coefficient of linear correlation was calculated to determine the relationship between age at neutering and age at diagnosis. Differences between groups were tested by use of χ^2 analysis or Student's *t* test. Prevalence of hyperadrenocorticism (percentage, confidence interval [CI]) in the survey population was calculated.[11] Differences were considered significant at $P < 0.05$.

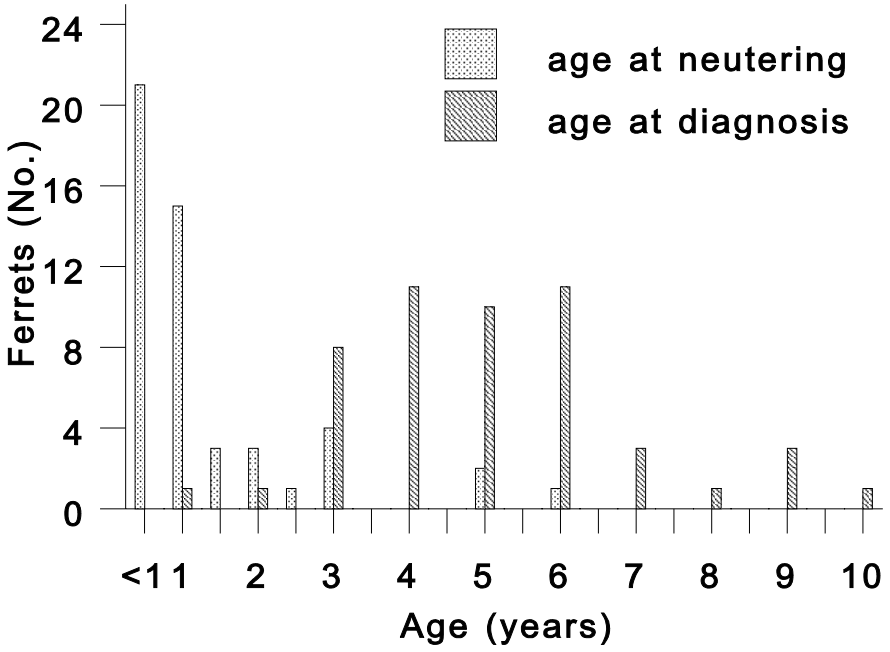

Figure 1. Age at neutering and age at diagnosis of hyperadrenocorticism in 50 ferrets.

Results

Owner survey

Of the 1,400 questionnaires sent, 492 (35%) were returned and provided data on 1,405 ferrets (782 male, 623 female) that had never had a diagnosis of hyperadrenocorticism. Data on age or age at neutering were lacking for 131 ferrets (88 male, 43 female); thus, data on 1,274 ferrets (694 male, 580 female) were used for analysis. Median age of this population was 3 years (mean ± SD, 3.2 ± 1.7 years; range, 0.5 to 10 years). Median age of neutered ferrets was 3 years (mean, 3.4 ± 1.7 years; range, 0.5 to 10 years). Most ferrets (91% of males, 82% of females) had been neutered between 0.5 and 1.5 years old; median age at time of neutering was 1 year (mean, 0.98 ± 0.65 years; range, 0.5 to 6 years). In 108 ferrets, neutering had been performed after 1.5 years of age. Eighty-seven ferrets (25 male, 62 female) had not been neutered. Of these, 27 ferrets (11 male, 16 female) were > 1.5 years old. Median age of sexually intact ferrets was 1 year (mean, 1.6 ± 1.3 years; range, 0.5 to 6 years). Within the total survey population of 1,274 ferrets, 7 cases of hyperadrenocorticism were confirmed during the survey period by histologic examination of adrenal glands after adrenalectomy. Seven suspected cases of hyperadrenocorticism were also identified, but the owners did not allow further studies. Thus, prevalence of confirmed

cases of hyperadrenocorticism in the survey population was 0.55% (95% confidence interval, 0.2 to 1.1%).

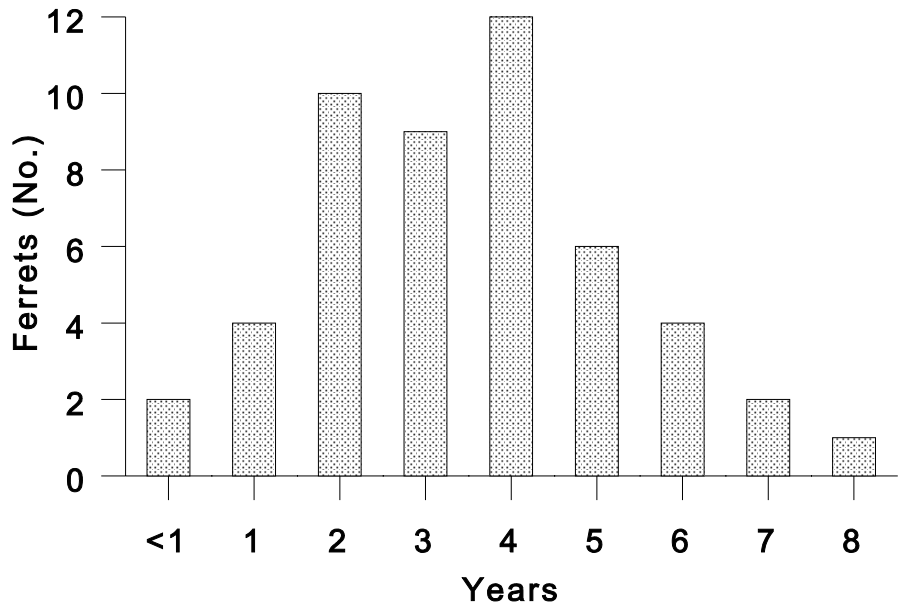

Figure 2. Interval between age at neutering and age at diagnosis of hyperadrenocorticism in 50 ferrets.

Retrospective study

For 46 of 50 ferrets with hyperadrenocorticism, the diagnosis was confirmed by histologic examination of surgical specimens obtained during unilateral adrenalectomy. Histologic diagnoses included intra- and extra-capsular hyperplasia, adenoma, and adenocarcinoma; distinctions between these differential diagnoses were difficult to determine, because adrenal tumors in ferrets seldom metastasize and microscopic characteristics overlap. For 4 ferrets, diagnosis was made on the basis of macroscopic appearance of adrenal glands and clinical improvement after adrenalectomy. All 50 ferrets (33 male, 17 female) with hyperadrenocorticism had been neutered. A significant difference in frequency distribution of sex between ferrets with hyperadrenocorticism and ferrets without hyperadrenocorticism was not detected. Median age at time of neutering was 1 year (mean, 1.4 ± 1.2 years; range, 0.5 to 6 years; Fig 1). Median age at diagnosis of hyperadrenocorticism was 5 years (mean, 5.1 ± 1.9 years; range, 1 to 10 years; Fig 1). Median interval between neutering and diagnosis of hyperadrenocorticism was 3.5 years (mean, 3.5 ± 1.8 years; range, 0.5 to 8 years; Fig 2). A significant linear correlation was found between age at neutering and age at diagnosis (Fig 3). Data on plasma estradiol and androgen concentrations were not available.

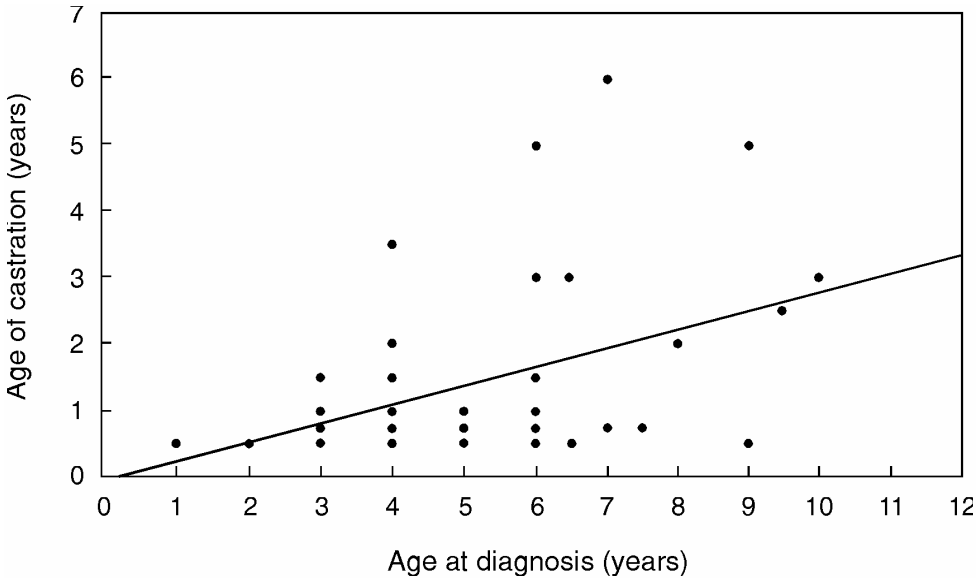

Figure 3. Scatterplot of age at neutering and age at diagnosis of hyperadrenocorticism in 50 ferrets (y= – 0.064 + 0.285x; r = 0.437; $P < 0.01$)

Discussion

To our knowledge, this is the first report of the prevalence of hyperadrenocorticism in ferrets. Weiss and Scott [10] reported that 20 to 25% of ferrets examined in their practice had hyperadrenocorticism, but this may not be a reliable indication of prevalence because it was based on a selected population. In our study of a Dutch ferret population, prevalence was estimated to be 0.55% (95% CI, 0.2 to 1.1%); 7 other ferrets were reported with typical clinical signs, but owners refused further diagnostic efforts. The fact that ferrets with hyperadrenocorticism seldom have increased plasma cortisol concentrations [7] prevents useful comparisons between hyperadrenocorticism in ferrets and hyperadrenocorticism in dogs, cats, or humans. Nevertheless, a prevalence of 0.55% must be considered high.

A significant correlation was found between age at neutering and age at diagnosis, suggesting that age at neutering may influence age of onset of this condition; however, the disease has also been reported in 7 sexually intact ferrets.[7,8,10] Many more cases in sexually intact ferrets may have gone unnoticed, because sexual behavior – a common clinical sign of the disease – may not be regarded as abnormal in a sexually intact ferret, whereas it is considered abnormal in a neutered ferret and may cause the owner to seek veterinary assistance.

Results reported from some institutions indicate almost twice as many female as male ferrets with adrenal tumors, but selection bias, rather than actual difference in prevalence,

was given as a possible explanation.[6] In the population described by Weiss and Scott,[10] the distribution between sexes was equal. Although two-thirds of the ferrets with hyperadrenocorticism in the study reported here were male, differences in the proportions of males and females among ferrets with and without hyperadrenocorticism were not significant; sex did not affect prevalence of the disease.

In our study, mean age of ferrets with hyperadrenocorticism was 5.1 ± 1.9 years; significantly older than affected ferrets (mean age, 3.4 ± 1.4 years; n = 50) reported by Rosenthal *et al*.[8] In the Netherlands, ferrets are neutered at 0.98 ± 0.65 years of age; American ferrets are commonly neutered at 4 to 6 weeks of age.[8] The interval between age at neutering and age at diagnosis in the Dutch ferret population (3.5 ± 1.8 years) was, however, similar to that in the population studied by Rosenthal *et al*. (3.3 ± 1.4 years),[8] assuming that those ferrets were neutered at 6 weeks of age. Although a significant correlation between age at neutering and age at development of hyperadrenocorticism is apparent, an explanation for this relationship has yet to be found. It has been speculated that, after neutering, the adrenal cortices are persistently stimulated by luteinizing hormone (LH) and follicle-stimulating hormone (FSH) as a result of the loss of negative gonadal feedback on hypothalamic gonadotropin releasing hormone (GnRH), and this results in adrenocortical hyperplasia or tumorigenesis [3,8]; Donovan and ter Haar [1] detected increased plasma concentrations of LH and FSH in spayed female ferrets. Luteinizing hormone receptors have been found in the adrenal glands of humans,[5] suggesting that increased LH concentrations in plasma could be a triggering factor for development of adrenal tumors. The successful use of the GnRH agonist leuprolide for medical control of hyperadrenocorticism strengthens the hypothesis that neutering plays a role in the development of hyperadrenocorticism in ferrets (Johnson-Delaney CA, personal communication, 1999).

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Chapter 8

The Role of Luteinizing Hormone in the Pathogenesis of Hyperadrenocorticism in Neutered Ferrets

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Summary

Four studies were performed to test the hypothesis that gonadotropic hormones, and particularly luteinizing hormone (LH), play a role in the pathogenesis of hyperadrenocorticism in ferrets: (I) adrenal glands of ferrets with hyperadrenocorticism were studied immunohistochemically to detect LH receptors; (II) gonadotropin-releasing hormone (GnRH) stimulation tests were performed in 10 neutered ferrets, with measurement of androstenedione, 17α-hydroxyprogesterone and cortisol as endpoints; (III) GnRH stimulation tests were performed in 15 ferrets of which 8 had hyperadrenocorticism, via puncture of the vena cava under anesthesia; and (IV) urinary corticoid/creatinine ratios were measured at 2-week intervals for 1 year in the same ferrets as used in study II.

Clear cells in hyperplastic or neoplastic adrenal glands of hyperadrenocorticoid ferrets stained positive with the LH receptor antibody. Plasma androstenedione and 17α-hydroxyprogesterone concentrations increased after stimulation with GnRH in 7 out of 8 hyperadrenocorticoid ferrets but in only 1 out of 7 healthy ferrets. Hyperadrenocorticoid ferrets had elevated urinary C/C ratios during the breeding season.

The observations support the hypothesis that gonadotropic hormones play a role in the pathogenesis of hyperadrenocorticism in ferrets. This condition may be defined as a disease resulting from the expression of LH receptors on sex steroid-producing adrenocortical cells.

Introduction

The most prominent, and initially seasonal, symptoms of hyperadrenocorticism in ferrets are symmetrical alopecia (Fig. 1), vulvar swelling in neutered jills, and recurrence of sexual behavior in neutered males.[15] There is no sex predilection.[18] The diagnosis is based upon increased plasma concentrations of androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate, and/or estradiol. In contrast plasma concentrations of cortisol are increased in a minority of cases.[16] Measurement of urinary corticoid/creatinine ratios [4] and ultrasonography of the adrenals [15] may contribute to the diagnosis. In approximately 85% of ferrets with hyperadrenocorticism only one adrenal gland is enlarged, without atrophy of the contralateral adrenal gland, and in the remaining 15% there is bilateral involvement.[17,24] After unilateral adrenalectomy there may be recurrence of the disease due to enlargement of the contralateral adrenal gland.[24] The histologic changes of the adrenals range from (nodular) hyperplasia to adenoma and adenocarcinoma.[17,24]

Figure 1. Symmetrical alopecia in a ferret with hyperadrenocorticism

These characteristics of hyperadrenocorticism in ferrets resemble those seen in some strains of mice in which nodular adrenocortical hyperplasia and adrenocortical tumors occur after neutering at an early age.[3,11,19] In recent years, independent observations have provided suggestive evidence that castration is an important risk factor in the development of hyperadrenocorticism in ferrets. First, in the USA and in The Netherlands hyperadrenocorticism is a common disease in ferrets,[15,18] whereas in the United Kingdom the condition is seldom diagnosed. This difference in incidence may be ascribed to the fact

that ferrets are usually not neutered in the United Kingdom,[10] whereas this is common practice in the USA and in the Netherlands. Second, a significant correlation has been found between the age at neutering and age at onset of hyperadrenocorticism in ferrets.[18] The observation that initially signs of hyperadrenocorticism occur only during the breeding season,[15] when plasma concentrations of gonadotropic hormones are high,[6] and the recently reported beneficial effects of treatment with leuprolide acetate[23] have led to the hypothesis that hyperadrenocorticism in ferrets is mediated by gonadotropic influences, for which castration may play a precipitating role.[9,17,18]

To test this hypothesis four studies were performed: (I) adrenal glands of ferrets with hyperadrenocorticism were studied immunohistochemically to detect luteinizing hormone receptors (LH-R); (II) a gonadotropin-releasing hormone (GnRH) stimulation test was performed in neutered ferrets via implanted venous catheters; (III) a GnRH stimulation test was performed in neutered ferrets via puncture of the vena cava under anesthesia; and (IV) urinary corticoid/creatinine (C/C) ratios were measured at 2-week intervals for 1 year in the ferrets of study II to investigate possible seasonal fluctuations.

Materials and Methods

(I) Immunohistochemical staining for the presence of LH receptors

Tissues

Adrenal glands of 6 neutered ferrets with signs of hyperadrenocorticism, and histologically diagnosed hyperplasia or adenoma, were examined for the presence of LH receptors. The adrenal glands were obtained during surgery. In all cases the contralateral adrenal gland appeared to be unaffected. Signs of hyperadrenocorticism disappeared after surgery in all cases. Two histologically normal adrenal glands of 2 intact ferrets without signs of hyperadrenocorticism were also examined for the presence of LH receptors. Ovaries and testes of healthy 6 to 9-month-old ferrets were used as positive controls.

Antibody and immunohistochemical staining

The murine LH-R monoclonal antibody (P1B4) was a gift from Dr. Wimalasena (Dept. Obstetrics and Gynecology, University of Tennessee, Knoxville, TN). The antibody had been raised against purified rat LH receptors, as described by Indrapichate *et al*..[5] The antibody binds specifically to LH receptors in various tissues of different species.[1,13]

All tissues were fixed in 4% buffered formalin. After at least 24 h of fixation the tissues were embedded in paraffin and cut into 4-µm sections. One section was routinely stained with hematoxylin and eosin. For immunohistochemical staining, sections were deparaffinized and endogenous peroxidase was blocked with 1% H_2O_2 in methanol for 30 min. The slides were washed in 0.01 M Tris-buffered saline (TBS, pH 7.4), incubated with 0.75% glycine in TBS for 30 min, and rinsed with TBS. The sections were blocked with 10% normal goat serum in TBS for 30 min, and then incubated overnight at 4 °C with the LH-R P1B4 monoclonal antibody at a 1:5000 dilution in TBS to which 0.05% acetylated BSA (BSA-c) was added (Aurion, Wageningen, The Netherlands). The next day the slides were rinsed with TBS and incubated for 60 min with a biotinylated goat-anti-mouse

antibody (Vector Laboratories, Burlingame, CA) [1:200 dilution in TBS with 0.05% BSA-c] at room temperature. Again, the slides were rinsed in TBS and subsequently incubated with the avidin (A) biotin (B) complex of the ABC staining kit (Vector Laboratories) for 60 min in a dilution of 1:1500 in TBS with 0.05% BSA-c. The ABC solution was prepared at least 15 min prior to use, to allow complex formation. Slides were rinsed again in TBS followed by Tris-HCl (0.05M, pH 7.6). Bound antibody was visualized using a 0.6 mg/ml solution of 3,3' - Diaminobenzidine tetrachloride (DAB, Sigma Chemical Co., St. Louis, MO) in Tris-HCl, to which 0.03% H_2O_2 was added. Sections were incubated with the DAB solution for approximately 50 sec and counterstained with Mayer's hematoxylin.

In control experiments the primary antibody was replaced by normal mouse serum.

(II) GnRH stimulation test in catheterized ferrets

Animals

For this study, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, 5 male and 5 female healthy ferrets were used in July and August 1999. All but one ferret were 2 years of age and had been gonadectomized at 6 weeks of age. One ferret, a 1-year-old male, had been gonadectomized at 9 months of age. The ferrets were either purchased at 6 weeks of age from a breeder or born and raised at the Department of Clinical Sciences of Companion Animals. The ferrets were individually housed in outdoor suspended cages with a night box. Both water and ferret pellets (FerRet, Hope Farms, Woerden, The Netherlands) were available *ad libitum*.

Unexpectedly, in two ferrets elevated basal plasma androstenedione and 17α-hydroxyprogesterone concentrations, as well as unilaterally enlarged adrenal glands visualized on ultrasonographic examination, indicated the presence of hyperadrenocorticism.

Catheterization

A jugular catheter was placed under isoflurane anesthesia in all ferrets. The catheters were tunneled subcutaneously to the base of the scull and connected to a 20-gauge cannula with an injection port (Vasofix® Braunüle®, Braun, Melsungen, Germany). The injection port was attached to the skin with polyglactin 910 (Vicryl®, Ethicon, Norderstedt, Germany) 2-0 USP sutures. The catheters (Silastic® Medical Grade Tubing [602-155], Down Corning Co. Midland, Michigan, U.S.A) had an inner diameter of 0.025 inch (0.64 mm) and an outer diameter of 0.047 inch (1.19 mm). The catheter was filled with a polyvinyl pyrrolidone (PVP) and heparin mixture (60 g PVP / 54 ml 0.9% NaCl + 6 ml heparin [5,000 IU/ml]) to keep it patent.

Sampling

Blood samples were collected 2 days after placement of the jugular catheter at –5, 0, 30, 60, 90, 120, 240, and 480 min after intravenous injection of 10 µg of a synthetic GnRH analogue (Fertagyl®, Intervet Nederland B.V., Boxmeer, The Netherlands), placed in pre-chilled EDTA-coated tubes and centrifuged. Plasma was divided into two portions and stored at – 20 ºC pending analysis. After the collection of each blood sample the catheter was flushed with 0.3 ml heparin solution (50 IU/ml).

(III) GnRH stimulation test with blood collection under anesthesia

We used a modified GnRH stimulation test for privately owned ferrets thought to have hyperadrenocorticism. Blood samples were collected under isoflurane anesthesia by puncture of the cranial vena cava, immediately before and 30 min after intravenous injection of 10 µg Fertagyl®. In between blood collections the ferrets were allowed to recover from anesthesia, which usually occurred within 5 minutes. Blood samples were placed in pre-chilled EDTA-coated tubes and centrifuged. Plasma was stored at – 20 ºC pending analysis.

Animals

GnRH stimulation tests with blood collection under isoflurane anesthesia were performed in 9 of the gonadectomized ferrets of study II (5 male and 4 female), including the 2 ferrets with hyperadrenocorticism, and in 6 privately owned neutered ferrets (4 male and 2 female; 3 – 7 years of age) with hyperadrenocorticism. The diagnosis of hyperadrenocorticism in the latter cases was based upon history, physical changes, and ultrasonographic examination of the adrenal glands. In the privately owned ferrets only plasma concentrations of androstenedione were measured because blood was needed for routine laboratory tests and the animals had to undergo surgery.

Reference plasma concentrations of androstenedione and 17α-hydroxyprogesterone

For the determination of reference values for basal plasma concentrations of androstenedione and 17α-hydroxyprogesterone, blood was collected between March 15 and September 29, 2000, from 18 healthy, privately owned ferrets, and 14 healthy ferrets kept under laboratory conditions. All ferrets (20 female and 12 male) had been neutered and were between 1.5 and 8 years old (median 3 years).

Hormone determinations

Androstenedione concentrations were measured by radioimmunoassay (RIA) as described previously.[22] The lower limit of detection was 0.1 nmol/l and the interassay coefficients of variation were 10.5%, 9.3%, and 11.6% at 1.43, 4.82, and 11.76 nmol/l, respectively. Steroids cross-reacted in this assay as follows: 0.4 % for testosterone, 0.3 % for dihydrotestosterone, 3.6 % for 11β-OH-androstenedione, 2.2 % for adrenosterone (11-keto-androstenedione), 5.5 % for 5α-andostanedione, and 2.2 % for 5β-andostanedione.

17α-Hydroxyprogesterone concentrations were measured after toluene extraction using a competitive RIA and a polyclonal anti-17α-hydroxyprogesterone-antibody (UCB i903, UCB Bioproducts, Brussels, Belgium). 17α-Hydroxy[1,2,6,7-^{3}H]-progesterone (TRK 611, Amersham Pharmacia Biotech Benelux, Roosendaal, The Netherlands) was used as a tracer after chromatographic purification. The lower limit of detection was 0.2 nmol/l and interassay coefficients of variation were 9.0%, 7.4%, and 9.9% at 0.89, 5.13, and 26.02 nmol/l, respectively. Steroids cross-reacted in this assay as follows: 0.4 % for progesterone, 0.3 % for pregnenolone and 20 % for 17α-OH-pregnenolone.

Cortisol concentrations were measured by RIA (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 1 nmol/l and the interassay coefficient of variation was between 4.0% and 6.4%.

(IV) Serial measurements of urinary corticoid/creatinine (C/C) ratios

Animals

One year after the GnRH stimulation test, 9 of the gonadectomized ferrets of study II (5 male and 4 female), including the 2 ferrets with hyperadrenocorticism, were used to monitor the urinary C/C ratios for 1 year. One of the ferrets with hyperadrenocorticism died 7 months after the last urine sample was collected. On postmortem examination a large (metastasized) adrenocortical tumor was found.

Sampling

Propylene litter boxes with Macrolon plates were placed underneath the cages for collection of overnight urine samples at 2-week intervals for a period of 1 year. A 2-mm space between the plate and the wall of the litter box allowed urine to drain away from the feces.[12] The litter boxes were underneath the cages from 17:00 – 8:00 hours. Urine was transferred to tubes and stored at 4 ºC pending analysis, which was performed within 5 days after collection of the sample.

Hormone determination

Urinary corticoid concentrations were measured by RIA for cortisol as described previously.[14] The cortisol antiserum was raised in rabbits against a cortisol-21-hemisuccinate-bovine serum albumin conjugate. This antiserum is known to cross-react with other endogenous corticosteroids such as 21-deoxycortisol (62%), corticosterone (11%), cortisone (2%), 11-deoxycortisol (1.3%), deoxycorticosterone (1.3%), and 17α-hydroxyprogesterone (0.1%).[21] The urinary corticoid concentration was related to the urinary creatinine concentration (Jaffé kinetic method, initial rate reaction) by calculation of its quotient (x 10^{-6}).[20]

Statistics

The increase in plasma androstenedione concentrations in the GnRH stimulation test in the two hyperadrenocorticoid ferrets was compared with the (mean + 2sd) increase in plasma androstenedione concentrations in the 8 ferrets with normal basal plasma androstenedione concentrations (study II). Significance at $P<0.025$ was assumed when the increase in plasma androstenedione concentrations in one of the hyperadrenocorticoid ferrets was higher than the mean increase + 2sd in the 8 other ferrets.

The reference values for plasma androstenedione and 17α-hydroxyprogesterone concentrations were established in percentiles.[2] The inner limits of the percentiles $P_{2.5}$ and $P_{97.5}$ are presented with a probability of 95%.

Student's t-test was used to compare the mean corticoid/creatinine ratio of the 2 ferrets with hyperadrenocorticism with the mean ratio for the 7 healthy ferrets during both the breeding and the non-breeding season. Statistical significance was assumed at $P < 0.05$.

Results

(I) Immunohistochemical staining for the presence of LH receptors

Thecal cells in the ovaries and Leydig cells in the testes of healthy control animals stained positively with the LH-R antibody. In the adrenal glands of the healthy ferrets there was positive staining for LH-R in the zona glomerulosa and a slightly less clear staining in the zona fasciculata (not shown).

The adrenal glands of ferrets with hyperadrenocorticism had a heterogeneous appearance on histology. Cells were either small with pyknotic nuclei or large with a clear cytoplasm. The latter cells are referred to as clear cells. Spindle-shaped cells were also observed in the adenomas. Cells staining positive for the LH receptor were seen in the hyperplastic and neoplastic adrenal glands of the ferrets with hyperadrenocorticism. These cells were mostly clear cells (**Color section**; Fig. 6).

(II) GnRH stimulation test in catheterized ferrets

No significant response was seen after the administration of GnRH to the 8 healthy ferrets. In the 2 ferrets with hyperadrenocorticism basal plasma concentrations of androstenedione (1.5 and 3.8 nmol/l) and 17α-hydroxyprogesterone (1.7 and 1.9 nmol/l) were significantly higher than those of the healthy ferrets. Moreover, the plasma concentrations of these hormones rose significantly ($P<0.025$) 30 min after GnRH administration, and returned to basal concentrations within 60 – 90 min. In all ferrets, including the ferrets with hyperadrenocorticism, plasma cortisol concentrations did not change significantly after GnRH stimulation (Fig. 2).

(III)GnRH stimulation test with blood collection under anesthesia

Reference plasma concentrations

Basal plasma androstenedione concentrations in 32 healthy neutered ferrets ranged from 0.1 to 0.5 nmol/l (median 0.2 nmol/l; $P_{2.5}$ - $P_{97.5}$: 0.1 – 0.4 nmol/l). Basal plasma 17α-hydroxyprogesterone concentrations in the same ferrets ranged from 0.3 to 1.2 nmol/l (median 0.4 nmol/l; $P_{2.5}$ - $P_{97.5}$: 0.3 – 0.7 nmol/l).

Ferrets used in study II

Basal plasma androstenedione and 17α-hydroxyprogesterone concentrations of the two ferrets with hyperadrenocorticism (number 4 and 7) were higher than the respective reference range. Concentrations of these hormones were significantly higher at 30 min after stimulation with GnRH. In the other 7 ferrets, basal plasma androstenedione and 17α-hydroxyprogesterone concentrations were all within the reference range (Table 1). The plasma androstenedione concentration was unchanged 30 min after stimulation with GnRH in 3 of the ferrets; it increased slightly, but remained within the reference range in 3 ferrets; and in 1 ferret it rose above the reference range (Table 1). The plasma 17α-hydroxyprogesterone concentration remained unchanged 30 min after stimulation with GnRH in 4 ferrets; it increased slightly, but remained within the reference range, in 1 ferret; and it rose above the reference range in 2 ferrets (Table 1).

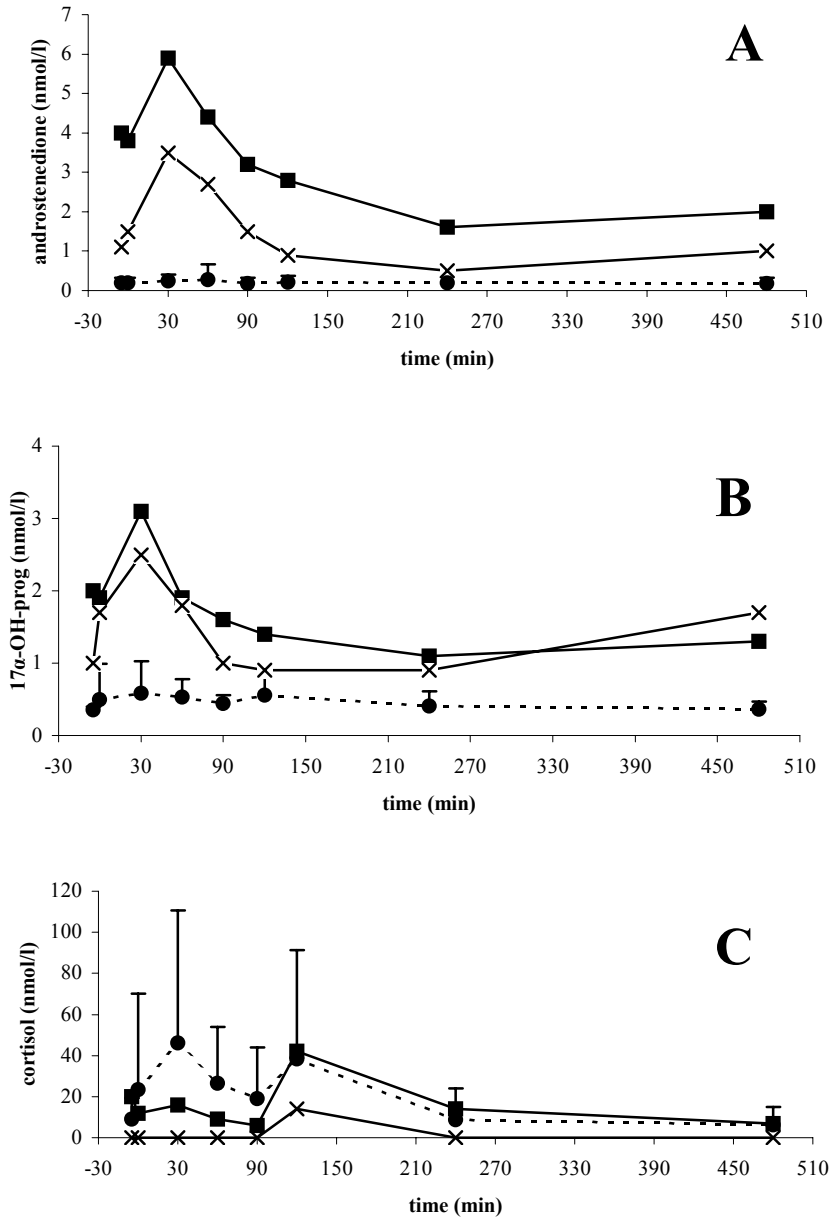

Figure 2. Mean (± sd) plasma concentrations of androstenedione (A), 17α-hydroxyprogesterone (B), and cortisol (C) before and after stimulation with 10 µg Fertagyl® (GnRH analogue) in 8 healthy neutered ferrets (●) and in 2 neutered ferrets (■, x) with hyperadrenocorticism.

Table 1. Plasma concentrations of androstenedione and 17α-hydroxyprogesterone (nmol/l) in 9 ferrets used in study II (1 – 9) and 6 privately owned ferrets with hyperadrenocorticism (C1 – C6) before and 30 min after and intravenous injection with 10 µg Fertagyl® (GnRH analogue). Blood samples were collected under isoflurane anesthesia. The same 2 ferrets which responded to GnRH stimulation when catheterized (number 4 and 7) responded in this modified stimulation test.

Ferret number	androstenedione		17 OH prog	
	t = 0	t = 30	t = 0	t = 30
1	0.2	0.2	0.4	0.4
2	0.2	0.3	0.4	0.3
3	0.2	0.2	0.4	0.4
4	8.5	13.8	5.1	10.5
5	0.2	0.2	0.4	0.4
6	0.3	0.4	0.4	0.5
7	4.2	16.9	7.5	35.0
8	0.3	0.4	0.5	0.8
9	0.4	1.3	0.6	1.0
C1	0.1	1.3		
C2	0.3	0.4		
C3	11.7	33.3		
C4	0.7	5.4		
C5	0.3	0.6		
C6	0.5	0.7		

Privately owned ferrets

In 3 of the 6 privately owned hyperadrenocorticoid ferrets, basal plasma concentrations of androstenedione were within the reference range. The plasma androstenedione concentration was within the reference range 30 min after stimulation with GnRH in 1 ferret; it was higher than the reference range in 2 ferrets (Table 1); and it was higher than the reference range at baseline and increased further after stimulation with GnRH in 3 ferrets (Table 1).

(IV) Serial measurements of urinary corticoid/creatinine ratios

During the breeding season (March to August) and the non-breeding season (September to February), the urinary corticoid/creatinine (C/C) ratio was measured on 12 occasions (Fig. 3). During the breeding season the mean (± sd) C/C ratio of the 2 ferrets with hyperadrenocorticism (both $3.5 \pm 1.1 \times 10^{-6}$) was significantly higher than that of the neutered control animals ($1.4 \pm 0.5 \times 10^{-6}$); however, during the non-breeding season the mean C/C ratio of only one ferret was significantly higher ($3.0 \pm 1.3 \times 10^{-6}$ and $1.8 \pm 0.3 \times 10^{-6}$ versus $1.8 \pm 0.7 \times 10^{-6}$ in the healthy control animals). The urinary C/C ratio of the latter ferret decreased at the end of October, after which the mean C/C ratio was no longer significantly higher than that of other animals ($2.5 \pm 1.1 \times 10^{-6}$ versus $1.9 \pm 0.7 \times 10^{-6}$).

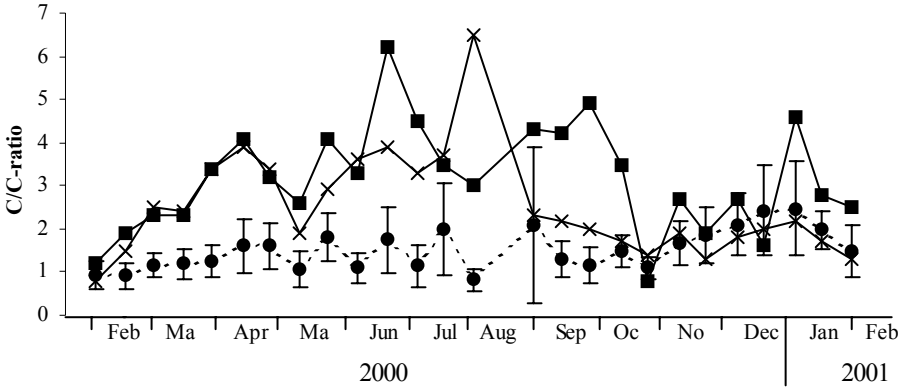

Figure 3. The mean (± sd) urinary corticoid/creatinine (C/C) ratio (x 10^{-6}) of 7 healthy neutered control ferrets (●) and the urinary C/C ratios of 2 neutered ferrets (■, x) with hyperadrenocorticism.
During the breeding season (March to August) the mean C/C ratio in both ferrets with hyperadrenocorticism was significantly higher than the mean C/C ratio in the 7 control ferrets ($P<0.025$)

Discussion

The detection of LH receptors in hyperplastic and/or neoplastic adrenal glands of ferrets with hyperadrenocorticism supports the hypothesis that LH plays a role in the pathogenesis of hyperadrenocorticism in these animals, and that this hormone is involved in the production of androstenedione and 17α-hydroxyprogesterone. The adrenal cortices of young intact healthy ferrets also stained positively for LH-R proteins. Since the LH-R antibody used in this study reacts with both intact LH-R protein and LH-R protein fragments,[5] it cannot be excluded that the immunohistochemical reactivity was due to staining of LH-R fragments, i.e. truncated forms of the receptor that are not functional. To investigate the functionality of this receptor, GnRH or LH stimulation tests should be performed either in vivo or in tissue culture.

Human chorionic gonadotropin (hCG) has been used for stimulation tests in women with endocrine tumors. In a woman with a virilizing adrenal adenoma testosterone levels increased after hCG,[8] and in a woman with LH-dependent Cushing's syndrome plasma cortisol levels increased after hCG.[7] However, we used GnRH as stimulating hormone, because it is not known which gonadotropic hormone stimulates the adrenal cortex in ferrets. Only the two ferrets (study II) with high basal androgen levels responded to stimulation with GnRH. Plasma concentrations of androstenedione and 17α-hydroxyprogesterone increased upon stimulation, whereas plasma cortisol concentrations

remained unchanged. The observation that the healthy ferrets did not have a response to GnRH stimulation indicates that the LH-R protein found in healthy ferrets is not functional.

In the modified GnRH stimulation test, 5 of the 6 privately owned ferrets with hyperadrenocorticism responded to stimulation. The increase in plasma androstenedione concentrations cannot be ascribed to isoflurane anesthesia because the increase in androstenedione concentrations did not occur in the healthy ferrets. The non-elevated basal androstenedione concentrations in 3 diseased ferrets were in accordance with the findings of Rosenthal and Peterson,[16] who observed that basal plasma androstenedione concentrations are not increased in approximately 25% of ferrets with hyperadrenocorticism.

Based on our findings, it would appear that the neoplastic transformation of the adrenal cortex is associated with activation of pre-existent receptor protein. It is not clear whether this activation is a prerequisite for hyperplasia and/or neoplasia or a consequence of cell transformation. The positive correlation between disease incidence and time elapsed since castration [18] makes it tempting to speculate that a persistent elevation of plasma gonadotropin concentrations plays a crucial role in both receptor activation and cell multiplication.

During the breeding season, the 2 ferrets with hyperadrenocorticism had a significantly higher C/C ratio than the other neutered ferrets. Earlier Gould *et al*.[4] had found that the C/C ratio in ferrets with hyperadrenocorticism was significantly higher than the C/C ratio in healthy ferrets. In the non-breeding season, when gonadotropic hormones are low,[6] the urinary C/C ratio of the 2 control ferrets with hyperadrenocorticism was no longer elevated. This finding further supports the hypothesis that hyperadrenocorticism in ferrets arises under the influence of gonadotropic hormones.

The use of the urinary C/C ratio as tool to aid the diagnosis of hyperadrenocorticism in ferrets has been questioned by Rosenthal,[15] because cortisol is not considered to play an important role in the development of the signs and symptoms of hyperadrenocorticism in ferrets.[16] The 2 ferrets with hyperadrenocorticism in study II also did not have increased plasma cortisol concentrations, whereas the C/C ratio was increased. This may be because the cortisol assay measures other steroids in urine because the antibody used cross-reacts with other steroid hormones.[21] Although it is uncertain whether the increased C/C ratio was due to an increased secretion of cortisol, we found that urinary steroid hormone excretion is increased during the breeding season in ferrets with hyperadrenocorticism, an increase which coincides with the reported increase in plasma LH concentrations.[6]

LH-dependent Cushing's syndrome and LH-dependent adrenal androgen secreting tumors have been described in humans.[7,8] Patients with LH-dependent Cushing's syndrome have bilateral macronodular adrenal hyperplasia with high plasma concentrations of cortisol and suppressed concentrations of ACTH.[7] Patients with LH-dependent adrenal androgen secreting tumors may have unilateral adrenal neoplasia and unaltered cortisol and ACTH concentrations.[8]

The results of the present study support the hypothesis that gonadotropic hormones, and particularly LH, play a role in the pathogenesis of hyperadrenocorticism in ferrets. This disease is not a counterpart of LH-dependent Cushing's disease in humans because GnRH does not elicit cortisol secretion in ferrets. There are similarities between

hyperadrenocorticism in ferrets and LH-dependent adrenal androgen-secreting tumors in humans, although the adrenal glands of diseased ferrets can also secrete estradiol.[16] Thus at this stage hyperadrenocorticism in ferrets is without a clear counter part in humans, and may probably be best defined as a disease resulting from LH-R expression by sex steroid-producing adrenocortical cells.

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Chapter 9

Gonadotropin Receptor Expression in the Adrenal Gland of Healthy Ferrets and Ferrets with Hyperadrenocorticism

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Summary

Hyperadrenocorticism is a common disease in ferrets and is characterized by excessive production of sex steroids, which gives rise to vulvar swelling in neutered females, recurrence of sexual behavior in neutered males, and alopecia in both sexes. It is thought to be caused by increased gonadotropin secretion as a result of neutering. In this study the presence and function of gonadotropin receptors in the adrenal glands of healthy ferrets and ferrets with hyperadrenocorticism was investigated.

The adrenal cortices of healthy ferrets stained positively with an antibody against the LH receptor (LH-R). Fifty-five hyperplastic and/or tumorous adrenal glands from 46 hyperadrenocorticoid ferrets also stained for the LH-R, with the exception of two metastasized carcinomas. RT-PCR analysis of 6 adrenal adenomas revealed the expression of the genes for LH-R and FSH-R.

Stimulation *in vitro* with ACTH and hCG of adrenal tissue from a healthy ferret led to significant increases in the concentrations of 17α-hydroxyprogesterone and cortisol in the culture medium, whereas the androstenedione concentration remained unchanged. FSH had no effect on steroid concentrations. In contrast, incubation of tumorous adrenocortical cells with hCG caused a sharp increase in androstenedione production in two of the three tumors investigated. FSH increased the production of 17α-hydroxyprogesterone and cortisol in the cells of one tumor, without having an effect on androstenedione release.

In vivo the plasma concentrations of 17α-hydroxyprogesterone, cortisol, and androstenedione of healthy ferrets did not change after stimulation with either FSH or hCG. In one of the two hyperadrenocorticoid ferrets studied, hCG but not FSH, increased the plasma concentrations of 17α-hydroxyprogesterone, cortisol and androstenedione. Plasma concentrations of 17α-hydroxyprogesterone and androstenedione increased after GnRH administration in 10 of 12 ferrets with hyperadrenocorticism, with concomitant increases in the concentrations of LH in 11 ferrets and FSH in 8 ferrets.

We conclude that, in healthy neutered ferrets, adrenocortical LH-Rs are not, or hardly, functional *in vivo*. In contrast, these receptors are functional in most neutered ferrets with hyperadrenocorticism. Although FSH-R mRNA is expressed in adrenal adenomas, FSH appears to have a less prominent pathogenetic role than LH.

Introduction

Hyperadrenocorticism is a common disease in neutered pet ferrets (*Mustela putorius furo*). The syndrome differs from hyperadrenocorticism in other species, such as humans, dogs, cats, and horses, in that glucocorticoid excess is much less pronounced in ferrets.[30] Instead, in ferrets the disease is characterized by excessive production of sex steroids, giving rise to vulvar swelling in neutered female ferrets (jills), recurrence of sexual behavior in neutered male ferrets (hobs), and alopecia.[19,29,30,31,34,46] In line with these physical and behavioral changes, plasma concentrations of androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate, and estradiol are often increased, whereas plasma cortisol concentrations only rarely exceed the upper limit of the reference range.[30] In approximately 85% of ferrets with hyperadrenocorticism there is unilateral adrenocortical enlargement without atrophy of the contralateral adrenal gland, while in the other 15% of cases bilateral enlargement is seen.[31,46] The disease may recur after unilateral adrenalectomy due to enlargement of the contralateral adrenal gland.[46] The histological changes of the adrenals range from (nodular) hyperplasia to adenoma and adenocarcinoma.[31]

There are several reasons to suggest a relationship between gonadotropic hormones and hyperadrenocorticism in ferrets. First, some symptoms of hyperadrenocorticism, such as alopecia and swelling of the vulva, initially occur only during the breeding season, when plasma concentrations of gonadotropic hormones are high.[13] Melatonin, which is released from the pineal gland during the dark period of the day, suppresses the release of gonadotropin-releasing hormone (GnRH). With increasing daylight melatonin release decreases accordingly, and when daylight exceeds 12 hours, melatonin exposure is insufficient to suppress the release of GnRH, leading to the start of the breeding season.[1] Second, as in some strains of mice [8,21,38] in ferrets the age of neutering is related to the age at which hyperadrenocorticism develops.[34] Thirdly, the similar plasma adrenocorticotropic hormone (ACTH) concentrations in ferrets with hyperadrenocorticism and in healthy ferrets makes it unlikely that the increased production of sex steroids is ACTH-dependent.[33] Fourthly, the depot GnRH agonist, leuprolide acetate, can be used successfully to treat ferrets with hyperadrenocorticism.[45] Finally, luteinizing hormone receptors (LH-R) have been detected in the adrenal glands of ferrets with hyperadrenocorticism. These receptors were considered to be functional because plasma concentrations of adrenal androgens increased after intravenous injection of a GnRH agonist.[35] These observations support the hypothesis that neutering leads to the persistent stimulation of the adrenal cortices, due to the loss of negative feedback, which may result in adrenocortical hyperplasia and tumor formation.[34] Daylight periods exceeding 12 h seem to promote this phenomenon.

The present study was designed to investigate the presence and functioning of LH and FSH receptors in healthy ferrets and in ferrets with hyperadrenocorticism by means of immunohistochemistry, RT-PCR, *in vitro* and *in vivo* stimulation tests.

Materials and Methods

1. Immunohistochemical staining for LH receptors

1.1 *Tissues*

Ovaries and testes from healthy, 6- to 9-month-old ferrets were used as positive controls. Adrenocortical control tissue was obtained from six intact (4 male, 2 female), 6- to 15-month-old ferrets that had died of diseases unrelated to hyperadrenocorticism.

Affected adrenal glands were obtained from 46 ferrets that had been presented with signs of hyperadrenocorticism. Most adrenal glands were obtained during adrenal surgery (n=31); the others were obtained post mortem (24 adrenal glands from 19 ferrets). From four ferrets, first an adrenal gland was obtained at surgery and later the contralateral adrenal gland was obtained post mortem. In total 55 affected adrenal glands were examined (39 left and 16 right).

For histological examination, all tissues were fixed for at least 24 hours in 4% phosphate-buffered formalin and then embedded in paraffin. Four-micrometer sections were cut: one section was routinely stained with hematoxylin and eosin (H&E) and the others were used for immunohistochemistry.

On the basis of the guidelines of Williams and co-workers, [48] the following criteria were used to categorize the adrenal cortices of diseased ferrets into hyperplasia, adenoma, or adenocarcinoma. Hyperplasia was defined as an increased tissue mass with maintenance of the normal cortical structure, with extra-capsular tissue or areas with ballooning cells (clear cells) within the cortex. Adenoma was defined as an increased tissue mass without the normal cortical structure; lesions usually contained a mixture of cell types ranging form clear cells to small cells with picnotic nuclei and spindle shaped cells. Adenocarcinoma was defined as infiltration of tumor cells into blood vessels and/or when metastases were found. Mitotic figures were not used as a selection criterion since they were absent in all slides examined.

Sixteen adrenal glands from 15 clinical cases were categorized as hyperplasia, 28 adrenal glands from 26 clinical cases were categorized as adenoma, and 11 adrenal glands from 10 clinical cases were categorized as adenocarcinoma. In three of the latter cases the tumor had metastasized.

1.2 *Immunohistochemical Staining*

Immunohistochemical staining for LH-Rs was performed as described previously.[35] The LH-R monoclonal antibody (P1B4), a gift from Dr. Wimalasena (Dept. Obstetrics and Gynecology, University of Tennessee, Knoxville, TN, USA), had been raised against purified rat LH-Rs, as described by Indrapichate *et al*..[12] The antibody binds specifically to LH-Rs in various tissues of different species.[4,25,41,42]

2. *In vivo* stimulation tests

2.1 *Stimulation tests in catheterized ferrets*

In a first study, which was approved by the Ethics Committee of the Faculty of Veterinary Medicine, Utrecht University, four stimulation tests were performed with catheterized ferrets. The order of the first three tests (ACTH, hCG, and FSH) was

determined by Latin square. The GnRH stimulation test was performed last as this test required twice the number of blood collections in comparison to the other tests. The tests were performed over two weeks with at least one day in between tests. The jugular catheter was placed 2 days before the first stimulation test. For the ACTH, hCG, and FSH stimulation tests, plasma concentrations of androstenedione, 17α-hydroxyprogesterone and cortisol were measured. For the GnRH stimulation test, plasma concentrations of LH and FSH were measured; the corresponding concentrations of androstenedione, 17α-hydroxyprogesterone, and cortisol are reported elsewhere.[35]

2.1.1 *Animals*

Twelve ferrets (6 male and 6 female) were used in July and August 1999. Eleven 2-year-old ferrets had been gonadectomized at 6 weeks of age. The twelfth ferret, a 1-year-old male, had been gonadectomized at 9 months of age. The ferrets were either purchased at 6 weeks of age from a breeder or born and raised in our department. The ferrets were individually housed in outdoor suspended cages with a night box. Water and ferret pellets (FerRet, Hope Farms, Woerden, The Netherlands) were available *ad libitum*.

Unexpectedly, two ferrets had increased basal plasma androstenedione and 17α-hydroxyprogesterone concentrations. This, together with ultrasonographically visualized unilateral adrenal gland enlargement, indicated the presence of hyperadrenocorticism, which was later confirmed by histological examination of the adrenal glands.

2.1.2. *Catheterization and sampling*

Jugular catheters were introduced, via vena section under isoflurane anesthesia, as described previously.[35] To keep the catheters patent they were filled with a mixture of polyvinyl pyrrolidone (PVP) and heparin (60 g PVP / 54 ml 0.9% sodium chloride + 6 ml heparin [5000 IU/ml]). Anesthesia was used only for catheter placement.

All blood samples were collected from the jugular catheter into pre-chilled EDTA-coated tubes and centrifuged. Plasma was stored in aliquots at – 20 ºC pending analysis. After collection of each blood sample the catheter was flushed with 0.3 ml heparin solution (50 IU/ml).

2.1.3 *Stimulation tests*

For the ACTH-stimulation test blood samples were collected at -5, 0, 30, 60, 120 and 180 min after an intravenous injection with 1 µg of synthetic $ACTH_{1-24}$ (corticotropin, Synacten®, Novartis Pharma B.V., Arnhem, The Netherlands). For the FSH-stimulation test blood samples were collected at -5, 0, 60, 120, 240, 480 and 1440 min after a subcutaneous injection of 5 IU follitropin alfa (Gonal-F® 75, Serono Pharma S.P.A., Bari, Italy). For the hCG stimulation test blood samples were collected at -5, 0 30, 60, 120 and 240 min after an intramuscular injection of 100 IU of the LH-R agonist hCG (Pregnyl®, Organon, Oss, The Netherlands). For the GnRH stimulation test blood samples were collected at -5, 0, 5, 10, 15, 30, 45, 60, 90 and 120 min after an intravenous injection of 10 µg of the GnRH agonist gonadorelin (Fertagyl®, Intervet Nederland B.V., Boxmeer, The Netherlands).

2.2 *GnRH stimulation test with blood collection under anesthesia*

In a second study a GnRH stimulation test was performed in 10 privately owned neutered ferrets (5 male and 5 female; 3 – 7 years of age) with hyperadrenocorticism. The diagnosis of hyperadrenocorticism was based upon history, physical changes, and ultrasonography of the adrenal glands.

Blood samples were collected under isoflurane anesthesia by puncture of the cranial vena cava, immediately before and 30 min after an intravenous injection of 10 µg Fertagyl®, as described previously.[35] Plasma concentrations of androstenedione, 17α-hydroxyprogesterone, LH, and FSH were measured.

2.3 *Hormone determinations*

Androstenedione concentrations were measured by radioimmunoassay (RIA) as described previously.[44] The lower limit of detection was 0.1 nmol/l and the interassay coefficients of variation were 10.5%, 9.3%, and 11.6% at 1.4, 4.8, and 11.8 nmol/l, respectively. 17α-Hydroxyprogesterone concentrations were measured after toluene extraction by RIA as described previously.[35] The lower limit of detection was 0.2 nmol/l and interassay coefficients of variation were 9.0%, 7.4%, and 9.9% at 0.9, 5.1, and 26.0 nmol/l, respectively. Cortisol concentrations were measured by RIA (Coat-A-Count® Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The lower limit of detection was 1 nmol/l and the interassay coefficient of variation was between 4.0% and 6.4%. FSH concentrations were measured by RIA as described previously.[47] This RIA has been validated for use in ferrets.[6] Rat FSH-RP2 (NIAMD, Bethesda, MD) was used as a standard. The lower limit of detection was 0.8 µg/l and the intra-assay coefficient of variation was 7.2%. LH concentrations were measured in duplicate 100-µl samples of plasma in one run with a heterologous RIA method as described before [5] with slight modifications. Anti-rat LH (1:11,200 CSU120; generously donated by Dr. G.D. Niswender, Colorado State University, Fort Collins, CO, USA), which recognizes ferret LH, was used. Radioiodinated rat LH (kindly donated by F.H. de Jong, Erasmus Medical Center, Rotterdam, The Netherlands) and canine LH LER 1685-1 (kindly donated by Dr. L.E. Reichert, Tucker Endocrine Research Institute LLC, Atlanta, GA, USA) were used as tracer and standard, respectively. The range was 0.39 to 50 µg/l at 94.6 to 6.5% relative bound with a maximum binding of 41%. Parallelism was tested by comparing the results of 50- and 100-µl samples of plasma from 5 ferrets (Fig 1). In four ferrets the difference between the 2 samples was 7.5%. In one ferret the results deviated by 43%. The intra- and interassay coefficients of variation were 5 and 10.5%, respectively. The limit of quantitation was 0.4 µg/l.

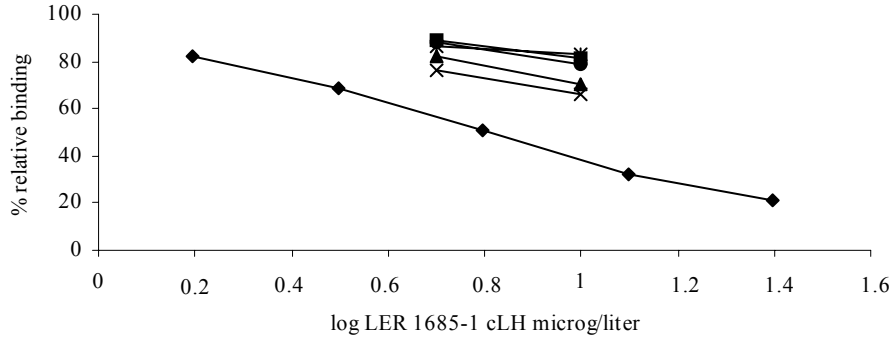

Figure 1. Ferret LH in plasma samples (n=5) of 50 and 100 µl plotted vs. canine LH (LER 1685-1) standards in a radioimmunoassay with iodinated rat LH and anti-rat LH antibody (CSU120).

3. *In vitro* stimulation test

3.1 *Adrenal tissue*

Adrenal tissue was obtained at surgery or within 30 min of death from a healthy intact male ferret, which died of a disease unrelated to hyperadrenocorticism, and three neutered ferrets with signs of hyperadrenocorticism. The adrenal glands of the healthy ferret (a 5-year-old male) had no macroscopic alterations. To be able to obtain sufficient adrenal cells no histological examination was performed on the latter glands. Histological examination of parts of the adrenal glands of the hyperadrenocorticoid ferrets (1 male, 2 female; aged 4 to 6 years) revealed two adenomas and one carcinoma.

3.2 *Tissue preparation*

The adrenal glands were collected in Hanks' balanced salt solution (Life technologies, Breda, The Netherlands) and placed on ice. Within 30 min of collection the adrenals were cut into pieces and incubated with 3 mg/ml collagenase (type 1, Sigma-Aldrich, St. Louis, USA) in Hank's solution at 37°C for 60 min under gentle agitation. Cells were collected by centrifugation at 250 g for 3 min and resuspended in DMEM (Sigma-Aldrich, St. Louis, USA) with 0.2% BSA before plating into 24-well culture dishes at a density of 500,000 cells/ml/well. The cells were incubated in medium without or with $ACTH_{1-24}$ (Synacten®) in a final concentration ranging from 10^{-6} to 10^{-9} M, GnRH (gonadorelin, Fertagyl®) at 10^{-6} to 10^{-9} M, FSH (follitropin alfa, Gonal-F® 75) at 2 and 20 IU/ml, and hCG (Pregnyl®) at 5 and 100 IU/ml. Incubations were performed in triplicate during 2 h at 37°C. Supernatants were collected and stored at – 20°C until measurement of cortisol, androstenedione, and 17α-hydroxyprogesterone concentrations as described above.

4. RT-PCR

4.1 *Primer design*

The primers for the LH receptor and FSH receptor (Table 1) were based on sequences with a high degree of homology among other animals and came from Invitrogen (Breda, The Netherlands) and Isogen (Maarsen, The Netherlands), respectively.

Table 1. Sequences of primers used for PCR amplification of ferret LH-R and FSH-R cDNA

Name	Sequence	Expected product size
LH-Forward	5′ ctaatgcctttgacaacctcctc 3′	208 bp
LH-Reversed	5′ tgtaagttatcacaaatttccagaatg 3′	
FSH-Forward	5′ ttggggacctggagaaaatagagatc 3′	447 bp
FSH-Reversed	5′ aggctccgtggaaaacatcattagg 3′	

4.2 *Tissues*

Nine surgically removed adrenal glands of ferrets with hyperadrenocorticism were divided in two. One part was immediately frozen in liquid nitrogen and stored at – 70°C, and the other part was fixed in 4% buffered formalin and submitted for histological examination, which revealed adrenocortical hyperplasia (n=2) and adenoma (n=7). All nine adrenal gland samples were examined for the presence of LH-R mRNA, while 6 adenomas were examined for the presence of FSH-receptor (FSH-R) mRNA. Ferret testes tissue, obtained during an elective castration and frozen in liquid nitrogen and stored at – 70°C, served as a positive control.

4.3 *RNA isolation and cDNA synthesis*

Total RNA was extracted from tissue using the RNAeasy mini kit (Qiagen, Valencia, CA), and 1 µg was reverse transcribed in 20 µl at 42°C for 60 min using the Reverse Transcription System (Promega, Leiden, The Netherlands). Each reaction contained 5 mM $MgCl_2$, 1x Reverse Transcription Buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 0.1% Triton X-100), 1 mM each dNTP, 1 U RNasin, 15 U AMV Reverse Transcriptase, and 0.5 µg Oligo$(dT)_{15}$ primer.

4.4 *PCR*

PCR to detect LH-R and FSH-R mRNA was performed on 1 µl cDNA in a 50-µl reaction mixture containing 2 mM $MgCl_2$, 1x reaction buffer (50 mM KCl, 10 mM Tris-HCl [pH9.0], 1% Triton X-100), 0.2 mM of each dNTP, and 1.25 U of Taq DNA polymerase.

Amplification was performed using the following thermo cycle parameters: 96°C for 4 min followed by 35 cycles of 30 sec at 96°C, 30 sec at 58°C and 1 min at 72°C. Final extension was performed at 72°C for 10 min. PCR products were analyzed on a 1% agarose gel. PCR products were also purified and sequenced for product verification.

Results

1. Immunohistochemical staining for LH receptors

Thecal cells in the ovary and Leydig cells in the testis of healthy ferrets (**Color section**; Fig 7) stained positive with the LH-R antibody. In the adrenal glands of healthy ferrets, the zona glomerulosa stained positively for LH-R, as did the zona fasciculata, but less intensely (**Color section**; Fig 8).

All adrenal glands from the clinical cases, except two adrenocortical tumors and their metastasis, stained positively for the LH-R. In another clinical case with a metastasizing adenocarcinoma, the primary tumor contained LH-R positive cells while the metastasis did not. Besides staining of the zona glomerulosa and zona fasciculata, clusters of clear cells in hyperplastic adrenal cortices stained for LH-R. Clusters of positively staining cells were found throughout the adenomas and adenocarcinomas (**Color section**; Fig 9).

2. *In vivo* stimulation tests

2.1 *Stimulation tests in catheterized ferrets*

Thirty minutes after intravenous administration of $ACTH_{1-24}$, plasma concentrations of cortisol and 17α-hydroxyprogesterone had increased in all healthy ferrets. In one of the ferrets with hyperadrenocorticism the cortisol response was in the range. The other hyperadrenocorticoid ferret had a low basal cortisol concentration and only a moderate response to stimulation with ACTH. In the healthy ferrets plasma androstenedione concentrations remained unchanged after stimulation with ACTH, whereas they increased in both hyperadrenocorticoid ferrets (Fig 2).

In the healthy ferrets, plasma concentrations of cortisol, androstenedione, and 17α-hydroxyprogesterone did not change after stimulation with recombinant FSH or hCG. In one hyperadrenocorticoid ferret, plasma concentrations of cortisol, androstenedione, and 17α-hydroxyprogesterone increased 60 minutes after administration of FSH and returned to baseline 60 minutes later. In the other ferret with hyperadrenocorticism hormone levels did not change after stimulation with FSH. Plasma concentrations of androstenedione and 17α-hydroxyprogesterone gradually increased after stimulation with hCG in the latter hyperadrenocorticoid ferret, but did not change in the former (Fig 3).

Within 10 minutes of intramuscular administration of GnRH, plasma LH concentrations increased in all ferrets and returned to baseline values after approximately 1 hour (Fig 4A), whereas plasma FSH concentrations did not change. In the two ferrets with hyperadrenocorticism plasma FSH concentrations increased slightly between 5 and 20 min after administration of GnRH (Fig 4B).

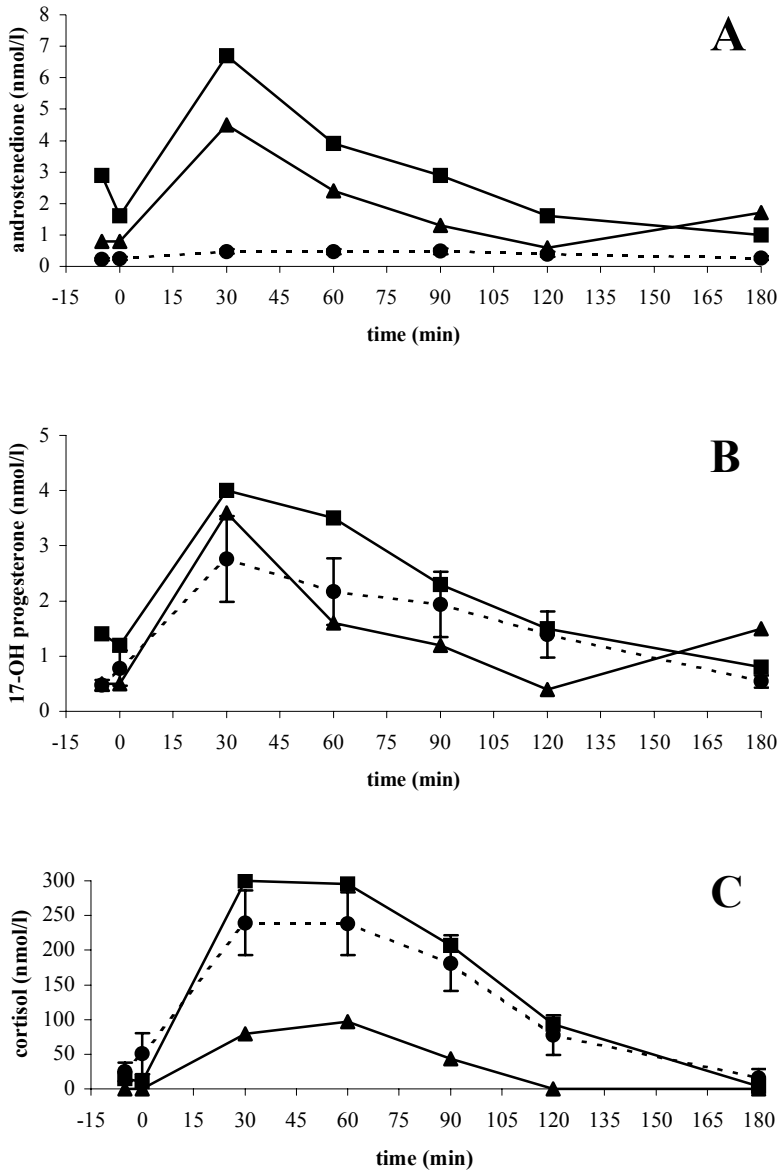

Figure 2. Mean (± SEM) plasma concentrations of androstenedione (A), 17α-hydroxyprogesterone (B), and cortisol (C) before and after the intravenous administration of $ACTH_{1-24}$ at t=0 min in 10 healthy neutered ferrets (●) and in 2 neutered ferrets (■, ▲) with hyperadrenocorticism.

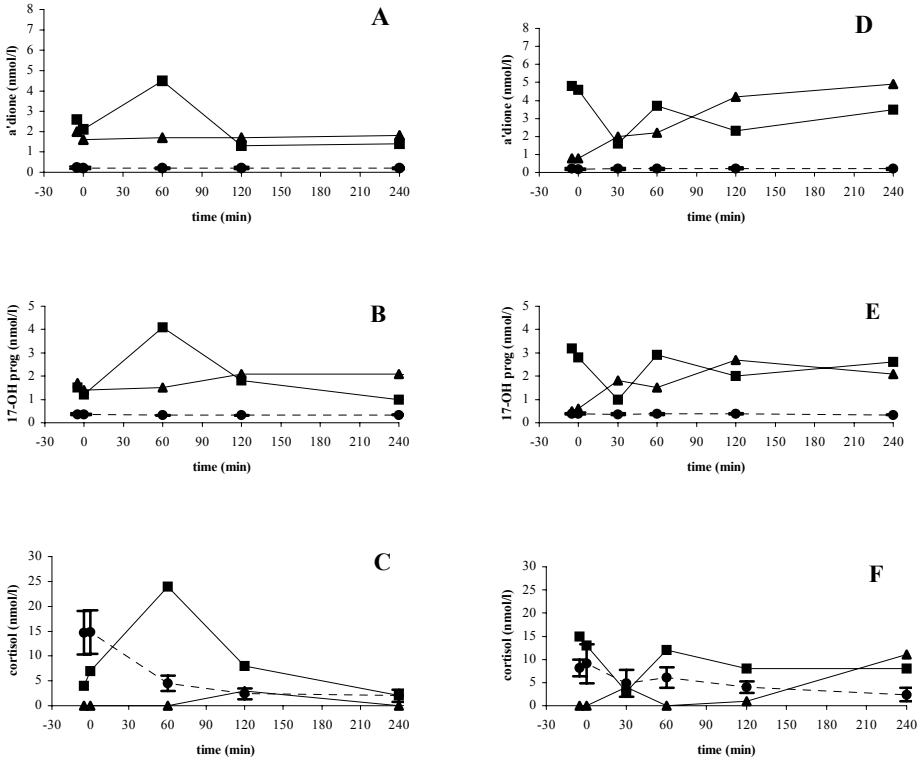

Figure 3. Mean (± SEM) plasma concentrations of androstenedione (A), 17α-hydroxyprogesterone (B), and cortisol (C) before and after the subcutaneous administration of recombinant FSH, and mean (± SEM) plasma concentrations of androstenedione (D), 17α-hydroxyprogesterone (E), and cortisol (F) before and after the intramuscular administration of hCG at t=0 min in 10 healthy neutered ferrets (●) and in 2 neutered ferrets (■, ▲) with hyperadrenocorticism. Note difference with figure 5 with regard to scale for cortisol.

2.2 *GnRH stimulation test with blood collection under anesthesia*

Intravenous injection of GnRH in 10 ferrets with hyperadrenocorticism increased the median plasma LH concentration from 1.6 µg/l (range, 0.7 – 2.2 µg/l) to 3.2 µg/l (range, 1.2 – 3.7 µg/l), and the median plasma FSH concentration from 23.8 µg/l (range, < 0.8 – 67.2 µg/l) to 34.8 µg/l (range, < 0.8 – 93.5 µg/l). In one ferrets the plasma LH concentration did not increase and in four ferrets the plasma FSH concentrations did not increase after GnRH administration. The median plasma androstenedione concentration increased from 1.1 nmol/l (range, 0.1 – 9.1 nmol/l) to 4.0 nmol/l (range, 0.1 – 22 nmol/l), and the median plasma 17α-hydroxyprogesterone concentration increased from 1.9 nmol/l (range, < 0.5 – 29 nmol/l) to 2.8 nmol/l (range, < 0.5 – 62 nmol/l). In 7 of the 10 hyperadrenocorticoid ferrets, basal plasma concentrations of androstenedione exceeded the reference range (0.1 –

0.4 nmol/l)[35]. In two of the ferrets with normal basal plasma concentrations of androstenedione, concentrations exceeded the reference range 30 min after stimulation with GnRH. In 6 of the 10 hyperadrenocorticoid ferrets basal plasma concentrations of 17α-hydroxyprogesterone exceeded the reference range (0.3 – 0.7 nmol/l)[35]. In three of the ferrets with normal basal concentrations, plasma 17α-hydroxyprogesterone concentrations exceeded the reference range 30 min after stimulation with GnRH.

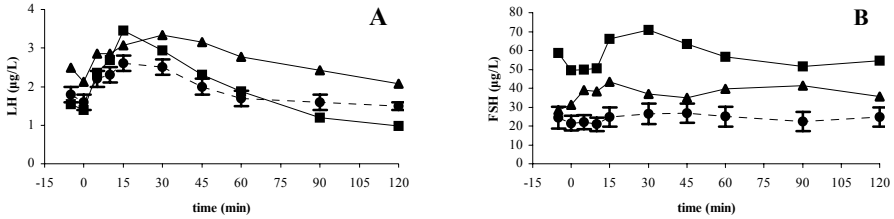

Figure 4. Mean (± SEM) plasma concentrations of LH (A), and FSH (B) before and after the intramuscular administration of a GnRH agonist at t=0 min in 8 healthy neutered ferrets (●) and in 2 neutered ferrets with hyperadrenocorticism (■, ▲).

3. ***In vitro* stimulation tests**

ACTH and hCG significantly stimulated the release of cortisol and 17α-hydroxyprogesterone from normal adrenal cells in culture medium as compared to the control incubations. This was also true for the dispersed cells from the adrenal adenoma A and carcinoma B. In the medium of cells obtained from adrenal adenoma C cortisol and 17α-hydroxyprogesterone concentrations only increased after incubation with hCG. The cells had been incubated with a far lower concentration of ACTH (10^{-11} M). Incubation with FSH only caused significant increases in cortisol and 17α-hydroxyprogesterone concentrations in adrenal cells from carcinoma B (Fig 5).

None of the hormones used stimulated the release of androstenedione from normal adrenal cells in culture medium as compared to the control incubations, whereas ACTH (10^{-6} M) significantly increased androstenedione concentrations in culture medium of cells from adenoma A and carcinoma B. Incubation with hCG led to a significant increase in androstenedione concentrations in culture medium with carcinoma B and adenoma C cells (Fig 5). GnRH did not stimulate the release of any of the hormones measured.

4. **RT-PCR**

RT-PCR analysis showed that LH-R and FSH-R mRNA is expressed in the ferret adrenal gland (Fig 6). Sequence analysis of the partially amplified LH-R mRNA revealed high sequence homology to the corresponding regions of the mRNA encoding LH-R in the polar bear (98%), pig (96%), cow (95%), human (92%), and rat (92%). Part of the ferret FSH-R mRNA was homologous to the published sequences of the polar bear (85%), cow (84%), pig (83%), human (80%), and rat (78%).

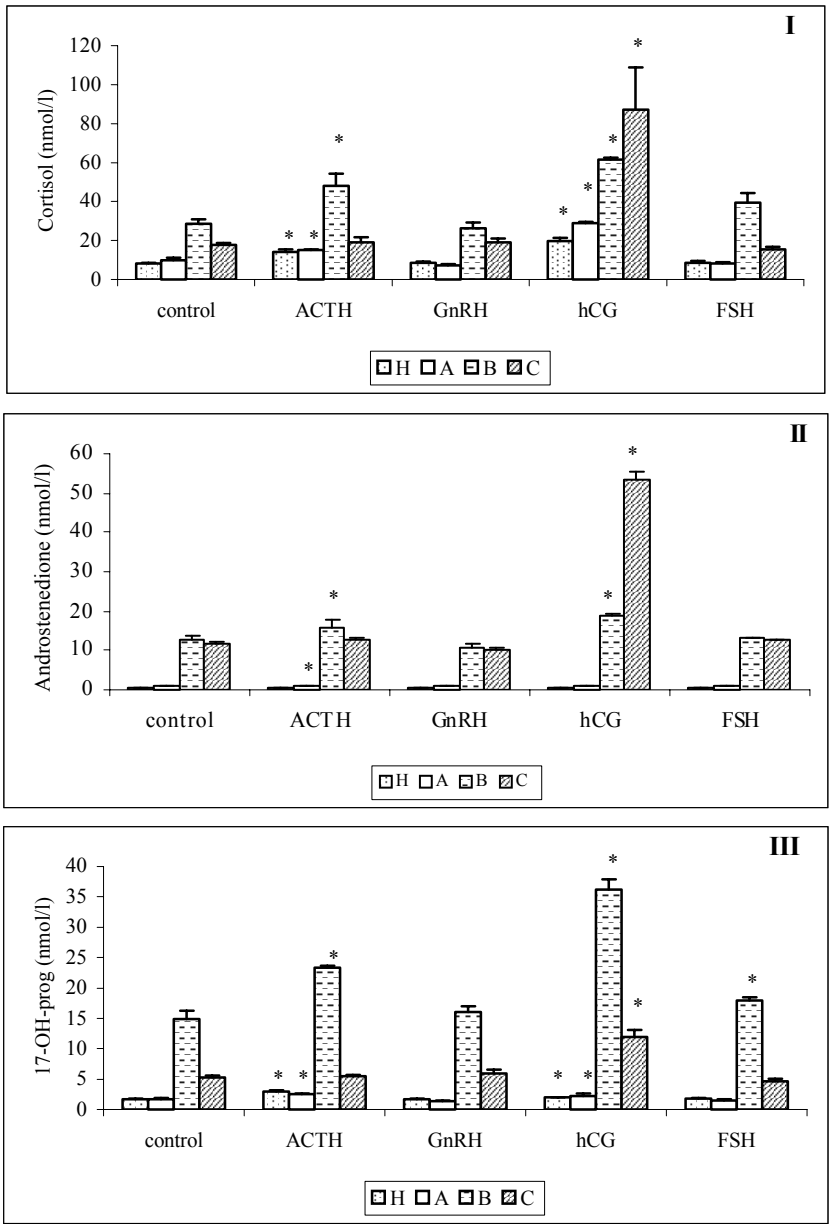

Figure 5. Concentrations of cortisol (I), androstenedione (II), and 17α-hydroxyprogesterone (III) in culture medium after a 2-h incubation of dispersed cells from 1 normal adrenal gland (H), 2 adrenal adenomas (A, C) and 1 adrenal carcinoma (B) with ACTH (10^{-6} M), GnRH (10^{-6} M), hCG (100 IU/ml), and FSH (20 IU/ml). The dispersed cells of adenoma C were incubated with ACTH in a concentration of 10^{-11} M. An asterisk indicates a significantly ($P < 0.05$) higher concentration than the control

Discussion

The present data provide further support for the concept that gonadotropic hormones play an important role in the development of hyperadrenocorticism in ferrets. The results of immunocytochemical studies, *in vivo* and *in vitro* stimulation tests, and RT-PCR studies point to the importance of the expression of receptors for gonadotropic hormones, and particularly LH-Rs, in adrenocortical cells. The results are discussed below according to the experimental approach used.

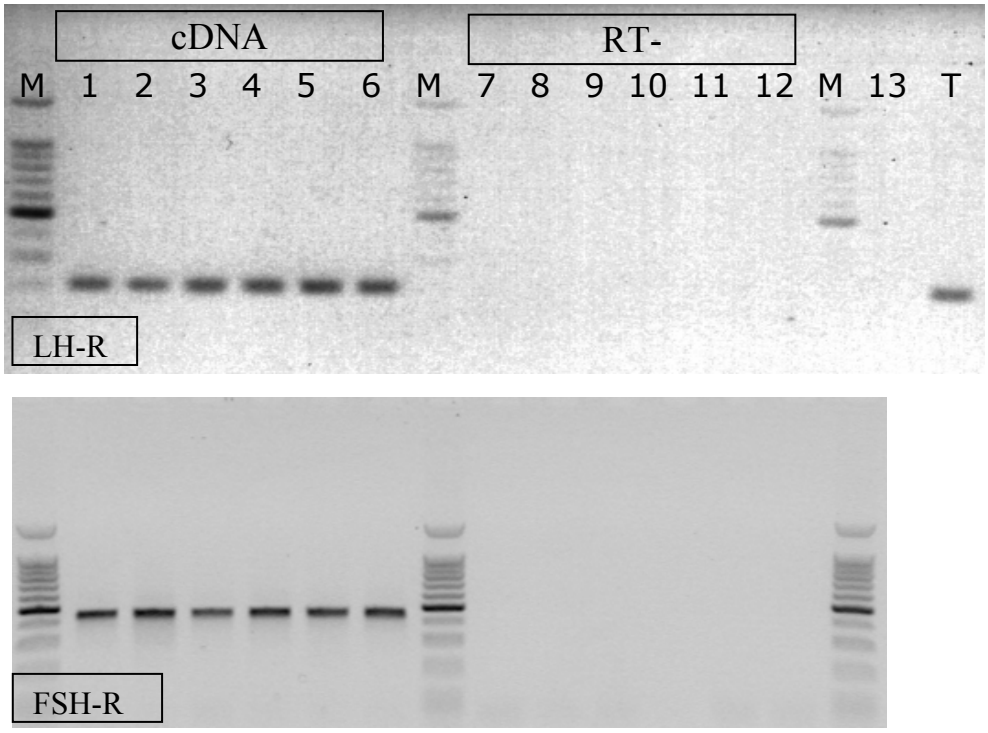

Figure 9. PCR-products obtained from 6 ferret adrenal adenomas (lanes 1-6) showing bands corresponding with the expected size for LH- and FSH-receptors. M = marker, T = testes as positive control. RT – = PCR reaction without the addition of reverse transcriptase.

Immunohistochemistry revealed the presence of LH-R protein in the adrenal cortices of intact 6- to 15-month-old ferrets. Thus, LH-R protein is already present in adrenal cortices of young, intact ferrets. The presence of LH-R protein in the zona glomerulosa and zona fasciculata is unexpected because LH-Rs are found in the zona reticularis and the distal zona fasciculata of the human adrenal gland.[23] These zones produce androgens, whereas the zona glomerulosa lacks the enzyme 17α-hydroxylase, which is essential to androgen synthesis.[40] The presence of LH-Rs in the zona glomerulosa, however, is not

unique to ferrets, and these receptors have been found throughout the adrenal cortex including the zona glomerulosa in mice with chronically elevated serum LH concentrations.[15] Hämäläinen *et al*. [11] detected positive β-galactosidase staining in the zona glomerulosa and zona fasciculata in transgenic mice carrying an LH-R promoter (7.4 kb) fused with β-galactosidase.

LH-R immunopositive cells were found in the adrenocortical lesions of 44 of the 46 ferrets with hyperadrenocorticism. Of the three ferrets with metastasizing adrenocortical adenocarcinomas only one had LH-Rs in the primary tumor but not in the metastases. These three ferrets had only minor signs of hyperadrenocorticism. In these animals LH-Rs may have been lost during dedifferentiation of the neoplastically transformed cells, as has been observed for steroid hormone receptors in malignant mammary tumors.[32]

The detection of LH-R mRNA by RT-PCR further confirms the local expression of LH-Rs in the ferret adrenal gland. However, the mRNA transcript and the locally produced protein may not be functional because the transcript may comprise truncated (iso)forms of the receptor.[11,12] In a previous study we demonstrated that plasma concentrations of androstenedione and 17α-hydroxyprogesterone increased in neutered ferrets with hyperadrenocorticism in response to intravenous administration of a GnRH agonist. However, no such rise occurs in healthy ferrets,[35] suggesting that LH-Rs may become functional only after form of metabolic/hormonal derangement.

In all eight healthy neutered ferrets plasma LH concentrations, but not FSH concentrations, clearly increased after intravenous administration of GnRH. In 11 of the 12 neutered ferrets with hyperadrenocorticism plasma LH concentrations increased after intravenous administration of GnRH, whereas plasma FSH concentrations increased in only 8 of the 12 ferrets. In the two ferrets with hyperadrenocorticism in which hCG was administered intramuscularly, one responded with increased plasma concentrations of androstenedione and 17α-hydroxyprogesterone. The other ferret appeared to respond only to subcutaneous administration of FSH. In the latter ferret, however, the response was seen only 60 min after administration, and then only after 60 min. Because ACTH can increase the plasma concentrations of these three hormones, this increase may well be due to the stress of handling before blood collection, rather than a direct effect of FSH administration. None of the healthy neutered ferrets responded to stimulation with either hCG or FSH, consistent with observations in healthy humans.[24,26] In a young woman with an adrenocortical adenoma testosterone secretion could only be stimulated with hCG before resection of the adrenal tumor.[2] Thus in humans[23] and ferrets, LH-Rs may become functional only under pathophysiological conditions.

ACTH has been implicated as an important factor controlling the adrenal androgen secretion.[9,24,27] In our study, however, only ferrets with hyperadrenocorticism responded to ACTH with an increased secretion of androstenedione; healthy ferrets showed no response. Adrenocortical cells can be categorized on the basis of their lipid content,[49] with lipid-rich cells producing more cortisol than androstenedione, and the reverse in lipid-poor cells. It is possible that the adrenal glands of ferrets with hyperadrenocorticism contain relatively more lipid-poor cells. However, in our ferrets the LH-R-positive cells seemed to be lipid-rich (clear cells). It is more likely that an alternative stimulatory pathway for the synthesis of androstenedione is activated in the hyperplastic or tumorous cells.

The *in vitro* stimulation tests gave the most cogent evidence for the existence of functional LH-Rs in the ferret adrenal glands. In the four adrenal glands, including a normal adrenal gland from an intact male ferret, cortisol secretion increased after incubation with hCG. In an adenoma and a carcinoma androstenedione concentrations increased after incubation with this hormone. Similar results have been reported for isolated guinea-pig adrenal cells, where hCG stimulated the production of cortisol and androstenedione.[22] The increase in cortisol concentrations after incubation of ferret adrenal glands with hCG may explain why the urinary corticoid/creatinine ratio is increased in ferrets with hyperadrenocorticism.[10, Schoemaker *et al.* submitted] However, *in vivo* this increase in cortisol secretion must be limited because plasma ACTH concentrations are not decreased in ferrets with hyperadrenocorticism.[33] An earlier study did not report an increase in cortisol after *in vivo* stimulation with hCG or GnRH in two ferrets with hyperadrenocorticism.[35]

The LH-R belongs to the group of G protein-coupled receptors (GPCRs). While the precise mechanism for GPCR activation after agonist binding remains to be defined, it is generally postulated that GPCRs are in equilibrium between an activated state and an inactive state. These states presumably differ in the disposition of the transmembrane helices and, in turn, the cytoplasmic domains that determine G protein coupling. Since continued exposure to an agonist leads to desensitization,[39] the adrenocortical LH-Rs may have become desensitized in the healthy neutered ferrets, as a result of continuous exposure to high LH concentrations.

Some mutations in GPCRs can cause loss of function, while others lead to a gain of function. Activating missense mutations are thought to disrupt normal inhibitory constraints that maintain the receptor in its inactive conformation,[14] shifting the equilibrium toward the activated state of the receptor. Some gain-of-function mutations are associated with increased sensitivity to the agonist.[50] As in mice, the tumorigenic consequences of the high LH levels [20,28] may be associated with or mediated by activated LH-Rs, which may be due to a mutation in either the receptor protein or the G protein.[3,43] In addition, receptor expression may be further increased after hormonal stimulation,[37] a supposition supported by studies in mice. In healthy intact mice LH-R mRNA is not expressed in the adrenal cortex. Increased LH concentrations following gonadectomy, however, resulted within 4 months in the expression of adrenal LH-R mRNA,[15] indicating that prolonged elevation of LH levels may result in the expression of LH-Rs in adrenal cortex.

Both LH-dependent hypercortisolism and LH-dependent adrenal androgen secreting tumors have been described in humans.[17] Patients with LH-dependent Cushing's syndrome usually have bilateral macronodular adrenal hyperplasia with high plasma concentrations of cortisol and suppressed concentrations of ACTH.[7,16] Hyperadrenocorticism in ferrets is usually associated with unilateral adrenal pathology. However, the contralateral adrenal cortex is not atrophic and plasma ACTH concentrations are not significantly different from those of healthy ferrets.[33] Thus hyperadrenocorticism in ferrets is not identical to LH-dependent Cushing's syndrome in humans. Hyperadrenocorticism in ferrets may, however, be comparable with LH-dependent adrenal androgen-secreting tumors in women, in whom, as in ferrets, usually only one adrenal gland is involved without alterations in plasma ACTH concentrations.[2,18]

To our knowledge, the presence of FSH-Rs in adrenal tissue has thus far only been reported in adrenocortical carcinoma cells from a rat.[36] Here we detected FSH-R mRNA in ferret adrenal adenomas by RT-PCR. We also observed a significant increase in cortisol and 17α-hydroxyprogesterone in response to FSH stimulation. However, since this response to FSH stimulation was demonstrated in only one of the three tumors examined, the response to an *in vivo* stimulation test was questionable, and plasma FSH concentrations did not increase during the GnRH stimulation test in 4 out of 12 ferrets, we speculate that the FSH-R plays a less prominent role than the LH-R in the development of hyperadrenocorticism in ferrets.

We conclude that the LH-Rs detected in the adrenal cortex of healthy neutered ferrets are hardly, or not, functional *in vivo*. In contrast, these receptors appear to be functional in neutered ferrets with hyperadrenocorticism. Although FSH-R mRNA is expressed in adrenal adenomas, these receptors appear to play a less prominent pathogenetic role than LH-Rs.

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Chapter 10

Current and Future Options for Non-Surgical Neutering of Ferrets (*Mustela putorius furo*)

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Summary

Female ferrets (jills) are commonly ovariectomized to prevent estrus-induced bone marrow suppression, and male ferrets (hobs) are castrated to reduce intraspecies aggression and skin odor. There is increasing evidence, however, that castration may precipitate the development of hyperadrenocorticism in ferrets. The detection of luteinizing hormone receptors in the adrenal cortex of ferrets with hyperadrenocorticism has strengthened this notion. Since surgical castration is a major risk factor for the development of hyperadrenocorticism in ferrets, this paper concentrates on the current and possible future options for neutering ferrets. Some of the alternatives, such as progestagen administration, seem very practical in jills but the effect in hobs is uncertain. Possible future alternatives may be the use of slow-release gonadotropin-releasing hormone (GnRH) implants, GnRH antagonists, or immunization against GnRH.

Introduction

Surgical castration of ferrets (*Mustela putorius furo)* is common practice in the USA and various European countries. Female ferrets (jills) are induced ovulators and therefore remain in estrus until they are mated, or for as long as daylight lasts longer than 12 hours. In the early 1980s several publications appeared concerning estrogen-induced bone marrow suppression in jills with prolonged oestrus.[8,36,43,66] Since then, preventive ovari(ohyster)ectomy of jills has been advised. In male pet ferrets (hobs) there is no medical need for castration. The main reason to castrate hobs is to reduce aggression so that they can be kept in groups, and to decrease the intensity of the musky odor produced by the sebaceous glands.[51] In the USA it is common practice to castrate ferrets at 6 weeks of age, before their delivery to pet shops.[58]

Hyperadrenocorticism is a common disease among pet ferrets and is characterized by signs of excessive production of sex steroids (androstenedione, 17α-hydroxyprogesterone, dehydroepiandrosterone sulfate and/or oestradiol), i.e., symmetrical alopecia, vulvar swelling in neutered jills, and recurrence of sexual behavior in neutered ferrets.[40,57,58,63,71] In recent years, evidence has accumulated that hyperadrenocorticism in ferrets is mediated by an increased secretion of gonadotropic hormones after castration.[40,58,63] First, the initial signs of hyperadrenocorticism occur only during the breeding season,[56] when plasma concentrations of gonadotropic hormones are high.[33] Second, in the USA and in The Netherlands, where the neutering of ferrets is common practice, hyperadrenocorticism is a common condition.[56,63] In contrast, hyperadrenocorticism is seldom diagnosed in the United Kingdom, where ferrets often remain entire.[41] Third, a significant correlation has been found between the age at neutering and age at onset of hyperadrenocorticism.[63] Fourth, the gonadotropin-releasing hormone (GnRH)-analogue leuprolide acetate has recently been reported to have beneficial effects in the treatment of this disease.[70] Finally, luteinizing hormone (LH) receptors have been detected in the adrenal cortex of ferrets.[64]

Because several lines of evidence point to surgical castration as a major risk factor in the development of hyperadrenocorticism in ferrets, this paper concentrates on current and future options for the neutering of ferrets.

Reproductive physiology

Gonadal activity is seasonal in both male and female ferrets, and more than 12 hours of light per day promotes reproductive activity.[10,25] The pineal hormone melatonin plays a central role in the regulation of these changes,[27] and plasma and pineal gland concentrations of melatonin are significantly higher during the dark phase of the day (scotophase) than in the light phase (photophase).[7]

Plasma concentrations of follicle-stimulating hormone (FSH) increased within a few days after male hamsters were transferred from a short to a long photoperiod. Plasma LH concentrations, however, increased only after exposure to a female hamster.[2] The different regulation of LH and FSH secretion can be explained by the fact that there are at least two types of GnRH receptors [30] and several GnRH isotypes, some of which may have specific FSH-releasing activity.[72] Anand *et al.* also found that both melatonin and a GnRH-antagonist (antide) could prevent the release of LH in male hamsters exposed to a female.[2]

These findings indicate that melatonin suppresses the release of GnRH in seasonal breeders.

During the breeding season, GnRH stimulates the production of the gonadotropic hormones LH and FSH, which stimulate the gonads to produce either oestradiol or testosterone. The latter two hormones exert a negative feedback on the hypothalamus and pituitary gland, thereby preventing excessive secretion of GnRH, LH, and FSH.

Definitions

In its classical definition, castration denotes the removal of gonads. Thus the term covers both the removal of the ovaries (ovariectomy or spaying) in females and the removal of the testes (orchiectomy) in males.[3] In recent years, this definition has been extended by the introduction of new methods to create non-functional gonads, e.g., chemical castration and immunological castration.[5] The term castration is controversial since it is sometimes used to indicate the removal of the gonads in male individuals only.[16] A term that encompasses all means of eliminating gonadal function in both males and females is neutering,[4] and this term is used in this article.

There are methods of contraception that do not affect gonadal function. For example, fertilization of oocytes can be prevented by vaccination against zona pellucida proteins [6,26,44] or vaccination against sperm proteins.[39,53] Another option to prevent oocyte fertilization is surgical sterilization, by means of tubal ligation. Immunization of female rats and monkeys with riboflavin carrier protein caused termination of pregnancy around the pre-implantation stage, while immunization of male rats and monkeys resulted in a reduced fertilizing potential of their spermatozoa.[1] Since these methods do not eliminate gonadal function and therefore do not prevent the resulting detrimental effects in ferrets, they are not discussed here.

Progestagens

Principle: Although not fully understood, the probable mode of action of progestagens is suppression of the secretion of gonadotropic hormones, thereby preventing ovarian cyclicity.[15]

Method: Several progestagens are used in veterinary medicine: medroxyprogesterone acetate, megestrol acetate, and proligestone. Megestrol acetate (Ovaban; Schering Plough) can be given orally, medroxyprogesterone acetate (Depo-provera; Pharmacia) can be given orally or by injection and proligestone (14α, 17α propylidene-dioxy progesterone) (Delvosteron; Intervet) is given by depot injection. The latter is recommended in the United Kingdom for the prevention of estrus in ferrets at a dose of 0.5 ml (100 mg/ml SC) just prior to the breeding season.[35,52] Proligestone can also be used in jills in estrus.

Effect: Return of estrus was reported in approximately 8% of ferrets 2 – 5 months after the initial dose of proligestone. In these cases, a second dose suppressed estrus for the rest of the breeding season.[52] Megestrol acetate has been used in ferrets to prevent estrus but is not recommended because of the assumed risk of pyometra.[19,60] However, this restriction is probably not justified because pyometra has not been described in ferrets after the use of megestrol acetate.[17,29]

Remarks: Reported side-effects associated with the use of progestagens in either dogs or cats are the development of cystic endometrial hyperplasia (CEH), prolonged pregnancy, hypersecretion of growth hormone (GH), diabetes mellitus, and increased risk of neoplastic transformation of mammary tissue.[62] Of these side-effects, only prolonged pregnancy (gestation 51 days, normal 38 – 44 days) has been reported after the use of proligestone. Proligestone had been given to these two ferrets when they were in estrus and had been mated.[35]

There is no information on the use of progestagens in hobs. In other species, including humans, progestagens have been used to suppress libido and fertility in males.[23,47,67] Progestagens are rarely used for contraception of human males since they cause loss of libido and incomplete suppression of spermatogenesis.[47] For this reason the combination of progestagens and androgens is often used: it provides a better contraceptive effect than progestagens alone and the libido is maintained.[22,47] This combination would not be an option in ferrets, because libido is an undesirable characteristic in hobs. Delmadinone acetate (Tardak; Pfizer) is used to suppress libido in dogs.[67] A recent study of Beagles, however, has revealed that this progestagen does not suppress plasma testosterone concentrations.[38] Thus delmadinone acetate, when used in ferrets, may not suppress the musky odor produced by the sebaceous glands. In humans, cyproterone acetate (Androcur; Schering) and medroxyprogesterone acetate have been used to suppress libido in sex offenders.[23] Both drugs suppress plasma testosterone concentrations.

Studies with progestagens are needed to determine whether these drugs can be used to control libido and odor in hobs. In addition, the effect of progestagen administration on GH release should be studied in jills, because progestin-induced expression of the mammary GH gene has now been demonstrated in dogs, cats, and humans.[49,50,65]

"Sham" mating

Principle: Ferrets, rabbits, and cats are all induced ovulators. When ovulation is achieved without fertilization, pseudopregnancy will occur.[19]

Method: In rabbit does, the proximity of an intact male, mechanical stimulation of the vagina, or mounting by a female rabbit can induce ovulation.[24] In cats, stimulation of the vagina will result in ovulation.[15,62] In ferrets, both vaginocervical stimulation and neck-gripping are necessary to induce ovulation.[9] Because of this elaborate procedure, it is not practical for owners to try to induce ovulation in jills. As an alternative, vasectomized hobs are used in the UK to induce ovulation.[32,60] One mating leads to cessation of estrus in about 75% of ferrets and two matings to cessation of estrus in 85% of ferrets.[60]

Effect: Termination of estrus followed by pseudopregnancy lasting about 42 days.[19]

Remarks: During pseudopregnancy, jills may display nesting behavior and enlargement of the abdomen and mammary glands.[19] The nesting behavior, which includes dragging cage mates around the cage and increased aggression towards the owners, does not make this an attractive option.

Vasectomized hobs remain aggressive and have a musky odor similar to that of intact hobs.

hCG or GnRH administration

Principle: After mating there is a preovulatory LH surge that may last to up to 12 hours.[12] This LH surge can be mimicked by the administration of either human chorionic gonadotropin (hCG) or indirectly by stimulating endogenous LH release with the hypothalamic releasing hormone GnRH.

Method: Ten days after the onset of estrus, 20 µg GnRH or 100 IU hCG is given intramuscularly.[19]

Effect: Approximately 35 hours after injection the ferrets ovulate, resulting in the formation of *corpora lutea* in 95% of the cases.[45] Vulvar swelling will start to decrease within 1 week of injection. Anoestrus (pseudopregnancy) will last for 40 – 60 days.[8]

Remarks: Fox *et al*. found that multiple injections of GnRH may sensitize the ferret to the drug, resulting in anaphylactic reactions shortly after administration.[19] Antihistamine administration ameliorates these reactions within minutes. The consequences of pseudopregnancy have been mentioned in the previous section and neutering ferrets with hCG or GnRH injections only applies to jills.

Manipulation of photoperiod and administration of melatonin

Principle: As described above, the ferret's reproductive season starts when there is more than 12 hours of light per day. During the scotophase, melatonin concentrations in plasma are high.[7,59] It has been speculated that keeping ferrets either under conditions with short photoperiods or giving them melatonin would suppress the pituitary-gonadal axis.

Method: Provision of a maximum of 8 hours of light per day, or daily administration of 1 mg melatonin 8 hours after the onset of light.[13]

Effect: Ferrets kept under 8h light : 16 h darkness (8L : 16D) come into estrus only 7 weeks later than ferrets exposed to long photoperiods (14L : 10D). When ferrets kept under long photoperiods (14L : 10D) received melatonin (1 mg/day) 8 hours after the onset of light, they come into estrus only 7 weeks later than ferrets kept under similar conditions and receiving oil injections.[13] Herbert *et al*. found that in the first year after ferrets were blinded they came into estrus at the expected time, but thereafter estrus synchrony was lost.[28] Estrus periods in blinded ferrets lasted from just a few weeks to up to 60 weeks.

Remarks: A limited light regimen and administration of melatonin are not effective in inhibiting the hypothalamus-pituitary-gonadal axis in ferrets.

Immunization against GnRH

Principle: There are several reports on the use of GnRH vaccines in mammals.[21,37,46,73] Depending on the species, these vaccines aim at contraception, the prevention of boar taint, or the control of hormone-dependent cancers, including breast and prostate cancers. In ferrets, use of a GnRH vaccine would serve two goals: inactive gonads in both sexes in combination with low plasma concentrations of gonadotropic hormones.

Method: Depending on the study inoculations were performed intramuscularly, subcutaneously or intranasally.

Effect: Decreased plasma gonadotropic hormone concentrations have been reported in male rats after immunization against GnRH.[37] Testosterone concentrations decreased in bulls, boars, male rats and dogs after immunisation,[21,37,46] while in female mice a sterilizing effect was seen.[73] Reduced testes size has also been reported in boars and rats after immunization against GnRH.[37,46]

Remarks: In a pilot study conducted at Utrecht University, eight out of twelve ferrets immunized against GnRH had to be euthanased (Schoemaker and others, in preparation). Post mortem examination disclosed aspecific lympho-plasmacellular infiltrations in multiple organs (liver, kidney, lung and intestines) suggesting an a-specific immune reaction. The background of these reactions has to be unraveled before GnRH immunization can be employed in ferrets.

Immunization with LH

Principle: Inhibition of LH secretion by immunization with heterologous LH.[18,34,55]

Method: Heterologous LH is injected intramuscularly or subcutaneously.

Effect: Injection of bovine LH causes a 90% reduction in the weight of rabbit testes, and genital atrophy in female rabbits and loss of receptiveness to males.[18] In another study with male rabbits, LH and testosterone plasma concentrations decreased significantly after immunization against LH; however, FSH concentrations increased significantly.[34] Similar, but less consistent, effects of LH immunization were seen in dogs.[42] In ewes estrus and pregnancy were prevented for 2 years after immunization against LH, although plasma LH concentrations were not lower than in control ewes.[55] In these ewes FSH concentrations were also increased.

Remarks: So far, there are no reports of LH vaccination in ferrets. Since not only LH but also FSH may influence the development of hyperadrenocorticism in ferrets, there is reason for caution with LH immunization in ferrets.

Immunization with LH receptor

Principle: Induction of LH receptor dysfunction by immunization with heterologous LH receptor.

Method: Immunization of bitches with 0.5 mg bovine LH receptor (bLH-R) encapsulated in a silastic subdermal implant, followed by intramuscular booster injections.[61]

Effect: In bitches immunization with bLH-R suppresses serum progesterone concentrations for approximately 1 year, while serum concentrations of oestradiol and LH are not affected. Although stimulation with GnRH in immunized dogs leads to a LH surge, serum progesterone concentrations do not increase. Thus bLH-R immunized bitches do not ovulate or do not produce active *corpora lutea*.[61]

Remarks: The main drawback of this approach is that ferrets may fail to ovulate. Prolonged estrus can be expected, which may result in bone-marrow suppression. In male mice, immunization against LH-R caused reduces androgen production.[54] In male ferrets, this might result in reduced aggressive behavior and decreased intensity of their musky

odor. Again some caution is warranted because high LH-R antibody titers in mice had an agonistic effect, resulting in hypertestosteronemia.[54]

Depot GnRH agonist

Principle: Depot GnRH agonists increase the levels of gonadotropic hormones, followed by a desensitization of gonadotroph receptors, resulting in decreased LH and FSH plasma concentrations.[69] The exact mechanism of the desensitization is still not clear.[30]

Method: Of the available formulations, leuprolide acetate (Lupron Depot 3.75 mg, TAP Pharmaceuticals Inc) is used to treat hyperadrenocorticism in ferrets.[70] Ferrets weighing less than 1 kg receive an intramuscular dose of 100 µg at monthly intervals and ferrets heavier than 1 kg receive 200 µg per month. A similar treatment protocol might also be effective for contraceptive purposes. Slow-release implants have been described in humans [14] and dogs.[68,69]

Effect: When leuprolide acetate is given to jills in estrus, ovulation will probably occur due to the initial LH surge seen after injection. Therefore leuprolide acetate should be administered before the breeding season, otherwise it has no advantage over regular GnRH. In dogs, however, leuprolide acetate induces estrus.[31] It is therefore uncertain whether this drug will be useful in neutering ferrets.

Remarks: Slow-release implants suppress reproductive function in dogs.[68] GnRH implants might therefore be an option for use in ferrets.

GnRH receptor antagonist

Principle: Competitive GnRH receptor occupancy with GnRH receptor antagonists results in a decreased release of gonadotropic hormones by the pituitary gland.[30]

Method: The available GnRH receptor antagonists have to be injected, but orally active non-peptide GnRH antagonists are currently being developed for use in humans.[48]

Effect: The initial increase in gonadotropic hormones, seen with GnRH agonists, are not seen with GnRH receptor antagonists. The use of these receptor antagonists will therefore result in an immediate decrease in gonadotrophic hormone concentrations.

Remarks: Until now, only a few GnRH receptor antagonists have been registered for use in humans.[30] New and longer-acting drugs are being developed. Degarelix (FE200486, Ferring) currently seems to be the most promising of these GnRH receptor antagonists.[11] While older GnRH antagonists caused increased histamine release after injection, the newer drugs do not have this side-effect. Nevertheless, local reactions at the site of injection are still common.[30] These drugs seem to be promising for future use in ferrets.

Conclusion

There are several potential ways to influence reproductive function in ferrets, other than surgical castration. Progestagens seem practical for use in jills but need to be studied for use in hobs. Possible future alternatives may be the use of slow-release GnRH implants, GnRH antagonists, or immunization against GnRH. Detailed studies are needed before these techniques can be recommended for neutering ferrets.

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Chapter 11

Summarizing discussion and conclusions

Hyperadrenocorticism is a common disease in ferrets.[9] In recent years evidence has accumulated that this disease differs from hyperadrenocorticism in humans (Cushing's syndrome), dogs, and cats, in that adrenocortical sex steroids, rather than cortisol play, an important role in the pathogenesis.[10] In this study attention was focused on the possible pathogenetic role of high plasma concentrations of gonadotropic hormones as a result of neutering. In addition, diagnostic dilemmas were addressed, such as the apparently increased urinary excretion of cortisol in the absence of hypercortisolemia.

First, the influence of sampling techniques on plasma concentrations of pituitary and adrenocortical hormones was studied **(chapter 3)**. Manual restraint resulted in a significant increase in the plasma concentrations of cortisol and ACTH, whereas anesthesia did not have a significant effect. Plasma concentrations of 17α-hydroxyprogesterone were unaffected by either manual restraint or anesthesia, whereas plasma concentrations of α-MSH were greatly influenced by both procedures. Manual restraint caused a decrease in plasma α-MSH concentrations, whereas isoflurane anesthesia resulted in an increase in α-MSH concentrations. Medetomidine anesthesia had no effect on plasma concentrations of α-MSH.

Although medetomidine anesthesia did not affect any of the hormones measured, isoflurane anesthesia was used to establish reference ranges for plasma concentrations of ACTH and α-MSH **(chapter 4)**, because the depth of anesthesia is easy to control and recovery is quick.[11] These reference ranges can be used in the clinic provided that the same sampling procedure is used. Reference ranges (percentiles $P_{2.5}$ and $P_{97.5}$ with a probability of 95%) for plasma concentrations of ACTH and α-MSH in healthy neutered ferrets were 2.9 – 22 pmol/l (13 – 100 ng/l) and 4.8 – 108 pmol/l (8 – 180 ng/l), respectively. Ferrets with hyperadrenocorticism had plasma concentrations of ACTH and α-MSH of 0.2 – 58.3 pmol/l (1 – 265 ng/l) (median 9.9 pmol/l [45 ng/l]) and 6 – 88.8 pmol/l (10 – 148 ng/l) (median 27.6 pmol/l [46 ng/l]), respectively. These values were not significantly different from those of healthy neutered ferrets. For this reason, we concluded that this disease should be regarded as a normocortisolemic and corticotropin-independent condition.

In order to increase insight into the significance of the commonly increased urinary corticoid/creatinine ratios (UCCR) in hyperadrenocorticoid ferrets, cortisol metabolism, and urinary steroid profiles were studied. In both healthy ferrets and a ferret with hyperadrenocorticism, 10% of [1,2,6,7 ^{3}H]cortisol was cleared via the urine, thus allowing accurate measurement of cortisol by radioimmunoassay **(chapter 5)**. High-performance liquid chromatography (HPLC) demonstrated that one third of the urinary corticoids was unconjugated cortisol, whereas the remaining peaks could be attributed to more hydrophilic conjugates or metabolites. None of the adrenal sex steroids appeared in the HPLC fractions. Together, this indicates that the UCCR in ferrets primarily reflects urinary cortisol excretion.

A distinct seasonal variation in the UCCR was observed in healthy intact ferrets, and in two neutered hyperadrenocorticoid ferrets, and an increased UCCR was measured in 27 of 31 privately owned ferrets with hyperadrenocorticism. However, no seasonal fluctuation in the UCCRs was seen in healthy neutered ferrets. Three explanations are postulated for the increased UCCR and the unaltered plasma concentrations of cortisol, namely (1) sexual arousal, (2) gonadotropin-dependent cortisol secretion, and (3) lowering of the plasma

concentration of cortisol-binding-globulin (CBG). Sexual arousal, which leads to activation of the pituitary-adrenocortical axis, may play a role in healthy ferrets and in the hyperadrenocorticoid ferrets with an elevated UCCR during the breeding season. This explanation would be in agreement with the largely unchanged plasma ACTH concentrations. However, in the hyperadrenocorticoid ferrets the resistance to dexamethasone suppression points to adrenocortical autonomy and/or ACTH-independent stimulation of the adrenal cortex. In these clinical cases, one has to assume that only small amounts of cortisol are produced by the adrenocortical hyperplasia and neoplasia, insufficient to cause suppression of ACTH. The latter effect may be reinforced by the increased concentrations of sex steroids affecting the binding capacity of CBG. A decrease in the binding capacity of CBG may be associated with an elevated fraction of free cortisol, while plasma total cortisol concentrations remain unchanged.

Further support for the finding that hyperadrenocorticism in ferrets is an ACTH-independent condition was provided by morphological studies of the pituitary gland of ferrets **(Chapter 6)**. The distribution of melanotropes and corticotropes in the pituitary glands of healthy ferrets appeared to be similar to that in dogs. Gonadotropes were found in the pars distalis of neutered, but not in intact, ferrets. Two of the ten ferrets with hyperadrenocorticism had a pituitary tumor. These tumors did not stain for any of the pituitary hormones. The low prevalence of pituitary tumors in hyperadrenocorticoid ferrets is in sharp contrast to the high number of pituitary tumors found in human, canine, and feline patients with Cushing's syndrome (60 – 80%). A large number of pituitary tumors are found in seemingly healthy humans (2 – 27%),[6] which may suggest that the pituitary microadenomas found in two hyperadrenocorticoid ferrets were not related to adrenal disease, but were rather an incidental finding. The pituitary tumors found in our ferrets, however, had characteristics of clinically non-functional gonadotrope tumors, as seen in humans.[4] Possibly the lack of gonadal feedback on hypothalamic GnRH, as a result of neutering, may have initiated pituitary tumor development.

In the United States of America, it has been postulated that early neutering, at 6 weeks of age, contributes to the high incidence of hyperadrenocorticism in ferrets.[9] In The Netherlands ferrets are neutered after 6 months of age. In a survey held among Dutch ferret owners **(Chapter 7),** a rather high prevalence of hyperadrenocorticism (0.55%: 95% confidence interval: 0.2 – 1.1%) was found, indicating that ferrets do not have to be neutered at an early age to develop hyperadrenocorticism. However, there was a linear correlation between age at neutering and age at time of diagnosis of hyperadrenocorticism, indicating that neutering did play a role in the development of this disease. On the basis of these findings we suspected that gonadotropic hormones play a role in the development of hyperadrenocorticism in ferrets.

The next step was to study the presence and function of gonadotropin receptors in the adrenal glands of healthy ferrets and of ferrets with hyperadrenocorticism **(Chapter 8 and 9)**. Positive immunohistochemical staining with a luteinizing hormone receptor (LH-R) antibody was found in the zona glomerulosa and the zona fasciculata of adrenal glands from healthy, intact 6 to 15-month-old ferrets. In addition, 44 adrenal hyperplasias and neoplasias from 46 ferrets with hyperadrenocorticism stained positive for the LH-R. The presence of LH-R-positive cells in the outer layers of the adrenal cortex was an unexpected

finding since adrenal androgens are produced in the inner layer of the adrenal cortex, the zone where LH-R-positive cells have been found in the human adrenal cortex.[7] The presence of LH receptors in the zona glomerulosa, however, is not unique to ferrets. In transgenic (TG) mice with chronically elevated serum LH concentrations, LH-R mRNA has been localized across the whole adrenal cortex, including the zona glomerulosa.[3] The histological characteristics of the LH-R-positive cells in the adrenal adenomas and carcinomas in hyperadrenocorticoid ferrets did not allow us to define the zone of origin. Because of the mixed cell character of the adrenal tumors in ferrets, however, it is tempting to speculate that they originate from the zona intermedia, which contains the progenitors of all the cell types of the adrenal cortex. To investigate the functionality of the LH-Rs, *in vivo* GnRH stimulation tests were performed with healthy neutered ferrets and neutered ferrets with hyperadrenocorticism. In the healthy ferrets, plasma concentrations of cortisol, androstenedione, and 17α-hydroxyprogesterone did not rise after the intravenous administration of a GnRH agonist, whereas plasma concentrations of androstenedione and 17α-hydroxyprogesterone did rise after the administration of this agonist in hyperadrenocorticoid ferrets. These findings indicate that although LH-Rs are present in the adrenals of young healthy ferrets, they are not functional.

To find out which gonadotropic hormone is responsible for the observed increase in plasma concentrations of sex steroids in ferrets with hyperadrenocorticism, plasma concentrations of LH and FSH were measured in the ferrets subjected to GnRH stimulation. LH plasma concentrations clearly increased after GnRH administration whereas no clear-cut increase in plasma FSH concentrations was seen. Administration of FSH did not promote the release of adrenocortical steroids, whereas hCG resulted in a gradual increase in the plasma concentrations of androstenedione and 17α-hydroxyprogesterone in one of two ferrets with hyperadrenocorticism. To rule out hormone production by other organs in the *in vivo* stimulations tests and the possibility of direct stimulation by GnRH of the adrenal cortex of ferrets, *in vitro* stimulation test were performed with adrenal cells suspended in culture medium. From these tests it became clear that neither GnRH nor FSH had a significant influence on the production of adrenocortical steroids, whereas hCG induced the production of these hormones.

As mentioned above, the LH-R is not always functional in ferret adrenal glands. A diminished or absent response to an agonist may be due to receptor desensitization. This would explain why in neutered ferrets, with persistently high LH concentrations, the LH-Rs were not functional. In neutered ferrets with hyperadrenocorticism, however, the receptors were functional. A mutation in the receptor may be responsible for this regain of function. It is also possible that the expression of LH-Rs increases after hormonal stimulation.[12] In mice there is increasing evidence that the LH-R can function as a tumor promoter when stimulated by the ligand hormone.[5]

Plasma FSH concentrations were extremely low in some ferrets with hyperadrenocorticism. This was unexpected since the loss of the negative feed-back on gonadotropic hormone secretion after neutering usually results in increased gonadotropin concentrations.[2] It is possible that inhibin, a hormone which is known to suppress FSH secretion, is produced by ferret adrenal tumors. The inhibin α subunit has been demonstrated in adrenal tumors of ferrets with hyperadrenocorticism.[8] In humans virilizing

adrenal adenomas secrete inhibin α in much higher amounts than normal adrenal glands do.[1] However, the possible role of this peptide or related peptides in the feedback control of FSH secretion in ferrets with hyperadrenocorticism remains to be elucidated.

Since surgical neutering seems to be a major risk factor in the development of hyperadrenocorticism in ferrets, the literature was reviewed for current and future alternatives for surgical neutering of ferrets **(Chapter 10)**. Of the alternatives, the use of progestagens seems the most practical in jills, but the effect of these drugs in hobs is uncertain. Other potential alternatives for the future may be slow-release GnRH implants, GnRH antagonists, or immunization against GnRH.

- Medetomidine anesthesia has less effect than manual restraint and isoflurane anesthesia on plasma concentrations of pituitary and adrenocortical hormones.
- Plasma concentrations of ACTH and α-MSH do not differ significantly between healthy ferrets and ferrets with hyperadrenocorticism.
- Cortisol is predominantly excreted through the hepatobiliary route. Only 10% of cortisol is excreted via the urine. This is, however, sufficient to allow accurate measurement of urinary cortisol concentrations.
- Urinary corticoid to creatinine ratios are increased in neutered hyperadrenocorticoid ferrets, and in intact ferrets during the breeding season.
- Three explanations are postulated for the increased urinary cortisol/creatinine ratios and unaltered plasma concentrations of cortisol, namely (1) sexual arousal, (2) gonadotropin-dependent cortisol secretion, and (3) lowering of the plasma concentration of cortisol-binding-globulin (CBG).
- Hyperadrenocorticism in ferrets is not associated with corticotropic adenomas. The tumors found in two of the 10 cases had similarities with clinically non-functioning gonadotrope tumors, which are known to occur in humans.
- Age at neutering and age at time of diagnosis of hyperadrenocorticism are significantly correlated, indicating that neutering plays a role in the development of this disease.
- Intravenous administration of a GnRH agonist results in elevation of plasma concentrations of androstenedione and 17α-hydroxyprogesterone in neutered ferrets with hyperadrenocorticism, but not in healthy neutered ferrets. This seems to indicate that cellular modification of LH-R is required for functionality, or that the cellular derailment is caused by (re)gain of functionality of the receptor.
- Adrenocortical steroid secretion can be stimulated *in vitro* by hCG, while neither GnRH nor FSH influences the secretion of these steroids, indicating that LH is the gonadotropic hormone which most likely plays a role in the development of hyperadrenocorticism in ferrets.
- The use of progestagens may provide a suitable alternative for surgical neutering in jills, but first of all possible effects on the release of growth hormone need to be studied. Currently there is no alternative for surgical neutering in hobs.

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Chapter 12

Samenvatting

Hyperadrenocorticisme is een veel voorkomende aandoening bij fretten.[10] In de afgelopen jaren is duidelijk geworden dat deze aandoening verschilt van hyperadrenocorticisme bij de mens (syndroom van Cushing), de hond en de kat. Bij mens, hond en kat zijn verhoogde cortisolconcentraties in het bloed verantwoordelijk voor de verschijnselen, terwijl de problemen bij het fret voornamelijk door geslachtssteroïden worden veroorzaakt.[11] In dit onderzoek is nagegaan of de hoge concentraties aan gonadotrope hormonen, ten gevolge van castratie, een rol spelen bij het ontstaan van deze aandoening bij fretten. Tevens is aandacht besteed aan diagnostische dilemma's zoals de verhoogde uitscheiding in de urine van als cortisol gemeten steroïden, in afwezigheid van verhoogde cortisolconcentraties in het bloed.

Allereerst werd onderzocht wat de invloed van verschillende methoden voor bloedafname was op plasmaconcentraties van hypofyse– en bijnierschorshormonen **(Hoofdstuk 3)**. Vasthouden van het fret resulteerde in een significante stijging van plasmaconcentraties van ACTH en cortisol, terwijl anesthesie hierop geen invloed had. Plasmaconcentraties van 17α-hydroxyprogesteron werden niet beïnvloed door vasthouden of anesthesie, terwijl plasmaconcentraties van α-MSH door deze benaderingen juist duidelijk veranderden. Vasthouden resulteerde in een daling van plasma α-MSH concentraties, terwijl isofluraananesthesie resulteerde in een stijging van dit hormoon. Medetomidineanesthesie had geen effect op plasmaconcentraties van α-MSH.

Ondanks dat medetomidineanesthesie geen effect had op de gemeten hormonen, werd voor isofluraananesthesie gekozen bij de bepaling van referentiewaarden voor plasmaconcentraties van ACTH en α-MSH **(Hoofdstuk 4)**. Het voordeel van isofluraananesthesie is dat de diepte hiervan eenvoudig is te regelen, en dat de fretten na bloedafname weer snel wakker zijn.[12] Als steeds dezelfde afnamemethode wordt gebruikt, zijn de referentiewaarden toepasbaar. De referentiewaarden (percentilen $P_{2.5}$ en $P_{97.5}$ met een waarschijnlijkheid van 95%) voor plasmaconcentraties van ACTH en α-MSH voor gezonde, gecastreerde fretten zijn vastgelegd op respectievelijk 2,9 – 22 pmol/l (13 – 100 ng/l) en 4,8 – 108 pmol/l (8 – 180 ng/l). De plasmaconcentraties van ACTH en α-MSH bij fretten met hyperadrenocorticisme waren respectievelijk 0,2 – 58,3 pmol/l (1 – 265 ng/l) (mediaan 9,9 pmol/l [45 ng/l]) en 6 – 88,8 pmol/l (10 – 148 ng/l) (mediaan 27,6 pmol/l [46 ng/l]). Deze waarden weken niet significant af van die van gezonde, gecastreerde fretten. Deze bevinding leidde tot de conclusie dat hyperadrenocorticisme bij fretten zich voltrekt onafhankelijk van het hypofysaire ACTH en met normale cortisolconcentraties in het bloed.

Om inzicht te krijgen in de betekenis van de verhoogde cortisol/creatinine ratio's in de urine van fretten met hyperadrenocorticisme, werd onderzoek gedaan naar het cortisolmetabolisme en de steroïdprofielen in de urine. Zowel bij gezonde fretten, als bij een fret met hyperadrenocorticisme werd 10% van [1,2,6,7 ^{3}H]cortisol geklaard via de urine, wat voldoende is om cortisol betrouwbaar te meten in een radioimmunoassay **(Hoofdstuk 5)**. Met behulp van high-performance liquid chromatography (HPLC) werd aangetoond dat een derde van de corticoïden in de urine ongeconjugeerd cortisol is. De andere pieken konden worden toegeschreven aan meer hydrofiele conjugaten of metabolieten. Geen van de uit de bijnier afkomstige geslachtssteroïden verscheen in de HPLC–fracties. Deze bevindingen rechtvaardigen de conclusie dat cortisol/creatinine ratio's in de urine van fretten een goede weergave zijn van de cortisolexcretie.

Bij gezonde, intacte fretten werd een duidelijke stijging van de cortisol/creatinine ratio in de urine gevonden tijdens het voortplantingsseizoen. Deze stijging werd ook gezien bij twee gecastreerde fretten met hyperadrenocorticisme. Bij de gezonde, gecastreerde fretten werd deze stijging echter niet waargenomen. De cortisol/creatinine ratio's in de urine van 27 van 31 particulier gehouden fretten met hyperadrenocorticisme waren ook verhoogd. Er worden drie mogelijke verklaringen voor de verhoogde cortisol/creatinine ratio's in de urine gepresenteerd: namelijk (1) opwinding samenhangend met de geslachtsdrift, (2) een gonadotrofine-afhankelijke cortisolsecretie en (3) een verlaging van plasmaconcentraties van cortisol-bindend-globuline (CBG). Bij gezonde, intacte fretten en fretten met hyperadrenocorticisme zou tijdens het voortplantingsseizoen de geslachtsdrift kunnen leiden tot activatie van de hypofyse-bijnierschorsas, met als gevolg verhoging van de cortisol/creatinine ratio's in de urine. Deze verklaring zou in overeenstemming zijn met de grotendeels onveranderde plasma ACTH concentraties. De resistentie tegen dexamethasonsuppressie bij fretten met hyperadrenocorticisme wijst echter op autonomie van de veranderde bijnierschors en/of op een ACTH-onafhankelijke stimulatie van de bijnierschors. Bij deze klinische gevallen moet aangenomen worden dat slechts een geringe hoeveelheid cortisol wordt geproduceerd door de afwijkende bijnierschors, die onvoldoende is om de ACTH-afgifte te remmen. Dit laatste zou nog versterkt kunnen worden door de toegenomen concentraties aan geslachtssteroïden die een invloed hebben op CBG. Een afname van de CBG-bindingscapaciteit leidt tot een toename van de vrije fractie van cortisol, terwijl de concentratie aan totaal cortisol niet hoeft te veranderen.

Aanvullende steun voor de bevinding dat hyperadrenocorticisme bij fretten ACTH-onafhankelijk is, werd verkregen door morfologische studies van de hypofyse van fretten **(Hoofdstuk 6)**. In de hypofyse van gezonde fretten lijkt de verdeling van de melanotrope en corticotrope cellen vergelijkbaar te zijn met die van de hond. Gonadotrope cellen werden in de pars distalis van gecastreerde fretten gevonden, maar niet in die van intacte fretten. Bij 2 van de 10 fretten met hyperadrenocorticisme werd een hypofysetumor gevonden. Deze tumoren kleurden voor geen van de hypofysehormonen. De lage prevalentie van hypofysetumoren bij fretten met hyperadrenocorticisme staat in schril contrast tot het grote aantal hypofysetumoren bij mensen, honden en katten (60 – 80%) met het syndroom van Cushing. Hypofysetumoren worden ook gevonden bij schijnbaar gezonde mensen (2 – 27 %).[6] Het is daarom mogelijk dat de bij 2 fretten gevonden hypofysetumoren niet gerelateerd zijn aan hyperadrenocorticisme, maar gezien moeten worden als een toevallige bevinding. De hypofysetumoren bij onze fretten vertoonden echter overeenkomsten met klinisch-niet-functionerende gonadotrope tumoren, zoals die bij de mens beschreven zijn.[4] De afwezige terugkoppeling vanuit de gonaden op hypothalamus en hypofyse, als gevolg van castratie, zou een initiërende rol gespeeld kunnen hebben bij het ontstaan van deze hypofysetumoren.

In de Verenigde Staten van Amerika is gesuggereerd dat het castreren op jonge leeftijd (6 weken) een bijdrage levert aan de hoge incidentie van hyperadrenocorticisme bij fretten.[10] In Nederland worden fretten vanaf een leeftijd van 6 maanden gecastreerd. Tijdens een enquête, die werd gehouden onder Nederlandse frettenhouders **(Hoofdstuk 7),** werd een hoge prevalentie voor hyperadrenocorticisme bij fretten van 0,55% (95% betrouwbaarheidsinterval: 0,2 – 1,1%) vastgesteld. Dit onderzoek liet zien dat castratie op

zeer jonge leeftijd geen voorwaarde is voor de ontwikkeling van hyperadrenocorticisme. Er bleek een lineaire correlatie te bestaan tussen de leeftijd waarop het fret werd gecastreerd en de leeftijd waarop hyperadrenocorticisme werd gediagnosticeerd. Deze bevindingen versterkte het vermoeden dat gonadotrope hormonen een rol spelen bij de ontwikkeling van hyperadrenocorticisme bij fretten.

De volgende stap was een onderzoek naar de aanwezigheid en functie van gonadotrofinereceptoren in de bijnieren van gezonde fretten en fretten met hyperadrenocorticisme **(Hoofdstuk 8 en 9)**. Met behulp van een antilichaam tegen de luteiniserendhormoon-receptor (LH-R) werd immunohistochemisch een positieve kleuring gevonden in de zona glomerulosa en de zona fasciculata van de bijnieren van gezonde, intacte 6 tot 15 maanden oude fretten. Daarnaast werd een LH-R-positieve aankleuring gevonden in 44 afwijkende bijnieren (hyperplasieën en tumoren) van 46 fretten met hyperadrenocorticisme. De aanwezigheid van LH-R positieve cellen in de buitenste lagen van de niet-afwijkende bijnierschors was een onverwachte bevinding, daar androgenen vooral in de binnenste laag van de bijnierschors (zona reticularis) worden geproduceerd. Dit is ook de zone waar bij mensen LH-R-positieve cellen zijn gevonden.[8] De aanwezigheid van LH-R-positieve cellen in de zona glomerulosa is echter niet uniek voor fretten. Bij muizen met chronisch verhoogde serum-LH-concentraties is LH-R-mRNA aangetroffen in de gehele bijnierschors met inbegrip van de zona glomerulosa.[3] Met behulp van de histologische kenmerken van LH-R-positieve cellen in de bijniertumoren van fretten met hyperadrenocorticisme was het niet mogelijk om aan te geven van welke zone de tumoren zijn uitgegaan. Daar deze tumoren een mengeling van celtypen bevatten is het echter verleidelijk om aan te nemen dat ze van de zona intermedia afkomstig zijn. Deze zone ligt tussen de zona glomerulosa en zona fasciculata en bevat de voorlopers van alle celtypen van de bijnierschors.

Om de functionaliteit van de LH receptoren *in vivo* te onderzoeken werden GnRH-stimulatietesten uitgevoerd bij gezonde gecastreerde fretten en gecastreerde fretten met hyperadrenocorticisme. Bij gezonde fretten stegen de plasmaconcentraties van cortisol, androsteendion en 17α-hydroxyprogesteron niet na de intraveneuze toediening van een GnRH-agonist, terwijl de plasmaconcentraties van androsteendion en 17α-hydroxyprogesteron wel stegen na toediening van deze agonist bij fretten met hyperadrenocorticisme. Deze bevindingen duiden erop dat in de bijnieren van jonge gezonde intacte fretten weliswaar LH-receptoren aanwezig zijn, maar dat deze receptoren niet functioneel zijn.

Om erachter te komen welke gonadotrope hormonen verantwoordelijk zijn voor de toegenomen plasmaconcentraties aan geslachtssteroïden bij fretten met hyperadrenocorticisme werden plasmaconcentraties van LH en FSH gemeten bij fretten die onderworpen werden aan een GnRH stimulatietest. Plasma LH concentraties stegen duidelijk na toediening van GnRH, terwijl er geen duidelijke stijging optrad van de plasma FSH concentraties. Toediening van FSH resulteerde niet in een stijging van bijnierschorshormonen, terwijl toediening van hCG resulteerde in een geleidelijke stijging van plasmaconcentraties van androsteendion en 17α-hydroxyprogesteron in één van de twee onderzochte fretten met hyperadrenocorticisme. Om na te gaan of tijdens de *in vivo* stimulatietesten andere organen een bijdrage leveren aan de hormoonproductie en voor

onderzoek naar een eventuele directe invloed op de bijnierschors van fretten, werden *in vitro* stimulatietesten uitgevoerd op bijniercellen in een kweekmedium. Uit deze testen werd duidelijk dat noch GnRH noch FSH een significante invloed hebben op de productie van bijnierschorshormonen, terwijl hCG de productie van deze hormonen stimuleert.

De LH-R is, zoals hierboven al vermeld, niet altijd functioneel in de bijnierschors van fretten. De afname of afwezigheid van een respons na toediening van een agonist kan veroorzaakt worden door desensitisatie van de receptoren. Dit zou verklaren waarom bij gecastreerde fretten, met aanhoudend hoge LH-concentraties, de LH-receptoren niet functioneel waren. De receptoren waren echter wel functioneel bij gecastreerde fretten met hyperadrenocorticisme. Een mutatie van de receptor zou verantwoordelijk kunnen zijn voor het functieherstel. Het is ook mogelijk dat een toename van de expressie van de LH-receptoren optreedt na blootstelling aan liganden.[13] Bij muizen worden steeds meer aanwijzingen gevonden dat LH-receptoren het ontstaan van tumoren kunnen bevorderen bij stimulatie door LH.[5]

Plasma-FSH-concentraties waren erg laag bij enkele fretten met hyperadrenocorticisme. Dit was een onverwachte bevinding, daar het verlies van de negatieve terugkoppeling op de gonadotrope hormonen door castratie gewoonlijk leidt tot een stijging van plasmaconcentraties van deze hormonen.[2] Een mogelijke verklaring voor de lage plasma-FSH-concentraties is dat bijniertumoren van fretten inhibine, een hormoon dat de FSH secretie kan remmen, produceren. De inhibine α subunit is aangetoond in bijniertumoren van fretten met hyperadrenocorticisme.[9] Androgeen-producerende tumoren bij de mens scheiden meer inhibine α af dan normale bijnieren.[1] De mogelijke rol van dit eiwit, of aanverwante eiwitten, op de terugkoppeling van de afgifte van FSH bij fretten met hyperadrenocorticisme moet echter nog verder opgehelderd worden.

Daar chirurgische castratie een belangrijke risicofactor lijkt te zijn bij het ontstaan van hyperadrenocorticisme bij fretten, is in de literatuur gezocht naar huidige en toekomstige alternatieven voor chirurgische castratie bij fretten **(Hoofdstuk 10)**. Van deze alternatieven lijkt het gebruik van progestagenen het meest toepasbaar voor de vrouwelijke fretten. De bruikbaarheid van deze middelen bij mannelijke fretten is echter onzeker. “Slow-release” implantaten met gonadotropin-releasing hormone (GnRH), GnRH antagonisten of immunisatie tegen GnRH zijn wellicht opties voor de toekomst.

- Bij de vergelijking tussen het effect van vasthouden en anesthesie (medetomidine of isofluraan) op plasmaconcentraties van hypofyse– en bijnierschorshormonen, blijkt dat anesthesie met medetomidine het minste effect heeft op deze hormonen.
- Er is geen significant verschil tussen plasmaconcentraties van ACTH en α-MSH van gezonde fretten en fretten met hyperadrenocorticisme.
- Cortisol wordt voornamelijk uitgescheiden via de lever en gal. Slechts 10% van het cortisol wordt uitgescheiden via de urine. Dit is echter voldoende om cortisolconcentraties in de urine betrouwbaar te kunnen meten.
- Bij gecastreerde fretten met hyperadrenocorticisme en bij intacte fretten is de cortisol/creatinine ratio in de urine is tijdens het voortplantingsseizoen verhoogd.
- Als mogelijke verklaringen voor de stijging van de cortisol/creatinine ratio in de urine, bij onveranderde plasma-cortisolconcentraties, worden genoemd: (1) opwinding samenhangend met de geslachtsdrift, (2) gonadotrofine-afhankelijke cortisolsecretie, en (3) verlaging van de plasmaconcentratie van cortisol-bindend-globuline (CBG).
- Fretten met hyperadrenocorticisme hebben geen corticotrope adenomen. De hypofysetumoren die werden gevonden bij 2 van de 10 fretten met hyperadrenocorticisme vertoonden overeenkomsten met klinisch niet-functionerende gonadotrope tumoren, zoals die bij de mens voorkomen.
- Er bestaat een significante correlatie tussen de leeftijd waarop het fret wordt gecastreerd en de leeftijd waarop hyperadrenocorticisme wordt gediagnosticeerd. Dit duidt er op dat castratie een rol speelt bij het ontstaan van deze aandoening.
- In tegenstelling tot bij gezonde gecastreerde fretten, leidt de intraveneuze toediening van een GnRH-agonist bij gecastreerde fretten met hyperadrenocorticisme tot een stijging van de plasmaconcentraties van androsteendion en 17α-hydroxyprogesteron. Dit wijst er mogelijk op dat een cellulaire verandering nodig is om de receptor te laten functioneren, of dat omgekeerd een ontsporing op cellulair niveau wordt veroorzaakt door de (weer) functionerende receptor.
- De afgifte van bijnierschorshormonen kan *in vitro* gestimuleerd worden door hCG, terwijl GnRH en FSH geen invloed hebben op de secretie van deze hormonen. Dit duidt erop dat vooral LH een rol speelt bij het ontstaan van hyperadrenocorticisme bij fretten.
- Het gebruik van progestagenen is mogelijk een geschikt alternatief voor chirurgische castratie van vrouwelijke fretten. Eerst moet echter onderzocht worden wat de mogelijke gevolgen van deze aanpak zijn voor de afgifte van groeihormoon. Op dit moment is er geen alternatief voor chirurgische castratie bij mannelijke fretten.

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Curriculum vitae

The author of this thesis was born in Amstelveen, The Netherlands, on March 20, 1967, and graduated from secondary school (Herman Wesselink College in Amstelveen) in 1985. He started his study in Veterinary Medicine at the Faculty of Veterinary Medicine, Utrecht University in 1986 and graduated in the beginning of 1994. After graduation, an internship in Companion Animal Medicine and a Residency in Avian Medicine and Surgery was followed at the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht University. This resulted in recognition as an Avian specialist in The Netherlands, Europe (Diplomate of the European College of Avian Medicine and Surgery [ECAMS]) and the United States of America (Diplomate of the American Board of Veterinary Practitioners [ABVP] – certified in Avian Practice). Since 1995 the author is employed as veterinarian at the Division of Avian and Exotic Animal Medicine of the Department of Clinical Sciences of Companion Animals, Faculty of Veterinary Medicine, Utrecht.

De schrijver van dit proefschrift werd op 20 maart 1967 geboren te Amstelveen. Het VWO-diploma (atheneum) werd behaald aan het Herman Wesselink College te Amstelveen (1979 – 1985). Van 1986 tot begin 1994 volgde de schrijver de studie Diergeneeskunde aan de Faculteit der Diergeneeskunde van de Universiteit van Utrecht. Direct aansluitend werd de opleiding tot Vogelspecialist gevolgd bij de Hoofdafdeling Geneeskunde van Gezelschapsdieren van de Faculteit Diergeneeskunde in Utrecht, wat leidde tot erkenning als specialist in Nederland, Europa (Diplomate of the European College of Avian Medicine and Surgery [ECAMS]) en de Verenigde Staten van Amerika (Diplomate of the American Board of Veterinary Practitioners [ABVP] – certified in Avian Practice). Vanaf 1995 is de schrijver werkzaam als dierenarts op de afdeling Vogels en Bijzondere Dieren van de Hoofdafdeling Geneeskunde van Gezelschapsdieren.

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Color section

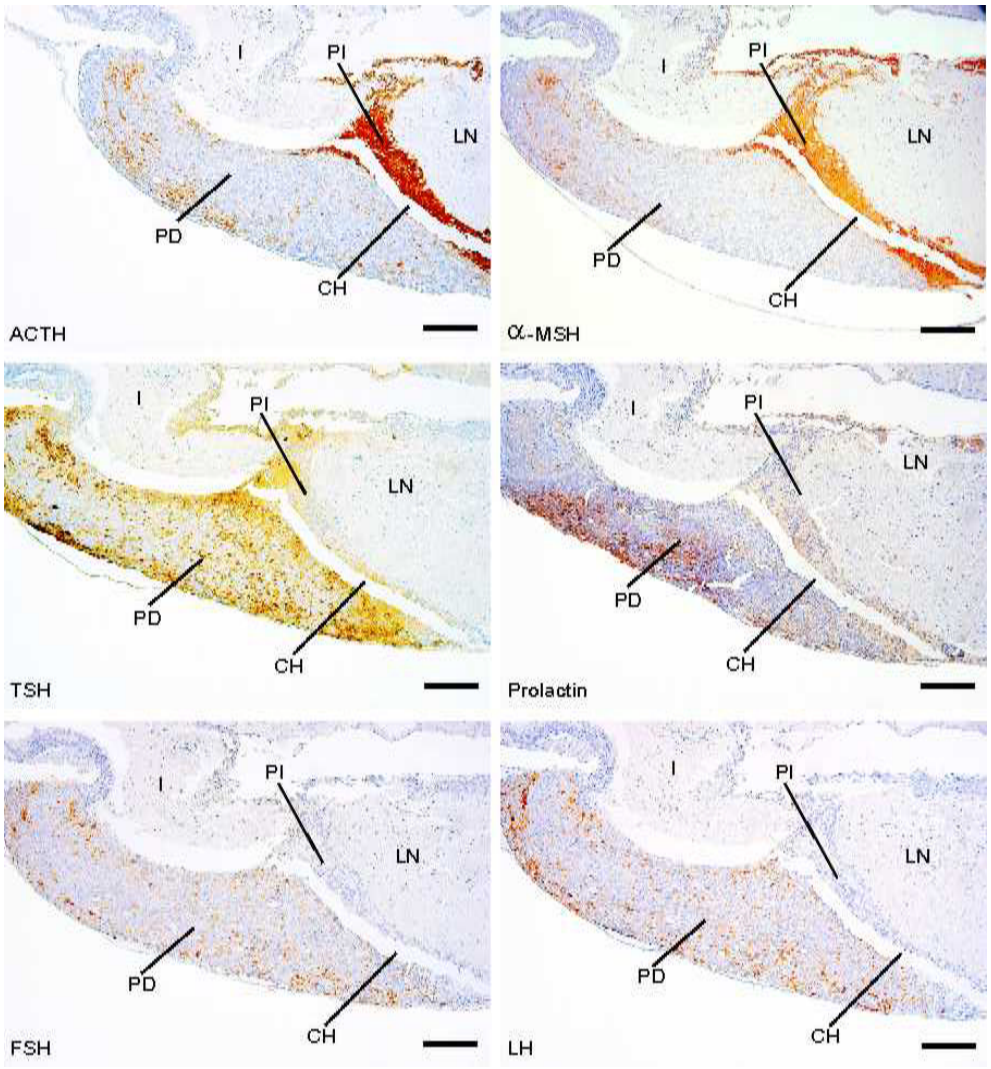

Figure 1. Midsagittal sections of the pituitary gland of a 2-year-old neutered female ferret stained for $ACTH_{1-24}$, α-MSH, TSH, prolactin, FSH and LH. For explanation of lettering see legend to Figure 1. Immunostain. Bar is 0.2 mm.

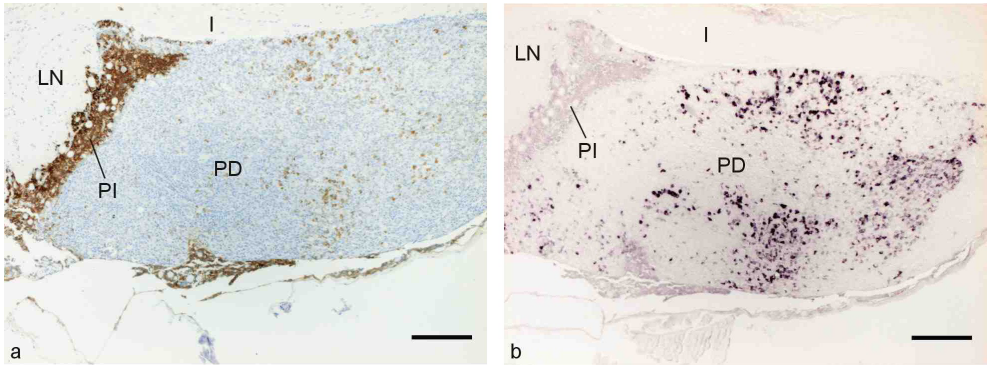

Figure 2. Midsagittal consecutive sections of the pituitary gland of a 5-year-old neutered male ferret immunohistochemically stained for adrenocorticotropic hormone (ACTH) with 3 different antibodies: [a] monoclonal mouse antibody to synthetic $ACTH_{1-24}$; [b] polyclonal rabbit antibody to human $ACTH_{18-39}$; [c] polyclonal rabbit antibody to synthetic carp $ACTH_{10-23}$. For explanation of lettering see legend to Figure 1. Bar is 0.2 mm.

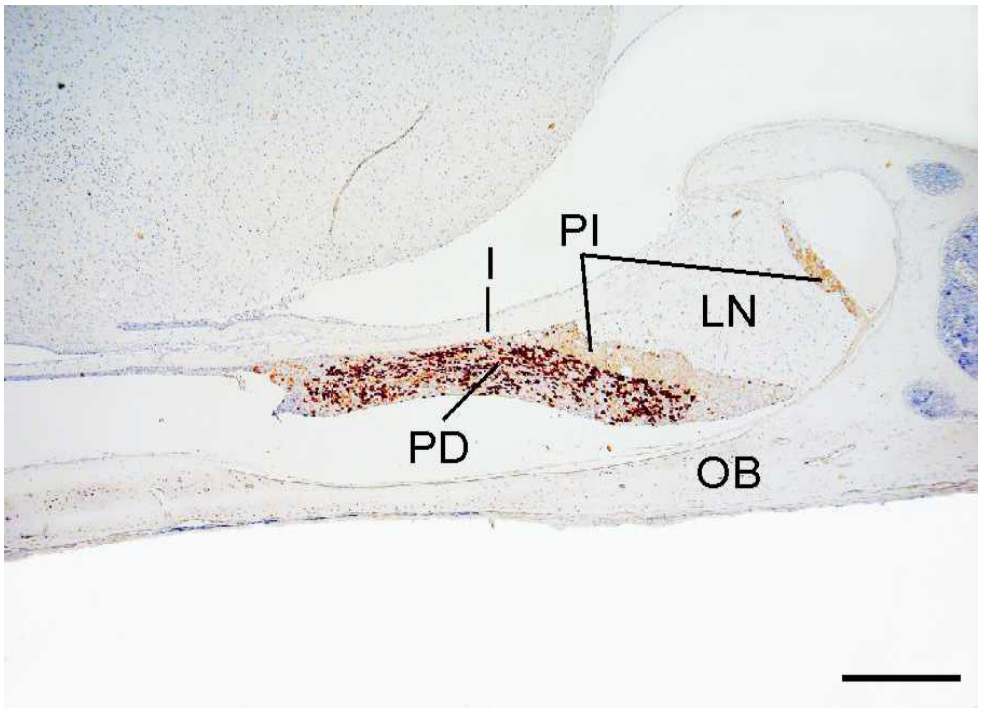

Figure 3. Midsagittal section of the pituitary gland of a 2-year-old neutered female ferret [surrounded by the *os basisphenoidale* (OB)] stained for growth hormone (GH). For explanation of lettering see legend to Figure 1. Immunostain. Bar is 0.5 mm.

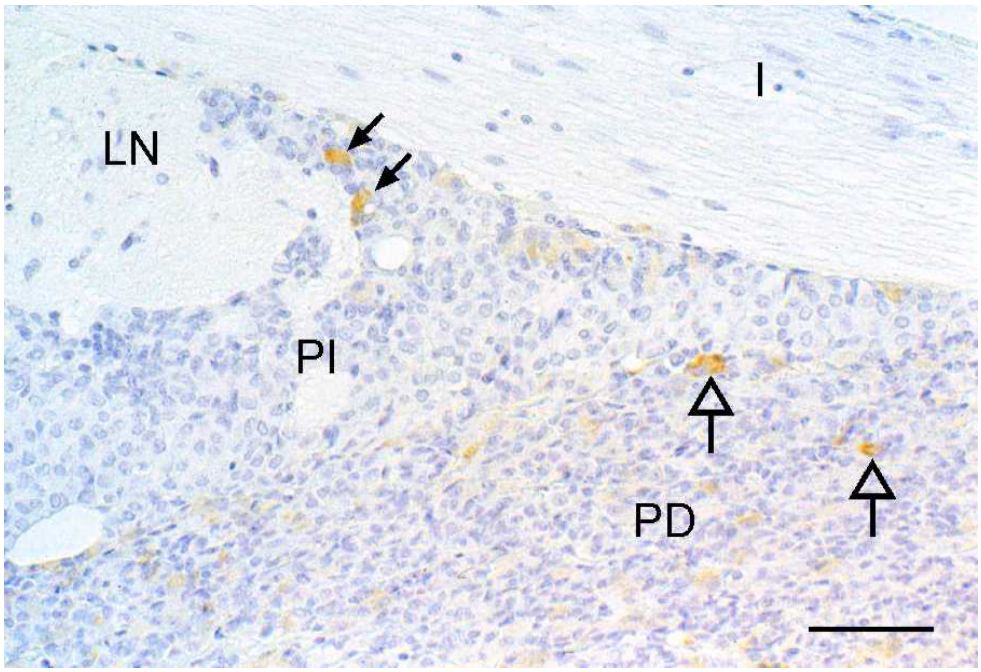

Figure 4. Midsagittal section of the pituitary gland of a 2-year-old neutered female ferret stained for luteinizing hormone (LH). Closed arrows indicate LH-positive cells within the *pars intermedia adenohypophysis* (PI). Open arrows indicate LH-positive cells within the *pars distalis adenohypophysis* (PD). For explanation of lettering see legend to Figure 1. Immunostain Bar is 0.05 mm.

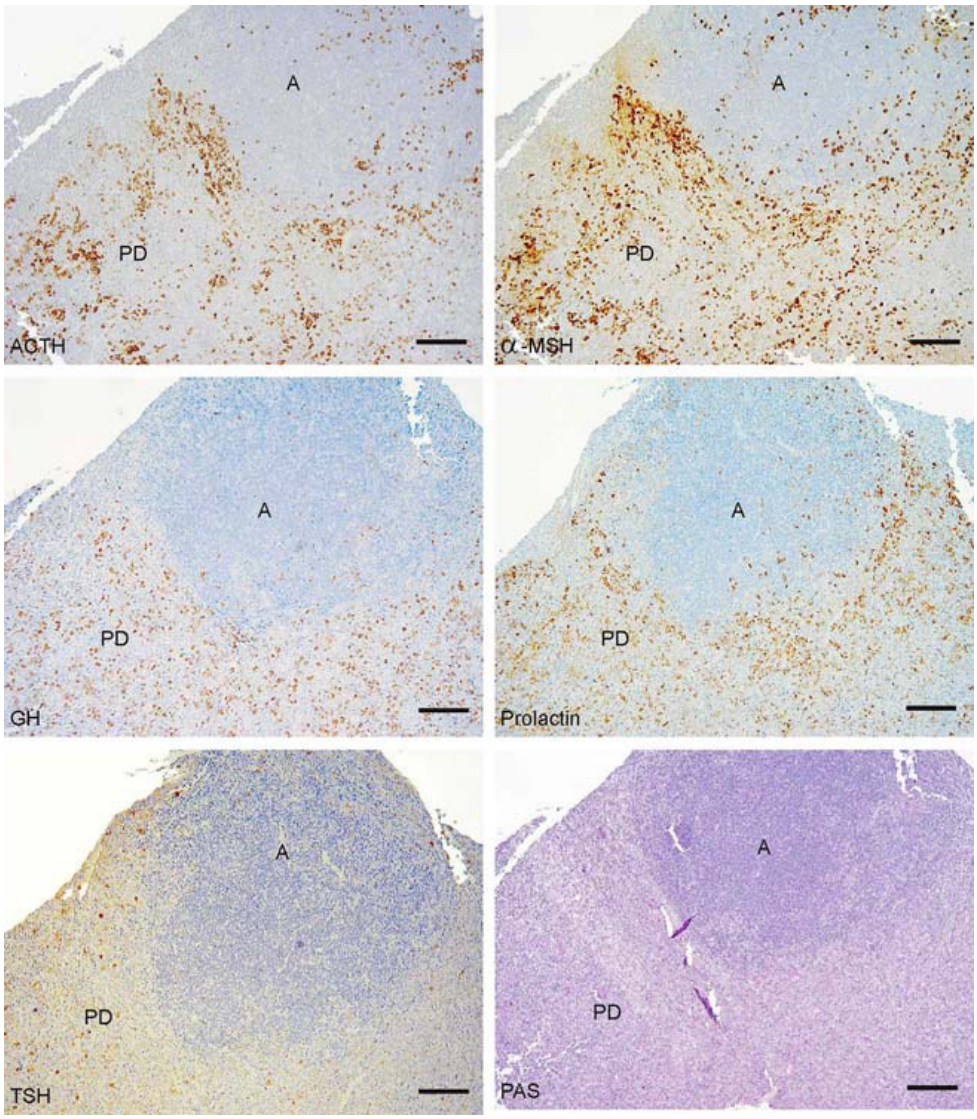

Figure 5. Horizontal sections of the pituitary gland of a 5-year-old male ferret (No 3) with hyperadrenocorticism due to adrenocortical tumors. At the top there is a round chromophobic adenoma with a diameter of 1.2 mm. This adenoma is stained with antibodies against $ACTH_{1-24}$, α-MSH, GH, prolactin, TSH and PAS. (PD: *pars distalis adenohypophysis*; A: adenoma). Immunostain. Bar is 0.2 mm.

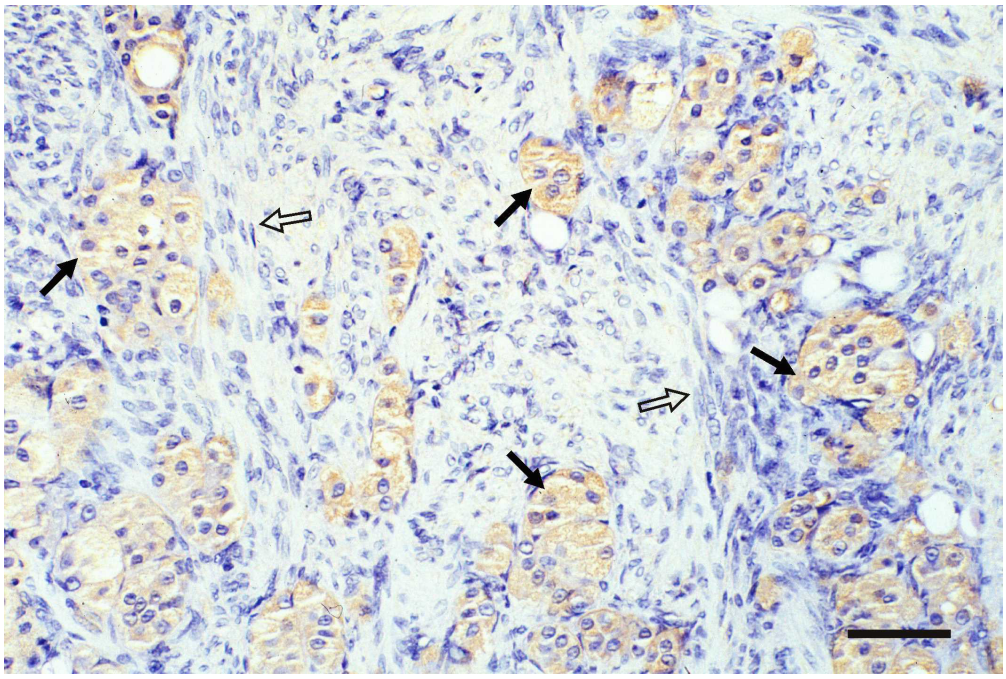

Figure 6. Photomicrograph of an adrenal adenoma from a neutered hyperadrenocorticoid ferret and immunohistochemically stained with the P1B4 LH-R antibody. Multiple LH receptor positive (brown) cells (closed arrows) are surrounded by LH receptor negative cells, some of which are spindle-shaped (open arrows). (Bar = 25 μm)

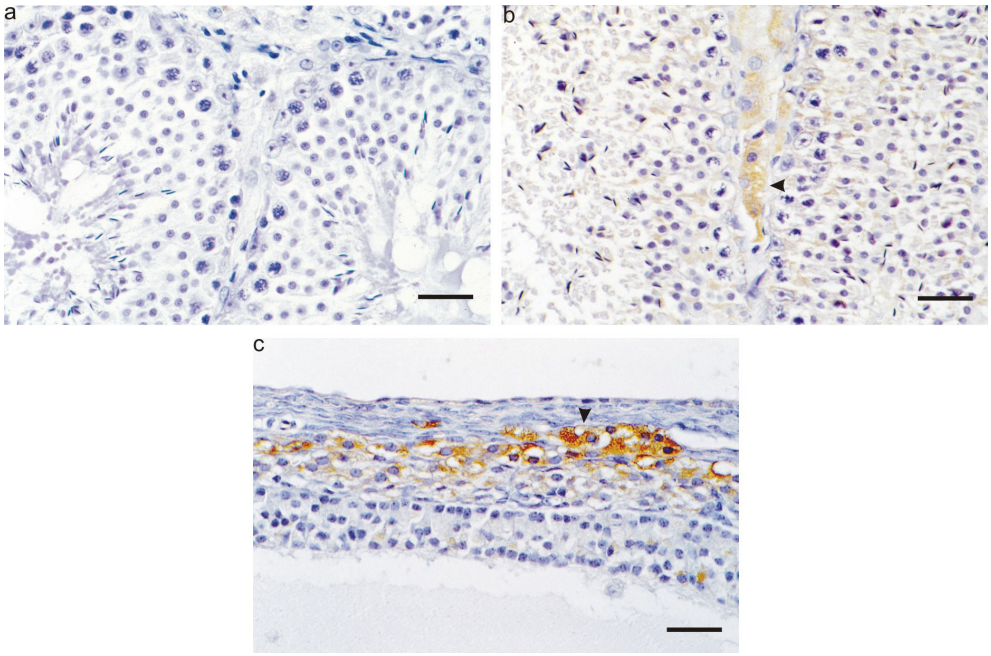

Figure 7. Histological sections of testes and ovaries of healthy ferrets

a. Serum control section of a ferret testes incubated with normal mouse antiserum. No background staining could be detected.
b. LH-receptor-positive staining (brown) of the Leydig cells (arrowhead) of the same testes immunohistochemically stained with the P1B4 LH-R antibody
c. Thecal cells (arrowhead) of a ferret ovary immunohistochemically stained with the P1B4 LH-R antibody

Bar = 60 µm

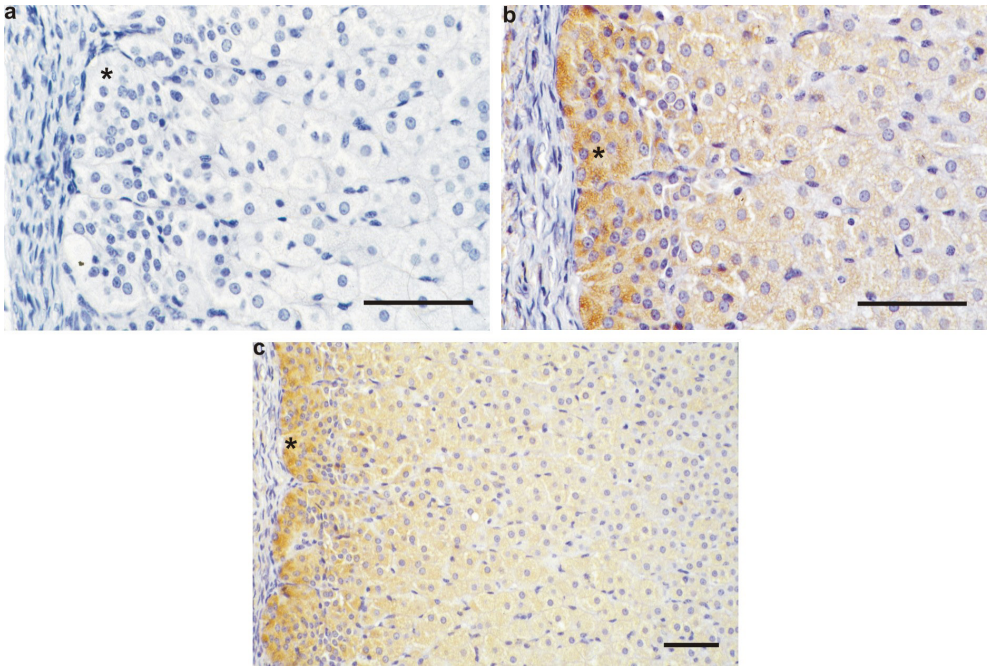

Figure 8. Histological sections of the adrenal cortex of a healthy 1-year-old ferret

a. Serum control section incubated with normal mouse antiserum. No background staining could be detected.

b. Immunohistochemically staining with the P1B4 LH-R antibody. Positive staining (brown) is seen throughout the entire cortex including the zona glomerulosa (asterisk) and zona fasciculata.

c. Immunohistochemically staining with the P1B4 LH-R antibody. Positive staining (brown) is seen throughout the entire cortex including the zona glomerulosa (asterisk) and zona fasciculata.

Bar = 60 µm

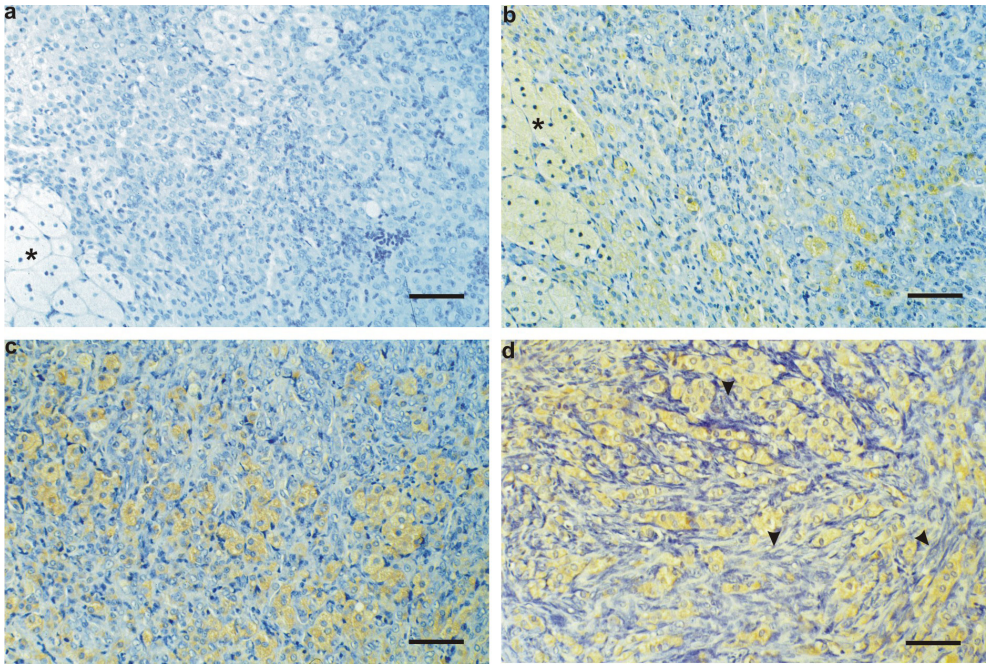

Figure 9. Representative microphotographs of different regions of an adrenal adenoma of a hyperadrenocorticoid ferret stained with the P1B4 LH-R antibody. (a) Serum control section incubated with normal mouse antiserum. No background staining could be detected. (b,c) LH-receptor positive (brown) cells are surrounded by small LH-R negative cells. The large clear cells (asterisk) of photos (a) and (b) are from the same region of the adenoma. (d) In between the LH-R-positive (brown) cells many spindle shaped cells (arrowheads) are visible in this part of the adenoma. Bar = 60 µm