Oestrus in the Mare

with Emphasis on Deviant Behaviour and Adrenal Gland Function

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Abstract

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Some owners appear to experience problems with their mares due to excessive oestrous signs and/or changes in their mare's behavioural characteristics across the oestrous cycle. Few such mares are examined at equine clinics and, thus, the cause of such behaviour is often unknown. Adrenal sex steroids have been suggested to cause oestrous behaviour in ovariectomised (OVX) mares and, if secreted in an aberrant manner, aggressive behaviour in intact mares. The aims of this study were to gain further knowledge regarding oestrus in the mare by (1) studying intact mares with a history of deviant behaviour ('problem' mares), intact control mares and OVX mares using objective methods and (2) by investigating mare adrenal gland function, specifically sex steroid hormone production after synthetic adrenocorticotropic hormone (ACTH) treatment (tetracosactide) in intact mares ('problem' mares and controls) and OVX mares, and adrenal steroid hormone receptors using immunohistochemistry in intact mares, OVX mares and oestradiol-treated OVX mares. In addition, since treatment with progestins to suppress oestrous cyclicity in competition mares is not endorsed by most European Community countries, a preliminary study was performed to determine if dioestrous ovulations and a subsequent prolonged luteal phase can be induced using human chorionic gonadotropin (hCG).

Neither oestrous-cycle stage nor whether a mare was a 'problem' or control mare affected the mare's response to a novel object test or isolation test. In the novel object test, mares (both control and 'problem') tended to touch the novel object earlier when tested in dioestrus first, indicating mares in the luteal phase may be more responsive to a novel environment. An effect of learning or habituation to the novel object test was found. OVX mares showed oestrous behaviour during both conventional teasing and during a 'paddock teasing' method, but without a normal cyclic pattern.

Adrenal production of progesterone, androstenedione and testosterone was demonstrated and the hormones were shown to be of both ovarian and adrenal origin in intact mares at oestrus. Ovariectomy affected the diurnal cortisol rhythm, with a more pronounced rhythm after ovariectomy. 'Problem' mares showed a lower cortisol response to ACTH treatment compared with controls. Staining for oestrogen receptor alpha (ERα) and progesterone receptor (PR) (weak) was found in cell nuclei of the adrenal cortex (all mares) and in the cytoplasm of medullary cells (for ERα, in OVX mares and one intact mare only).

Ovulation was induced in three of four hCG-treated mares that also developed prolonged luteal phases, as assessed by progesterone analysis. However, although no control mare ovulated after saline treatment, two control mares showed prolonged luteal activity during the study period. Further studies are needed to determine the method's efficacy and applicability in practice.

The present study confirms that mares after ovariectomy show oestrous signs, but without normal cyclicity. The adrenal gland produces a significant amount of androgens, which may be involved in such paradoxical sexual behaviour. The presence of ERα and PR in the adrenal cortex indicates a direct effect of ovarian steroids on adrenal gland function. 'Problem' mares showed few deviations from control mares, but the low number of animals and their heterogenicity may have confounded the results.

Key words: mare, oestrus, behavioural tests, ovariectomy, adrenal gland, steroid hormones, steroid hormone receptors, human chorionic gonadotropin, prolonged luteal phase.

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About the horse…

“It is the difficult horses that have the most to give you.”

- Lendon Gray

“You can tell a gelding, but you have to ask a mare.”

- Unknown

“Horses can't talk but they can speak if you listen.”

- Unknown

“Care, and not fine stables, makes a good horse.”

- Danish Proverb

Contents

Appendix

List of original papers I-V.
The thesis is based on the following papers, which will be referred to in the text by Roman numerals:

I. Hedberg, Y., Dalin, A.-M., Öhagen, P., Holm, K.R. & Kindahl, H. 2005. Effect of oestrous-cycle stage on the response of mares in a novel object test and isolation test. *Reproduction in domestic animals 40*, 480-488.

II. Hedberg, Y., Dalin, A.-M., Forsberg, M., Lundeheim, N., Hoffmann, B., Ludwig, C. & Kindahl, H. 2006. Effect of ACTH (tetracosactide) on steroid hormone levels in the mare. Part A: Effect in intact normal mares and mares with possible estrous related behavioral abnormalities. *Animal reproduction science*, in press (e-published ahead of print 2006).

III. Hedberg, Y., Dalin, A.-M., Forsberg, M., Lundeheim, N., Sandh, G., Hoffmann, B., Ludwig, C. & Kindahl, H. 2006. Effect of ACTH (tetracosactide) on steroid hormone levels in the mare. Part B: Effect in ovariectomized mares (including estrous behavior). *Animal reproduction science*, in press (e-published ahead of print 2006).

IV. Hedberg, Y., Sukjumlong, S., Kindahl, H. & Dalin, A.-M. Steroid hormone receptors ERα and PR characterised by immunohistochemistry in the mare adrenal gland. *Submitted for publication.*

V. Hedberg, Y., Dalin, A.-M., Santesson, M. & Kindahl, H. 2006. A preliminary study on the induction of dioestrous ovulation in the mare – a possible method for inducing prolonged luteal phase. *Acta veterinaria scandinavica 48*: 12. (Published online July 26, 2006).

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Abbreviations

17α-OH-P4	17-α-hydroxyprogesterone
17α-OH-P5	17-α-hydroxypregnenolone
17β-HSD	17-β-hydroxysteroid dehydrogenase
3β-HSD	3-β-hydroxysteroid dehydrogenase
A4	androstenedione
ACTH	adrenocorticotropic hormone
AVP	arginine vasopressin
CRH	corticotropin-releasing hormone
DHEA	dehydroepiandrosterone
DHEA (S)	sulphated dehydroepiandrosterone
ER	oestrogen receptor
ERα	oestrogen receptor alpha
hCG	human chorionic gonadotropin
HPA	hypothalamo-pituitary-adrenal
HPG	hypothalamo-pituitary-gonadal
i.m.	intramuscularly
IU	international units
LH	luteinising hormone
mRNA	messenger ribonucleic acid
Oe	oestradiol benzoate
OVX	ovariectomised
P450	cytochrome P450 family
P450aldo	P450 aldosterone synthetase
P450c11	P450 11-β-hydroxylase
P450 c17	P450 17-α-hydroxylase/17-20 lyase
P450c21	P450 21-hydroxylase
P450scc	P450 cholesterol side-chain cleavage
PR	progesterone receptor
SD	standard deviation
Stb.	Standardbred trotter
S-Tferase	sulphotransferase
Sw. Wbl.	Swedish Warmblood

General introduction

Background

Excessive oestrous behaviour and behavioural problems related to the oestrous cycle in the mare (sometimes referred to as nymphomania) can cause problems with riding and handling, particularly if the mare is intended for competition use (Christensson, 1991; Taugbøl, Andresen & Thomassen, 1991; McDonnell, 1992; Jorgensen et al., 1996). In a survey performed at the Swedish University of Agricultural Sciences by the Department of Clinical Sciences (return-rate 79%), the most common complaints of mares with abnormal oestrous behaviour were a refusal to move forward and frequent urination, with some mares also showing aggressive behaviour (Vikström, 2001). Diagnostic differentials for such behavioural changes include hormone producing ovarian tumours (e.g. granulosa-theca cell tumours) (Meagher et al., 1978), musco-skeletal or neurologic disorders and urinary tract infections. However, often no pathological reason for the disturbed behaviour is found. Moreover, the diagnosis of deviant oestrous behaviour is frequently set by the owner, without thorough evaluation of the animal, and few mares appear to be examined at equine clinics (Lindström, 2000). When still permitted, long-acting progesterone therapy was frequently used in Standardbred trotters in both Sweden and Norway (Taugbøl, Andresen & Thomassen, 1991; Lindström, 2000). However, if the hormonal treatment was used in mares with behavioural abnormalities, or simply to suppress normal oestrous signs, is not known. Nonetheless, the treatment of competing mares with long-acting progesterone to control undesired sexual behaviour is no longer endorsed by most European Community countries.

Although poorly investigated, an aberrant production of adrenal sex steroid hormones, such as testosterone, has been suggested to be a cause of temperamental changes in some mares, e.g. an increased aggressiveness (Beaver & Amoss, 1982; Roberts & Beaver, 1987). Further, adrenal gland hormone production has been implicated as a possible cause of oestrous behaviour in ovariectomised (OVX) and seasonally anovulatory mares, since treating such mares with dexamethasone, and thus inhibiting adrenal gland hormone production, suppressed oestrous signs (Asa et al., 1980b). Additionally, an interaction between the hypothalamo-pituitary-adrenal (HPA) axis and the hypothalamo-pituitary-gonadal (HPG) axis has been described in various species, such as rats, sheep and non-human primates (Viau & Meaney, 1991; Xiao et al., 1994; Van Lier, Perez-Clariget & Forsberg, 2003), but limited knowledge is available in the horse. Considering the potential of an adrenal influence on sexual behaviour in the mare, it is of interest to study this interaction between the HPA and HPG axes also in the equine.

Oestrous-related behavioural problems – where to begin?

In view of the scant scientific literature available on behavioural problems associated with the oestrous cycle in mares, with subjective evaluations and owner diagnosis abundant, there is a need to objectively study such mares. Therefore mare behaviour during the oestrous cycle should be investigated using objective methods that have been previously validated. Secondly, in view of the possible role of adrenal gland steroid hormones in mare oestrous behaviour, studies on mare adrenal function are needed.

Oestrous behaviour in mares

Mares may show a wide variation in oestrous intensity (Ginther, 1992a), which can make it difficult to distinguish normal oestrous behaviour from abnormally intense signs. The mare is a poly-oestrus seasonal breeder and, in the Northern Hemisphere, the ovulatory season extends from approximately March/April to September/October, with a shorter season in ponies compared with horses (Ginther, 1992b). During the winter months, there is usually a period of ovarian non-activity (anovulatory period), although some mares may ovulate throughout the year (Hughes, Stabenfeldt & Evans, 1972; Colquhoun et al. 1987; Fatallah, Younis & Jawad, 1988). The oestrous cycle is on average 19-24 days with an oestrous phase of approximately 6.5 days and a dioestrous phase of 15 days (Ginther, 1992b). The majority of mares ovulate on the penultimate or last day of oestrus and the increase in progesterone from the developing corpus luteum causes cessation of oestrous behaviour (Hughes, Stabenfeldt & Evans, 1975). During the transition from the anovulatory period to cyclic activity, the mare often shows longer and irregular periods of oestrus not accompanied by ovulation (Hughes, Stabenfeldt & Evans, 1972; Ginther, 1974; Hughes, Stabenfeldt & Evans, 1975). Normal oestrous behaviour in the mare includes posturing (squatting), tail raise, flashing clitoris and urination (Fig. 1). Some mares tend to show silent oestrus (that is, no behavioural receptive signs).

Fig. 1. Normal behaviour at oestrus.

Unlike females of other non-primate species, OVX mares and mares with inactive ovaries can show symptoms of oestrus (Ginther, 1974; Hughes, Stabenfeldt & Evans, 1975; Asa et al., 1980a; Hooper et al., 1993; Dalin, Andresen & Malmgren, 2002). Such behaviour is most likely not a learned response, since fillies ovariectomised at four months of age can still show oestrus at the time when they would have reached puberty (Wesson & Ginther, 1981). Interestingly, low levels of exogenous oestradiol (0.5 mg) will elicit oestrous behaviour in OVX mares, which may indicate that such mares are primed with steroid hormones of non-ovarian origin (Hillman & Loy, 1975). Moreover, oestrous behaviour in OVX mares suggests that oestrogens of ovarian origin are not necessary for behavioural oestrus in the mare. In addition, Munro, Renton & Butcher (1979) could not show a consistent correlation between oestrous intensity and plasma levels of oestrogens and androgens in intact mares. Nevertheless, the ovary seems to have a regulatory role in controlling the mare's oestrous cycle through the inhibitory effects of progesterone secreted by the corpus luteum, with OVX and seasonally anovulatory mares showing irregular oestrous signs of greater frequency compared with intact mares (Asa, Goldfoot & Ginther, 1979; Asa et al., 1980a).

Assessment of temperament – use of objective behavioural tests

Behavioural tests have recently begun to be used in an attempt to objectively describe the temperament of horses (Le Scolan, Hausberger & Wolff, 1997; Wolff, Hausberger & Le Scolan, 1997; Visser et al., 2001, 2002, 2003). One aspect of temperament, emotionality, can be defined as the capacity of an individual to perceive and react to (potentially) dangerous events (Boissy, 1995). This perception of (potential) danger elicits fear or anxiety, emotional states that many behavioural tests attempt to investigate in the exposed individual (i.e. the individual's fearfulness or nervousness). Subjective ratings of horses correlated well with objective behavioural scores and heart rates derived from objective behavioural tests (Le Scolan, Hausberger & Wolff, 1997; Visser et al., 2003). However, although it is often regarded that mares have a less stable temperament than geldings, attributed to the hormonal fluctuations of the oestrous cycle, few behavioural studies of the horse have addressed this issue. In cattle (Bouissou, 1990; Boissy & Boiussou, 1994) and sheep (Vandenheede & Bouissou, 1993a; Vandenheede & Bouissou, 1993b), sex steroid hormones appear to affect other behaviours than sexual behaviour. In order to understand more fully what effect the oestrous cycle may have on other aspects than receptive behaviour alone in the mare, subjecting mares to the same behavioural test in both phases of the oestrous cycle (oestrous phase and luteal phase) is required.

Temperamental characteristics may be assessed using a variety of methods. Novel object tests have been used to assess behaviour in a variety of species, such as pigs (Lawrence, Terlouw & Illiys, 1991; Spoolder et al., 1996), heifers (Boissy & Bouissou, 1995), sheep (Vandenheede, Bouissou, & Picard, 1998) and horses (Le Scolan, Hausberger & Wolff, 1997; Wolff, Hausberger & Le Scolan, 1997; Visser et al., 2001). This behavioural test investigates exploratory behaviour of an unknown object, which is either introduced as a surprise effect (sudden appearance) (Lawrence, Terlouw & Illiys, 1991; Vandenheede, Bouissou, & Picard, 1998; Visser et al., 2001) or is already present in the test arena when the animal is introduced (Boissy & Bouissy, 1995; Spoolder et al., 1996; Le Scolan, Hausberger & Wolff, 1997; Wolff, Hausberger & Le Scolan, 1997). Behavioural variables recorded in novel object tests vary between studies and may include posture, locomotion, vocalisation and attention directed towards as well as exploration of the object (including latencies to approach and touch the object). Short-term consistency of most behavioural variables studied in a novel object test in young horses was found, indicating reliability of the test and accuracy of the identified behavioural traits (Visser et al., 2001).

Isolation from conspecifics is stressful to gregarious species, such as sheep (Syme, 1981; Vandenheede & Bouissou, 1993a; Vandenheede & Bouissou, 1993b) and domestic fowl (Mills et al., 1993). Isolation tests have been used in sheep to assess individual differences in sociability (that is, the animal's response to separation from a group) (Syme, 1981). Although few objective isolation tests have been performed in the horse, social contact is important to this herd-living species and has been related to a reduced risk of behavioural abnormalities (McGreevy et al., 1995).

Heart rate is another method to measure an individual's emotionality and it was demonstrated that an increase in the heart rate of horses was positively correlated with an increase in nervousness (McCann, 1988). By using heart rate measurements, it was possible to distinguish sheep of different sociability, even when no behavioural differences were observed (Syme & Elphick, 1982/83). It was found that horses' heart rates, but not behavioural responses, in a novel object test correlated well with the riders' assessments of the horses (Visser et al., 2003).

Adrenal gland function

The adrenal cortex

The equine adrenal cortex consists of three zones that have different histological morphology and steroid producing capacity (Almahbobi et al., 1985). The outer zona glomerulosa produces the mineralcorticoid aldosterone, whereas both the middle zona fasciculata and inner zona reticularis are sources of glucocorticoids. In the horse, the main glucocorticoid is cortisol (James et al., 1970; Larsson et al., 1979; Alexander, Irvine & Donald, 1996). Corticosterone is also produced, although to a lesser extent (Hoffis et al., 1970; James et al., 1970; Bottoms et al., 1972). Cortisol secretion is regulated by adrenocorticotropic hormone (ACTH) secreted by the anterior pituitary gland, which in the horse, like other species, is released in frequent peaks of irregular amplitude and time (Alexander, Irvine & Donald, 1996). Regulation of cortisol secretion in the horse has been extensively studied by Alexander, Irvine & Donald (1996), who used a special non-surgical technique for collecting pituitary venous blood. In the unstressed horse, arginine vasopressin (AVP) seemed to be the main stimulus for ACTH release, whereas the major role of corticotropin-releasing hormone (CRH) was to 'fix the cortisol setpoint' (that is, to keep the cortisol levels stable). During stress, the secretagogues that stimulated ACTH secretion depended on the type of perturbation. Alexander, Irvine & Donald (1996) also suggested that there is an ACTH-release inhibiting factor that during chronic stress can counteract the stimulatory effects of CRH, leading to hypocortisolaemia.

The adrenal cortex not only produces mineralcorticoids and glucocorticoids, but in several species studied was also found to be a source of sex steroid hormones. For example, ACTH treatment resulted in increased plasma levels of progesterone in OVX cows, zebu cows and heifers (Watson & Munro, 1984; Bolaños, Molina & Forsberg, 1997; Båge et al., 2000) and in intact sows (Tsuma et al., 1998; Brandt et al., 2006) and intact sheep (Van Lier et al., 1998), as well as in increased plasma levels of testosterone in OVX mares (Watson & Hinrichs, 1989). Adrenal androgen production was also observed when mare cortical tissue was incubated with pregnenolone (Silberzahn et al., 1984). In species that produce adrenal androgens, these are secreted predominately from the zona reticularis (McKenna & Cunningham, 1991).

It is the relative enzyme distribution as well as the characteristics of the enzymes (such as catalytic activity, substrate specificity and degree of induction by ACTH) in the adrenal cortex that determine which steroid hormones are produced and species differences exist [Hornsby & Aldern, 1984; Conley & Bird, 1997 (review)]. Metabolism of pregnenolone can occur via two different pathways, either to progesterone (Δ4 pathway) or to 17-α-hydroxypregnenolone (17α-OH-P5) (Δ5 pathway) (Fig. 2). For example, the human and primate zona reticularis secrete a substantial amount of dehydroepiandrosterone (DHEA) (Δ5 pathway) [Cutler et al., 1978b; Hornsby & Aldern, 1984; Bélanger et al., 1990; Labrie, 1991 (review); Martel et al., 1994; Conley, Pattison & Bird, 2004 (review)] whereas adrenal androgen levels were found to be relatively low in other mammals, including the horse (Cutler et al., 1978b; Hornsby & Aldern, 1984; Bélanger et al., 1990). Also, in some species, such as the pig, guinea pig and hamster, 17-α-hydroxyprogesterone (17α-OH-P4) is readily converted to androstenedione (Δ4 pathway) (Tremblay et al., 1994; Lee-Robichaud et al., 1995; Mathieu, Auchus & LeHoux, 2002), but only negligible conversion of 17α-OH-P4 to androstenedione occurs in human and bovine adrenal glands [Hornsby & Aldern, 1984; Zuber, Simpson & Waterman, 1986; Lee-Robichaud et al., 1995; Miller, Auchus & Geller, 1997 (review); Mathieu, Auchus & LeHoux, 2002]. To our knowledge, the distribution of enzymes in the equine adrenal cortex has not yet been studied.

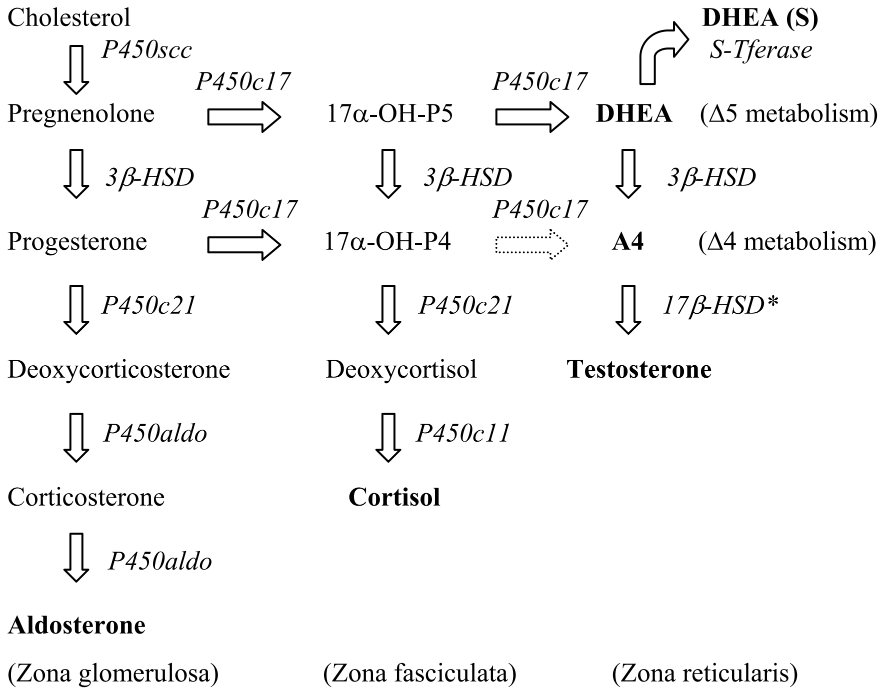

Fig. 2. Adrenocortical steroid biosynthesis in higher mammals (not rodents). The broken arrow indicates an enzymatic conversion available in e.g. pigs, but not humans or ruminants. DHEA: dehydroepiandrosterone; DHEA (S): sulphated DHEA; A4: androstenedione; P450: cytochrome P450 family; P450scc: P450 cholesterol side-chain cleavage; 3β-HSD: 3-β-hydroxysteroid dehydrogenase; P450c17: P450 17-α-hydroxylase/17-20 lyase; P450c21: P450 21-hydroxylase; P450c11: P450 11-β-hydroxylase; P450aldo: P450 aldosterone synthetase; S-Tferase: Sulphotransferase; 17β-HSD: 17-β-hydroxysteroid dehydrogenase. * = demonstrated in rhesus monkey (Martel et al., 1994). Adapted from Conley & Bird, 1997.

The adrenal medulla

The equine adrenal medulla consists of two types of chromaffin cells. In the outer medullary (juxtacortical) zone, there are large columnar chromaffin-positive cells, whereas the deeper medullary regions are composed of chromaffin-positive cells that are smaller and of irregular round to polygonal shape. Both cell types contain two types of cytoplasmic secretory granules (Gelberg, Cockerell & Minor, 1979). In all species, adrenal medullary secretion of adrenaline and noradrenaline by chromaffin cells appears to be continuous, but in response to sympathetic stimulation, excessive amounts are secreted (Dickson, 1993). In the horse, there is evidence that noradrenaline, but not adrenaline, is involved in stimulating ACTH secretion, although this effect may be counteracted during stress by the negative feedback exerted by the concomitant hypercortisolaemia (Alexander, Irvine & Donald, 1996).

Ovarian effects on adrenal function

It is well known that activation of the HPA axis can affect the HPG axis, often in an inhibitory manner [Rivier & Rivest, 1991 (review); Tilbrook, Turner & Clarke, 2002 (review)]. However, in some species, the interaction appears to be two-way, with gonadal hormones also affecting the HPA axis. This interaction has predominantly been studied in the rat, sheep and rhesus monkey. For instance, oestradiol, both endogenous and exogenous, acted stimulatory on both basal corticosterone concentrations and the corticosterone response to ACTH in intact and OVX female rats (Kitay, 1963a; Kitay, 1963b; Lésniewska, Nowak & Malendowicz, 1990; Viau & Meaney, 1991). In sheep, sex differences in the HPA axis response exist, but seem to depend on the type of stressor. For example, rams showed a greater cortisol response to hypoglycaemia, whereas ewes displayed a greater cortisol response to isolation/restraint stress (Turner et al., 2002). Van Lier, Perez-Clariget & Forsberg (2003) subsequently demonstrated a sex difference in the cortisol response to ACTH in sheep, with a greater cortisol response to ACTH treatment in ewes compared with rams. In the rat (Cutler et al., 1978a), sheep (Van Lier et al., 2003) and rhesus monkey (Hirst et al., 1992) oestradiol receptors (ER) have been found in the adrenal gland, supporting the notion of a direct effect of ovarian steroids on the adrenal gland. The possibility of oestradiol enhancement of the HPA axis in the mare has not yet been investigated and extrapolating data from other species is inappropriate.

An effect of progesterone on adrenal function has received much less attention. Adrenal progesterone receptors (PR) have been found in the rat (Uotinen et al., 1999) and PRmRNA was measured in the adrenal gland of sheep (Van Lier et al., 2003). In addition, a progesterone-binding protein was demonstrated in guinea pig adrenocortical nuclei (Demura, Driscoll & Strott, 1989). These findings indicate a direct effect of progesterone on the adrenal gland. However, the role of progesterone on HPA function is still unclear and results from studies in the rat diverge. In one study, progesterone appeared to exert a modulating effect on the oestradiol induced augmentation of HPA activity (Carey et al., 1995), whereas Viau & Meaney (1991) suggested a purely inhibitory role of progesterone on such oestradiol enhanced HPA activity. Again, studies in the mare are lacking.

Suppression of oestrous behaviour

Even though ovarian hormones are not necessary for the mare to display signs of oestrus, progesterone secretion by the corpus luteum seems essential for the mare to have a normal cyclic pattern by suppressing oestrous behaviour. This inhibitory effect of progesterone has been utilised in the mare to suppress unwelcome oestrous signs [Loy & Swan, 1966; Neely, 1988 (review); Christensson, 1991; Jorgensen et al., 1996]. However, since exogenous progestins are often banned by the authorities for equine sports, other alternative methods for suppressing unwanted cyclicity in mares have emerged, such as herbal remedies (Vikström, 2001), intrauterine ball devices (Nie et al., 2001; Rivera del Alamo, Reilas & Katila, 2005) and the crushing of embryos (Lefranc & Allen, 2004). The latter two methods both result in elevated endogenous progesterone levels. However, some of these methods lack scientific evaluation in the mare (e.g. herbal treatment) or may be considered unethical (e.g. the deliberate crushing of singleton embryos). There is thus a need for alternative, less invasive, methods to suppress unwanted oestrous signs in the mare.

Prolonged luteal phase and dioestrous ovulations

The incidence of prolonged luteal phase in the mare is relatively common (Stabenfeldt et al., 1974; Hughes, Stabenfeldt & Evans, 1975). Severe endometritis and embryonic loss after maternal recognition of pregnancy have been shown to cause prolonged luteal activity in the mare (Kooistra & Ginther, 1976; Hughes et al., 1979; Neely et al., 1979, Stabenfeldt et al., 1981; Ginther et al., 1985). The mare is unique in that it can ovulate during high endogenous progesterone concentrations and, if such ovulations take place late in the luteal phase, a prolonged luteal phase can occur (Ginther & Pierson, 1989). This phenomenon has been attributed to the refractoriness of corpora lutea to prostaglandin for up to four days, as demonstrated by both the use of exogenous prostaglandins (Douglas & Ginther, 1975) and stimulation of endogenous prostaglandin $F_{2\alpha}$ release via intrauterine saline infusions (Arthur, 1975; Neely et al., 1975). At ovulations occurring during dioestrus no oestrous signs are observed, due to the inhibitory effect of progesterone (Hughes, Marcelo & Stabenfeldt, 1985). In order to mimic dioestrous ovulations, human chorionic gonadotropin (hCG) has been used to induce ovulation in mares receiving altrenogest treatment (Daels et al., 1996). The treatment resulted in five out of six mares ovulating and developing a subsequent prolonged luteal phase; i.e. it was possible to artificially induce a prolonged luteal phase in the mare. However, Daels et al.’s method involved the use of exogenous progesterone treatment, which is prohibited during equestrian competitions in many countries. This problem could be overcome by inducing ovulation during the natural luteal phase of the mare.

Aims of the study

The general aim of this thesis was to gain further knowledge regarding oestrous behaviour and adrenal gland function in the mare by studying:

(1) Mare behaviour, using objective methods, in:

- intact mares
- mares that, according to their owners, suffered from oestrous-related behavioural problems
- OVX mares

(2) Adrenal steroid hormone production in:

- intact mares
- mares that, according to their owners, suffered from oestrous-related behavioural problems
- OVX mares

(3) Adrenal sex steroid hormone receptors

Another aim was to study a method to suppress oestrous cyclicity in mares by evaluating:

(4) the effect of hCG on dioestrous ovulations and luteal phase length

Methodological considerations

Animals

A summary of the data of the mares used in each study is shown in Table 1. Seven mares used in papers I and II were selected from a survey using questionnaires, which was performed by the Department of Clinical Sciences (‘problem’ mares). Another three mares intended to be included could not be used in the study due to being too dangerous to handle. The survey was sent to owners who believed their mares suffered from oestrous-related behavioural problems. Inclusion criteria were behavioural problems perceived by the owner to be related to the oestrous cycle or very frequent/strong oestrous symptoms. In brief, the questionnaire contained questions related to what type of activities the mare was used for, type of feeding, stable and pasture environment, previous pregnancies, type of behavioural abnormality, association of the behavioural abnormality with the oestrous cycle and if any treatment had been tried. The use of a survey sent to interested owners was regarded as a better way to obtain information on such mares than through veterinary practices, since very few mares with behavioural problems are examined at equine clinics in Sweden (Lindström, 2000). Paper III was performed on five of the mares used in the first two studies (two ‘problem’ mares and three control mares), and at least four weeks after ovariectomy had been performed. In paper IV, adrenal glands were collected from both intact and OVX mares (with or without prior oestradiol benzoate treatment). Finally, in paper V, nine intact mares were used.

Table 1. Summarised data of mares used in the respective studies.

Study	Group	Number of mares	Age-span (years)	Weight (kg)	Breed
I and II	‘Problem’	7	5-16	250-600	Various
	Control	5	5-13	500-550	Stb.
III	Intact/OVX	5	6-12	400-550	Various
IV	Intact	5	3-20*	500-600	Various
	OVX	2	8-10	400-550	Various
	OVX+Oe	3	5-12	500-550	Stb.
V	hCG	5	8-17	450-550	Stb. and Sw Wbl.
	Control	4	4-20	450-550	Stb.

* = age unknown for one mare
OVX = ovariectomised; OVX+Oe = ovariectomised with oestradiol benzoate treatment;
Stb. = Standardbred trotter; Sw. Wbl. = Swedish Warmblood

Questionnaire data regarding ‘problem’ mares (papers I and II)

Data of the ‘problem’ mares included in the study, as reported in the questionnaire, is summarised in Table 2. The mares’ undesirable behaviours had either been displayed during the entire ownership (range 1-3 years) (mares An, Ev, F and Z) or begun with a change in environment or circumstance [e.g. change of trainer (mare Be), at start of training (mare C) and when lost a foal (mare W)]. All mares, except for mare An, were actively used for riding and/or driving activities, with one mare (mare Be) competing at the time of the survey. According to the mare's owner, mare An could not be ridden due to the behavioural problem.

Table 2. Summary of questionnaire data.

Mare ID	An	Be	C	Ev	F	W	Z
Behaviour							
Frequent urination/tail raise	X	X	X		X	X	
Uncooperative	X		X		X	X	X
Aggressive					X	X	X
Screaming and hitting hind-end against box wall				X			
Pressing against handler/cart	X	X					
Time aberrant behaviour occurred							
Always			X*		X*		X*/ **
Regular intervals	X			X		X*	
At competition		X					

* behaviour most frequent at exercise; ** better when lactating

Housing and management

During the studies, the horses were kept at the Department of Clinical Sciences, Division of Comparative Reproduction, Obstetrics and Udder Health. They were stabled on straw in individual boxes indoors at night and were turned out during the day (approximately, 08.00 to 15.00 h). All mares were fed three times daily with hay (morning, noon and evening) and once daily in the afternoon with a small amount of oats, sugar beet and minerals. Water was provided ad libitum. In papers I and II, four of the 'problem' mares were lent to the department for a period of at least two oestrous cycles. During the daytime, these client-owned mares were for safety reasons kept in individual outdoor pens (approx. 10 m x 20 m), but within sight and hearing contact of other horses. All other mares were during the day turned out together in a larger field. In paper V, after at least one month of experimental study, seven of the mares were transported to a larger pasture 30 km away from the department, where they were kept outdoors 24 hours per day until the end of the study. The mares always had free access to water.

Clinical examination (papers I and II)

In papers I and II, mares were initially subjected to thorough clinical examinations. This was done in order to rule out other possible, pathological, causes for the disturbed behaviours described in the 'problem' mares. Included was a lameness examination (walking and trotting in a straight line on a hard surface and flexion tests of all limbs), back palpation, blood test [haemoglobin, haematocrit, total white cell count (with differential count) and fibrinogen] and urine sample (to check for bacterial growth). Urine was sampled using a sterile catheter inserted into the urethra and applied to a three-partitioned selective culture plate (SELMA, National Veterinary Institute, Uppsala, Sweden) which was incubated overnight at 37 ˚C before being checked for bacterial growth. Urinary infections may cause frequent urination, which can be misinterpreted by the owner as oestrous signs. A careful gynaecological examination was also performed, including vaginoscopy. The mares that according to their owners were difficult to ride or drive also had their teeth checked.

Dexamethasone-suppression test (paper II)

In order to evaluate HPA axis function, a dexamethasone-suppression test was performed in all mares used in paper II (for details, see paper II). This test was done after the main study had been completed, in order not to influence the results.

Evaluation of behaviour

Oestrous detection (papers I, II, III and V)

As a daily routine, mares were teased by leading the mare to the stallion's box, which had a small opening allowing some contact between the mare and the stallion. Oestrous signs were scored as follows: + 1 for standing in a straddled posture, +1 for flashing clitoris/urination, +1 for raising tail, -1 for kicking, -1 for switching tail, and –1 for ears back. The mare was considered to be in oestrus if she showed at least two positive scores. In papers I and II mares were teased twice daily, before being let out at pasture and when brought in. Teasing in papers III and V was performed once daily, except for on the saline and ACTH treatment days in paper III, when the mares were also teased in the afternoon. For paper V a simplified protocol was used, with oestrous scores designated as follows: +1 for standing in a straddled posture, + 1 for flashing clitoris/urination and + 1 for raising tail. Behavioural oestrus was in this paper defined as a score of +2 or above.

'Paddock teasing' (paper III)

In paper III, the OVX mares were, in addition to conventional teasing, also teased by letting each mare and the stallion loose together in a paddock for 10 min. This was in order to allow stallion and mare to get more contact, as well as providing a more natural setting, which was anticipated to stimulate oestrous behaviour. In order to allow more accurate comparisons between studies, full and weak oestrus were defined as described by Asa et al. (1980a), although they used 20 minute long teasing periods (for details, see paper III).

Objective behavioural tests (paper I)

The novel object test was chosen because it has been well validated for use in the horse (Le Scolan, Hausberger & Wolff, 1997; Visser et al., 2003). An isolation test was also included since oestrous behaviour may be related to the gregariousness of a mare. Behavioural tests in oestrus were performed when mares had a follicle ≥ 30 mm, oedema in the uterus and/or displayed behavioural oestrus. The tests in dioestrus were carried out between days 5 and 12 post-ovulation when the concentration of progesterone was high (Ginther, 1992c). For more details of the methods used, see paper I.

Isolation test

In the isolation test the mare was alone in its box and in the stable unit for three minutes, unable to see or hear another horse. Due to practicalities, this test was not video recorded; only heart rate recordings were taken.

Novel object test

The test procedure was based on a model used by Visser et al. (2001), with some minor modifications. The behavioural variable ‘head low’ used in Visser et al.’s (2001) study was excluded, since it was regarded as difficult to distinguish from the variable ‘exploring other’. The behavioural variable ‘tail up’ was not included since it was not displayed by the mares in the present study. The test was video recorded by a person out of view from the mare being tested. Because a cross-over design was used, with each mare tested twice, mares were not only habituated to the test arena before the first test occasion, but also before the second test. An inflated ball was used as a novel object since it was an object unlikely for the mares to have come across previously and also, was unlikely to cause injury (Fig. 3). The ball was let down using an attached weight, causing also a surprise effect.

Fig. 3. The test arena used in the novel object test.

Data acquisition

Heart rate was recorded using Polar Vantage (Polar Electro OY, Kempele, Finland) with the receiver set to record the instantaneous heart rate every five seconds (Fig. 4). This method to register heart rate was used since it was found to work well in a novel object test using young horses (Visser et al., 2001). However, in the present study, the electrodes failed to properly register the heart rate in three of the mares (two 'problem' mares and one control mare) in the novel object test, due to rolling on the ground or exuberant behaviour. In addition to heart rate recordings, seven behavioural variables were also measured in the novel object test (Table 3).

Fig. 4. Equipment used for heart rate registration.

Gynaecological and transrectal ultrasound examinations (papers I, II, III and V)

In papers I, II and V, mares were rectally examined with ultrasonography (six MHz linear-array scanner, 485 Anser, Pie Medical, Maastricht, the Netherlands) to determine follicle size, presence of a corpus luteum and the presence of oedema in the uterus. Oedema in the uterus was graded as follows: grade one indicated a heterogenic appearance, but no defined endometrial folds; grade two indicated an increase in heterogenicity with some endometrial folds visible; and grade three indicated extreme heterogenicity with large anechoic areas. In paper III, ultrasonic examination of the uterus was not performed in the OVX mares. However, since one mare was mated by the stallion during 'paddock teasing', it was checked for fluid in the uterus. Then it was observed that the mare had oedema in the uterus and similar findings were subsequently made when the remaining four OVX mares were examined. Because of this unexpected oedema, mares were again examined using ultrasound on five separate occasions in November.

Table 3. Behavioural variables measured in the novel object test. (Adapted from Visser et al., 2001).

Behavioural variable	Abbreviation	Definition
Snorting[a]	Snort	Forceful expulsion of air
Exploring object[b]	Expo	Eyes, ears and head pointed towards object, nose below belly line and horse within 2 meters of object
Exploring other[b]	Expoth	Eyes, ears and head pointed forwards but not towards object, nose below belly line and horse not within 2 meters of object
Focused on object[b]	Foc	Eyes, ears and head pointed towards object
Latency to enter circle[b]	Latcirc	Latency to come within 2 meters of object
Latency to touch object first time[b]	Lattou	
Trotting or cantering[b]	Tr/Ca	

[a] Frequency
[b] % of total test time

Blood sampling and hormonal analyses

Non-frequent blood sampling – progesterone and oestradiol analyses

In each of the papers, blood samples for progesterone and/or oestradiol content were taken by jugular venipuncture for various purposes. In papers I and II, blood samples were collected daily for progesterone analysis, as an additional test to determine normal oestrous cyclicity. In paper I, blood samples on the behavioural test days were, in addition to progesterone, also analysed for oestradiol content. In paper III, in order to confirm high endogenous oestradiol levels, samples at 14:00 h (before ACTH or saline treatment) and at 02:00 h (after treatment) in intact mares at oestrus were analysed for oestradiol content. In paper IV, one blood sample was collected from each mare prior to euthanasia and analysed for progesterone and oestradiol content. In paper V, starting on the hCG treatment day, blood samples were collected for progesterone analysis twice weekly for one month, and thereafter once weekly, in order to determine luteal phase length. Since blood samples after the initial month were obtained only once weekly, a higher cut-off level, than that normally used for progesterone (3 nmol/l; Ginther, 1992c), was used for these samples to define luteal phase (10 nmol/l) (Koskinen et al., 1990; Koskinen, Huhtinen & Katila, 1996).

Frequent blood sampling and ACTH treatment (papers II and III)

In papers II and III, blood sampling was performed frequently over two 26-hour periods per mare (i.e. one control period with saline treatment and one ACTH-treated period). The blood sampling in intact mares was performed when the mares had a follicle ≥ 30 mm, oedema in the uterus and/or displayed behavioural oestrus. An indwelling catheter was inserted into the jugular vein to minimise the stress of frequent blood sampling (Milacath®, 14 gauge x 3.5", MILA International Inc.). For ACTH treatment, tetracosactide [2 ml (0.5 mg); Synachten®, 0.25 mg/ml, Novartis] diluted in saline to a volume of 5 ml was used. Tetracosactide is a synthetic polypeptide containing the first 24 of the 39 amino acids of naturally occurring ACTH and is reported to have all of the pharmacological properties of endogenous ACTH (Novartis, Täby, Sweden).

All blood samples during the 26-hour periods were analysed for cortisol, progesterone and androstenedione content (30 samples/mare/occasion). Pre-treatment samples and samples obtained during the first eight-hour period post-treatment were analysed for testosterone content (14 samples/mare/occasion). In paper III, one pre-treatment sample and samples taken during the first five hours post-treatment were analysed for oestradiol content in OVX mares only (10 samples/mare/occasion).

Hormone assays

Cortisol (papers II and III)

Plasma cortisol was determined by a solid-phase radioimmunoassay (Coat-A-Count Cortisol, Diagnostic Products Corporation, Los Angeles, USA). The kit was used according to the manufacturer's instructions with some minor modifications (for details, see paper II). All samples were run in duplicates.

Progesterone

Plasma progesterone was determined by a solid-phase radioimmunoassay kit (Coat-a-Count Progesterone, Diagnostic Products Corporation, Los Angeles, USA) that was used according to the manufacturer's instructions (see papers I, II, IV and V).

Androstenedione (papers II and III)

Plasma androstenedione was determined by a liquid-phase radioimmunoassay after ether extraction (DSL-4200, Diagnostic Systems Laboratories, Inc., TX, USA). The procedure used has been described in ewes (Viñoles et al., 2003), although some modifications were made (see paper II). Parallelism of the standard curve with serial dilution of equine plasma samples was demonstrated.

Testosterone (papers II and III)

The concentration of testosterone was analysed using a liquid-phase radioimmunoassay after toluene extraction. All samples were run in duplicates. For details, see paper II.

Oestradiol-17β (papers I, III and IV)

Oestradiol concentration was determined by a radioimmunoassay using a DPC kit (Diagnostic Product Corporation, Los Angeles, CA, USA) after ether extraction, as reported for use in bovine plasma (Sirois & Fortune, 1990). The method has previously been validated (Meikle et al., 1997). All samples were run in duplicates (see papers I, III and IV).

Oestradiol treatment (paper IV)

Three of the OVX mares were treated with 0.5 ml of oestradiol benzoate (5 mg/ml) i.m. 18 to 22 hours before euthanasia and adrenal gland tissue collection (OVX+Oe). Oestradiol has been shown to up-regulate both the progesterone receptor (PR) and oestrogen receptor alpha (ERα) in uterine tissue in e.g. the sow (Sukjumlong et al., 2003) and the mare (Aupperle et al., 2000; Hartt et al., 2005), as well as receptor genes in most uterine cells in the ewe (Ing & Tornesi, 1997).

Immunohistochemical procedures (paper IV)

The immunohistochemical procedure has been described previously by Sukjumlong et al. (2003). Tissue samples from adrenal glands and uteri were fixed in 10 % formaldehyde for up to two days and thereafter embedded in paraffin prior to performing immunohistochemistry. A standard avidin-biotin immunoperoxidase technique (Vectastain® ABC kit, Vector Laboratories, Inc., USA) was applied to detect the steroid receptors (ERα and PR) using two different mouse monoclonal antibodies as primary antibodies (ERα, C311-sc787, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA and PR-2C5, Zymed Laboratories, Inc.). For additional details of the procedure, see paper IV.

For classification of positively stained cells, a blind evaluation of ERα and PR positive cells was carried out by the same person (Sayamon Sukjumlong). In uterine tissue, staining intensity was graded as described by Sukjumlong et al. (2004). Because of the weaker staining intensity observed in the adrenal gland, it was not possible to use the same criteria when grading adrenal tissue as that used for uterine tissue. Adrenal staining was thus presented with a different definition; proportion of positively stained cells was classified as major (> 50 % of cells) or minor (< 50 % of cells) and staining intensity as either weak or moderate.

hCG treatment (paper V)

In paper V, mares were treated i.m. with either saline or hCG (3000 IU) when a dioestrous follicle ≥ 30 mm was detected. The hCG treatment was chosen since, as stated in the introduction, it has been shown that hCG induced ovulation during exogenous progesterone treatment may cause a prolonged luteal phase (Daels et al., 1996). The mares were followed with rectal palpation of the ovaries and uterus, including ultrasound examinations, from approximately day eight after ovulation in dioestrus. During breeding management, a follicle size ≥ 35 mm is generally considered a prerequisite for hCG treatment (Wilson et al., 1990; Kilicarslan et al., 1996; Barbacini et al., 2000). However, since it was expected that only few large dioestrous follicles would develop, a smaller follicle size (≥ 30 mm) as compared with hCG treatment at oestrus, was chosen for the present study.

Statistical analyses

In paper I, statistical analyses were calculated using Minitab®Release 14.11, 2003, and non-parametric statistical tests chosen due to the low number of animals in the study. To test the difference between the oestrous and dioestrous value for each variable, Wilcoxon Signed Rank Confidence Interval was used. To determine the effect of a mare being a 'problem' mare, compared with a control mare, analysis of variance (GLM-procedure) was calculated for each variable (behavioural and heart rate variables) where the response variable was the difference between the oestrus and dioestrus value for each variable and the model variable was either 'problem' mare or 'control' mare. The same procedure was then used to calculate the effect of *test order* of how the mares reacted in the tests (the model variable for *test order* was 'oestrus first' or 'dioestrus first'). The mares were then divided into two groups, those tested in dioestrus first and those tested in oestrus first, and one-sample Wilcoxon test used testing the median (median not equal to 0) of the difference between the values in oestrus and dioestrus for each group. In addition, to evaluate if the effect of *test order* was in part due to differences between *test occasion* (i.e. an effect of learning or habituation), one-sample Wilcoxon test was again used, testing the median (median not equal to 0) of the difference between the values in test one and test two for each variable studied.

In papers II and III, statistical analyses were carried out using the SAS software (Ver. 8; SAS Institute Inc., Cary, NC, USA). For the hormone concentrations, repeated measurement analyses of variance were performed using the MIXED procedure. The observations were for the statistical analyses grouped into five time periods: 12:00-14:00 h (period one; pre-treatment samples); 14:30-17:00 h (period two); 18:00-23:00 h (period three); 24:00-05:00 h (period four); 06:00-14:00 h (period five). The analyses were performed for each treatment day (saline or ACTH), as well as for the difference between treatment days (ACTH minus saline, within mare and sampling time). The fixed effects included in the statistical models were group of mares [control or 'problem' (paper II); intact or OVX (paper III)], time period (five periods), sampling time nested within time period, and the interaction between group of mares and time period. Least-squares means were estimated, and pair-wise tests of significance of the difference between Least-squares means were performed.

In paper II, procedure TTEST was used to determine if there were differences between the morning and afternoon oestrous detection scores for each mare group ('problem' and control), on both the saline treatment day and the ACTH treatment day. To compare the proportion of oestrous days different behaviours were displayed in each mare group during both oestrous periods studied, the GLM procedure was used. The proportion was calculated as a percentage of the total number of oestrous days the behaviour was displayed (days with behaviour/total oestrous days x 100). The statistical analyses were performed for both oestrous periods (saline treatment oestrus and ACTH treatment oestrus). The fixed effects were mare group (‘problem’ or control) and behaviour (i.e. ‘tail raise’, ‘clitorial winking/urination’, ‘straddled posture’, ‘kicking’, ‘switching tail’ and ‘ears back’). Least-squares means were estimated, and pair-wise tests of significance of the difference between Least-squares means were performed.

Due to the low number of mares used, no statistical analyses were performed in papers IV and V.

Results

Clinical examination (papers I and II) and dexamethasone-suppression test (paper II)

No sign of infections could be found in either the blood or urine samples. Except for minor reactions to the flexion tests of the limbs, none of the mares showed any musco-skeletal problems. The mares were all found to have normal ovaries and uteri, with no pathological findings. The daily blood samples confirmed that all mares were cycling normally during the experimental period. One ‘problem’ mare (mare Be) had an oestrous period of 17 days in her first cycle. This was, however, considered within normal limits for the time of year (March). The dexamethasone-suppression test to evaluate HPA function (performed at the end of the main study) showed normal suppression of the adrenal gland.

Behavioural results (papers I, II and III)

Subjective observations and conventional teasing (papers I, II and III)

In the subjective evaluation, only three of the seven ‘problem’ mares were considered to show the abnormal behaviours associated with oestrus that their owners had previously described (papers I and II). Mare An would refuse to move forward as well as press against and show oestrous symptoms towards her handler. Another mare, mare Ev, would, when in oestrus, scream and hit her hind-end on the box wall. The third mare, mare Be, showed very strong oestrous signs. Interestingly, during conventional teasing, two of the ‘problem’ mares, mare F and mare Z, tended to show ‘silent oestrus’, that is, no outward signs of behavioural oestrus. The control mares showed no abnormal behavioural changes across the oestrous cycle. However, one control mare (mare Ae) tended to show weak oestrous signs at teasing. In papers II and III, conventional teasing in the afternoon on the treatment days (saline and ACTH) was always performed during time period two, when also maximal hormone concentrations after ACTH treatment were found. There were no statistical differences between the morning and afternoon oestrous detection scores in either mare group (‘problem’ mares and control) on either sampling occasion (saline and ACTH) (t-test, $p > 0.2$). Also, no statistical differences in oestrous behaviour between ‘problem’ mares and controls, as assessed by conventional teasing, were found in either of the two oestrous periods studied (difference between Least-squared means, GLM procedure). None of the OVX mares showed oestrous behaviour during the afternoon teasing on the ACTH treatment day (paper III).

In paper III, all five OVX mares showed oestrus during conventional teasing in July-September, although the number of days varied considerably between mares (2, 3, 30, 39 and 78 days respectively). In general, OVX mares showed more days of oestrus compared with when they were intact, the year before (44 days of oestrus for all intact mares compared with 152 days for all OVX mares during June-September). For technical reasons, daily teasing was not performed on all days of the experimental period in all of the mares and thus complete comparison between years is not possible. Oestrous behaviour after ovariectomy did not follow the normal cyclic pattern seen in intact mares.

'Paddock teasing' (paper III)

This more intense teasing method was performed during the breeding season (July) and non-breeding season (November). In July, three of the five mares showed at least two occurrences of full oestrus during the three teasing occasions, with one of the mares allowing the stallion to mount and ejaculate (mare B). Two of the mares showed no symptoms of oestrus in July (mares An and Ae). In November, only one mare showed full oestrous signs (mare Z), but did not allow the stallion to mount. The other four mares showed no symptoms of oestrus.

Objective behavioural tests (paper I)

None of the behavioural variables or heart rate scores differed according to stage of oestrous cycle (oestrus versus dioestrus) in either test situation (novel object and isolation). There were also no significant differences in any of the measured variables in either of the two tests between 'problem' mares and controls. However, in the novel object test, there was a significant effect of *test order* for exploring the object ($P < 0.05$), latency to touch the object ($P < 0.05$) and a tendency, for latency to enter the circle ($P = 0.06$). *Test order* denotes which oestrous cycle stage a mare was tested in first and this order affected her behavioural response to the test situation. When separating the mares into two groups, it was found that the mares that were tested in dioestrus first tended to touch the novel object earlier in the first test ($P = 0.06$), whereas no significant differences could be found between test occasions with the mares tested in oestrus first. For all mares, the mares explored the object more, as well as entered the circle and touched the object earlier on the first test occasion as compared with the second ($P < 0.05$). The mares also had a significantly higher mean heart rate in the first test ($P < 0.05$).

Gynaecological findings (papers I, II, III and V)

Intact mares were used in papers I, II and V. The mares in papers I and II had normal cyclic patterns in the ovaries (i.e. follicular development and ovulation) and in the uteri (i.e. oedema) as observed during the rectal palpation combined with ultrasound examinations. The same 12 mares were used in papers I and II and these had a mean cycle length of 22.3 (± SD 3.5) days ($n = 11$) during the study period. One mare was treated with prostaglandin $F_{2\alpha}$ on the 23rd day of dioestrus to induce oestrus prior to behavioural testing (and was not included in the calculation of mean cycle length). Another mare had a long oestrus (17 days), but this was considered normal for the time of year (March). In paper V, mares had a mean cycle length of 25.3 (± SD 3.8) days prior to treatment ($n = 9$). The longer cycle length in this study was likely due to time of year (February-March); the study commenced in early April.

In paper III, all OVX mares had oedema in the uterus [average oedema score of 1.8 (± SD 0.3)] when the ultrasound examination was performed in July, the day after the last 'paddock teasing' was carried out, regardless of the mare having been mounted by the stallion or not. Over the next two days, the oedema decreased to 1.7 (± SD 0.3) (day two after 'paddock teasing') and 1.2 (± SD 0.4) (day three after 'paddock teasing'). In contrast, no mare had uterine oedema in early November with the uteri of all mares being thin and flaccid.

Non-frequent blood sampling (papers I, II and III)

The daily samples confirmed the mares' cyclicity as observed at oestrous detection. (papers I and II). The plasma hormone concentrations (oestradiol and progesterone) during behavioural testing showed high individual variation (paper I). However, all mares had progesterone concentrations above 20 nmol/l, when tested in the luteal phase, and levels equal to or below 1 nmol/l during tests performed at oestrus (except for one mare with a progesterone level of 3.6 nmol/l). As expected, oestradiol concentrations during the objective tests were more varied; in oestrus the mean value of oestradiol was 24.5 (± SD 11.5) pmol/l and in dioestrus the mean value was 11.7 (± SD 7) pmol/l. In paper III, analysis for oestradiol confirmed high endogenous levels in the intact mares at oestrus [mean levels of 46.2 (± SD 13.1) pmol/l and 31.6 (± SD 18.4) pmol/l on the saline treatment day and ACTH treatment day, respectively] and low concentrations in OVX mares (mean oestradiol level below the minimal detection concentration of the assay).

Effect of ACTH-treatment on plasma steroid hormone levels – intact and OVX mares (papers II and III)

Cortisol

In both intact mares (paper II) and after ovariectomy (paper III), there was a significant effect of time period on the saline treatment day, indicating a diurnal rhythm ($P < 0.001$). 'Problem' mares did not differ from controls, but the five OVX mares had lower cortisol levels in time period three (18:00 to 23:00 h) ($P < 0.001$) and higher cortisol levels in time period four ($P < 0.05$), compared with when intact. In both intact (paper II) and OVX mares (paper III), ACTH treatment resulted in significantly elevated cortisol levels in time periods two and three ($P < 0.01$). Control mares showed a greater response compared with 'problem' mares in time period two ($P < 0.001$) (paper II), but upon scrutinising the data, one mare in the control group (mare Ex) had visibly higher cortisol levels on the ACTH treatment day as compared with the other four control mares. However, excluding her from the data did not alter this result. In paper III, there was no overall significant effect of mare group (intact and after OVX) in cortisol response to ACTH (i.e. difference between treatment days).

Progesterone

In intact mares, the mean progesterone level was low (≤ 0.4 nmol/l) on the saline treatment day (paper II), and paper III showed that levels were even lower after ovariectomy in time period four (24:00-05:00 h) ($P < 0.05$). In paper II, there was a significant effect of time period on the saline treatment day, showing progesterone levels changed with time ($P < 0.001$). Such an effect was not found after ovariectomy (paper III). On the ACTH treatment day, OVX mares had significantly lower progesterone values at all time periods as compared with when intact ($P < 0.05$) (Paper III). The ACTH treatment caused significantly increased progesterone levels in time period two in intact mares ('problem' and control) ($P < 0.001$) and in time periods two and three in OVX mares ($P < 0.05$). The 'problem' mares showed a significantly higher response in progesterone levels after the ACTH treatment in time period two as compared with the control mares (difference between treatment days; $P < 0.05$) (paper II).

Androstenedione

A significant effect of time period was found on the saline treatment day in intact mares (paper II) ($P < 0.001$), but not after ovariectomy (paper III). Levels of androstenedione in both the saline and ACTH sampling periods were significantly higher in intact mares as compared with after ovariectomy at all time periods ($P < 0.001$) (paper III). The ACTH treatment resulted in a significant increase in plasma androstenedione concentrations in all intact mares ('problem' and control) and in OVX mares, with levels significantly higher in time period two, as compared with the saline treatment day ($P < 0.001$). No significant effect of mare group in intact mares (control vs. 'problem' mare) was found in androstenedione response to ACTH (difference between treatment days; $P = 1.0$). However, androstenedione values increased significantly more in time period two as a result of the ACTH treatment in intact mares as compared with after ovariectomy (difference between treatment days; $P < 0.05$).

Testosterone

Levels of testosterone in both the saline and ACTH sampling periods were significantly higher in intact mares as compared with after ovariectomy at all time periods (one to three) ($P < 0.001$) (paper III). The ACTH injection resulted in a significant increase in plasma testosterone concentrations in time period two in all intact mares ('problem' and control) and in mares after ovariectomy ($P < 0.001$). Neither 'problem' mares nor mares after ovariectomy showed a difference in testosterone response to ACTH as compared with intact controls (papers II and III).

Oestradiol (paper III)

The concentration of oestradiol was only measured in selected samples from OVX mares (one pre-treatment sample and samples up to and including five hours post-treatment) (paper III). On both the saline- and ACTH treatment days, the majority of samples had levels of 1 to 3 pmol/l, with a maximum of 6 pmol/l after ACTH treatment. Since the minimal detection limit of the assay was 2.1 pmol/l and the intra-assay variation at 3.2 pmol/l was very high (45%), all samples were considered equal to null.

Immunohistochemical localisation of ERα and PR (paper IV)

ERα staining in uterus and adrenal gland

In uterine tissue, positive staining for ERα was observed in the cell nuclei of all compartments of the endometrium (surface epithelium, glandular epithelium, connective tissue stroma) and the myometrium. The highest proportion of ERα staining was, in general, found in the glandular epithelium and myometrium. For intact mares, the mare in oestrus (mare 526) showed the strongest staining intensity and highest proportion of stained cell nuclei in all tissue compartments, as compared with mares in metoestrus/dioestrus. When intact mares in metoestrus/dioestrus were compared with OVX and OVX+Oe mares, there was a general tendency for stronger ERα staining and/or higher proportion of stained cells in the latter two mare groups. No clear differences in staining intensity or proportion of stained cells were found between OVX and OVX+Oe mares. The negative controls showed no staining for ERα.

In the OVX and OVX+Oe mares, there was a major proportion (> 50 %) of cell nuclei with moderate positive staining for ERα in all zones of the adrenal *cortex*. In addition, in OVX mares, cytoplasmic staining of moderate intensity for ERα was also observed in a major proportion (> 50 %) of cells in the adrenal *medulla*. In the cell nuclei of the adrenal *cortex* of intact mares, the ERα staining intensity was weak, but observed in a major proportion (> 50 %) of cells. No specific staining was found in the adrenal *medulla* of intact mares, except for mare 502, where a low proportion of weak to moderate cytoplasmic ERα staining was observed.

PR staining in uterus and adrenal gland

In uterine tissue, positive staining for PR was found in the nuclei of all types of uterine cells as for ERα immunostaining. The lowest intensity of PR staining and proportion of stained cells were found in mare 478, a mare considered to be in metoestrus, because although a corpus luteum was observed after euthanasia, the plasma level of progesterone was still low. For the other mares (intact in oestrus/dioestrus, OVX and OVX+Oe), no clear differences were observed. No PR staining was found in the negative controls.

For PR immunostaining most of the adrenal *cortex* cells stained negatively, but a minor proportion (< 50 %) of weak positive cells was found in the zona fasciculata and zona reticularis. Moreover, in all mare groups, a major proportion (> 50 %) of weak cytoplasmic staining was observed in the cells of the adrenal *medulla*.

Effect of hCG-treatment on dioestrous length (paper V)

In total, in the first luteal phase examined, only three of the nine mares [experimental group (n=5) and control group (n=4)] developed follicles that were above 30 mm (and thus could be treated). The remaining mares developed large enough follicles in the second (n=4), third (n=1) or in none (n=1) of the luteal phases studied. Three of four hCG-treated mares (experimental group) ovulated and developed prolonged luteal phases lasting for 58, 68 and 82 days post-treatment, respectively. A fifth mare never developed dioestrous follicles ≥ 30 mm and could thus never be treated. None of the control mares treated with saline ovulated after the injection and in three of these mares progesterone values dropped below 3 nmol/l within nine days post-injection. The fourth control mare, although no ovulation was detected after the saline treatment, nevertheless developed a prolonged luteal phase that lasted for 72 days. Progesterone analysis of all of the samples also showed that one of the control mares that came into oestrus after the saline injection thereafter developed a prolonged luteal phase lasting for 76 days. Thus, in total, two of the control mares showed prolonged luteal activity during the study period.

General discussion

General aspects

The present study was initially intended to explore a clinical problem that, until now, has not received thorough scientific investigation, using both behavioural studies and sex steroid hormone analyses. As stated in the introduction, the diagnosis of deviant behaviour associated with the oestrous cycle in mares is commonly set by the owner. Clinical evaluation of the animal seems seldom to occur, perhaps since behavioural complaints are not redeemed by insurance companies in Sweden. Thus, since a low number of mares with oestrous-related behavioural problems are examined at Swedish veterinary clinics, the mares in the present studies were selected from a survey. One problem with using surveys, however, is relying on the owners' abilities to interpret oestrous signs correctly. The present study showed that mares with true behavioural abnormalities clearly associated with the oestrous cycle were, in fact, difficult to find. In addition, some mare owners were unwilling to lend their mares to the department for the period of time required (at least two complete oestrous cycles). Thus, because of the low number of mares with possible oestrous-related behavioural problems available, the study was designed to investigate behaviour (both during the oestrous cycle and after ovariectomy) and adrenal gland function also in normal mares.

The 'problem' mares that were studied had heterogenic histories, with a wide variety of behaviours believed to be associated with the oestrous cycle. However, few behavioural deviations were displayed by the mares during the experimental period (papers I and II). This may be due to owners misinterpreting their mare's behaviour and erroneously linking the behaviour to the mare's oestrous cycle. In addition, since the mare has a naturally long oestrous period, normal oestrous behaviour may be experienced as a problem *per se*. According to McDonnell (2003), most performance problems believed to be oestrous-related are not associated with the mare's reproductive cycle. Instead, submissive behaviour, urogenital discomfort, or simply inability of the owner to detect true oestrus, are often associated with the term nymphomania. In the present study, however, pathological conditions, such as urogenital infections and disturbances in the locomotor apparatus, were ruled out by careful clinical evaluation of the mares.

Apart from owner misconception, another reason for the lack of observed behavioural abnormalities in the present study may be that the environmental change (i.e. the move to the department) attenuated the mares' deviating behaviour, which would suggest that handling and milieu factors are important considerations when dealing with oestrous-related behavioural issues. At the department, mares were handled by experienced handlers and according to a regular routine, and not exposed to potential stressors, such as training and competing. In a survey (Christensson, 1991), 50 % of mare owners only experienced oestrous-related problems in association with competition, which most often also involves a change of environment for the horse.

The present study (papers I and II) also showed that mares believed to suffer from deviant oestrous behaviour may in fact show only weak signs during conventional teasing methods. Interestingly, it has been proposed that high emotionality in mares can have a negative effect on oestrous signs, since Thoroughbreds, a breed often regarded as highly excitable, showed weaker sexual receptive signs at teasing as compared with a more sedate primitive breed (Konik polski) (Górecka, Jezierski & Sloniewski, 2005). Further, other behaviours than sexual appear to be altered in some mares during the oestrous cycle (that is, a difference between oestrus and dioestrus). Such behavioural changes may only become apparent when the mare is used, e.g. ridden or driven. An attitude change, increased aggressiveness and diminished cooperativeness are behaviours often described in mares with oestrous-related behavioural changes (Jorgensen et al., 1996; Vikström, 2001).

Behaviour

Behavioural tests

Since oestrous-associated behavioural deviations may be more related to temperamental changes in mares, rather than strong oestrous signs, methods for behaviour evaluation other than the traditional method of stallion teasing are required. Moreover, although studies using objective behavioural tests to evaluate the temperament of the horse are emerging, few have considered the effect of sex differences and none, to date, appear to have looked at the effect of oestrous-cycle stage. In paper I, objective behavioural tests were used to determine whether oestrous-cycle stage would affect exploratory behaviour, emotionality (i.e. fearfulness and nervousness) and gregariousness in mares. Changes in such behaviours could potentially affect how the mare reacts and interacts with her environment and lead to undesirable behaviours affecting manageability. The tests employed have previously been used in the equine species (novel object test) (Le Scolan, Hausberger & Wolff, 1997; Wolff, Hausberger & Le Scolan, 1997; Visser et al., 2001, 2002, 2003) or in other animal species (isolation test) (Syme, 1981; Mills et al., 1993; Vandenheede & Bouissou, 1993a). The novel object test is reported to show good validity in horses (Le Scolan, Hausberger & Wolff, 1997; Visser et al., 2003). In mature horses (> 3 years), this test appears to measure the degree of nervousness (Le Scolan, Hausberger & Wolff, 1997), whereas in youngsters (≤ 3 years), the test seems to assess fear (Wolff, Hausberger & Le Scolan, 1997; Visser et al., 2001). This notion is supported by the present study where mature mares reacted with an increased nervousness (i.e. an elevated heart rate), but not fear (as indicated by their curiosity and willing exploration of the object) on their first encounter with the novel object.

In the present study (paper I), objective behavioural tests failed to show any significant effect of oestrous-cycle stage on exploratory behaviour and reaction to social isolation. However, mares tested in the luteal phase first appeared to be more receptive to a novel environment in that they tended to touch the novel object earlier, as compared with when tested in oestrus. This is an interesting finding that may indicate that mares in dioestrus are more responsive to environmental changes. In the rat, a species which is often used in behavioural tests, studies have shown that oestrous-cycle stage can affect the response to various test situations, e.g. inhibited conditioned avoidance responses, increased ambulation and decreased defecation at oestrus (Wade, 1972; Hyde & Jerussi, 1983; Diaz-Veliz et al., 1991). In this species, oestrus has been suggested to result in diminished emotionality (Gray, 1987), although some behavioural changes (e.g. increased ambulation and decreased defecation) may be more related to changes in metabolism (Birke & Archer, 1975). Similarly, in cows, oestrogen treatment seemed to reduce fear (Bouissou, 1990). In the present study, mares in oestrus tended to have longer latencies to exploring the object compared with when in dioestrus, indicating instead an increased level of nervousness or emotionality during oestrus. The reason for this finding is unclear. However, we found that elevated progesterone levels during dioestrus were accompanied by increased levels of allopregnanolone in two mares (Hedberg et al., 2006), a neurosteroid that appears anxiolytic in rats and mice (Wieland et al., 1991; Brot et al., 1997; Gomez et al., 2002) and has been related to mood changes and premenstrual syndrome in women (Freeman et al., 1993; Bicikova et al., 1998; Monteleone et al., 2000; Andréen et al., 2005). Moreover, in OVX rats, administration of progesterone increased exploratory behaviour, an effect related to increased levels of allopregnanolone in the cerebral cortex (Bitran, Purdy & Kellogg, 1993). Further studies are, however, required to confirm if oestrous cyclicity, and the accompanying hormonal changes, is related to changes in emotionality and exploratory behaviour in mares.

In the present study (paper I), the 'problem' mares showed no significant differences in behaviour in either test situation as compared with control mares. Due to the low number of animals available, it was not possible to match groups for age and breed and this diversity, as well as differences in the mares' previous experiences, may have confounded the results. In horses, breed differences in emotionality appear to exist. For example, Thoroughbreds were rated as more emotional than Quarter Horses and, interestingly, also showed a slower discrimination-learning rate (Mader & Price, 1980). However, individual ratings of emotional reactivity and learning rate were not correlated. In another study, although mature horses (3-17 years) in general reacted in a similar manner to a novel object test, regardless of breed, there were clear individual differences, as demonstrated by the high coefficients of variation in the overall sample (Le Scolan, Hausberger & Wolff, 1997). Thus, although the present study was unable to demonstrate differences in test response between 'problem' mares and controls, further studies with a larger number of mares are needed.

It appears that the horse's ability to remember depends on which type of memory skill is being evaluated. For example, horses were unable to recall a feeding event involving two choices after a 10 second delay, indicating the horse has a limited short-term prospective memory (McLean, 2004). However, long-term memory, although not thoroughly investigated in McLean's study, seemed excellent. Procedural memory (that is, the memory of skills and procedures, or 'how to' knowledge) also appears well-developed in the horse (Wolff & Hausberger, 1996). The present result revealed that mature mares may become accustomed to a novel situation, when re-exposed after a period of at least one month. If a stimulus does not lead to any consequence for the horse, repeated exposure leads to a ceased or decreased response, i.e. habituation, which can be regarded as a simple form of learning (Voith, 1986). In a study using 2-year old Danish Warmblood stallions, some horses failed to habituate to a novel stimulus, even though the test was repeated 20 times (Christensen, 2006). The horses were, however, untrained youngsters as compared with the mature trained horses used in the present study and it has been shown that handling can increase learning performance in the horse (Heird, Lennon & Bell, 1981). However, the quick habituation in the present study, after only one previous exposure, was nonetheless unexpected and may have masked differences in behaviour according to oestrous-cycle stage. By using two diverse novel object tests, where the same mare can be tested in different test orders, such an effect might be avoided. However, this would have required a larger number of mares.

Ovariectomy

Ovariectomy in the mares resulted in continued displays of sexual receptiveness, although with the loss of normal cyclicity (paper III). This finding is supported by earlier observations that an active ovary is not essential for oestrous behaviour in the mare (Ginther, 1974; Asa et al., 1980a; Hooper et al., 1993; Dalin, Andresen & Malmgren, 2002). As was suggested by Asa et al. (1980a), the phenomenon may be a way to keep a strong social bond between a stallion and a mare also during the anovulatory season. However, as stated in the introduction, the ovary nonetheless appears to be necessary for regulating cyclic sexual behaviour (Asa, Goldfoot & Ginther, 1979; Asa et al., 1980a). In the present study, mares in general displayed more days of oestrus after ovariectomy as compared with when they were intact, the year before (paper III). Similarly, it was demonstrated that OVX mares and seasonally anovulatory mares showed oestrous signs of moderate intensity approximately 50 % of the time, as compared with 33 % in intact mares during the ovulatory season (Asa, Goldfoot & Ginther, 1979; Asa et al., 1980a).

The ‘paddock teasing’ in the present study (paper III) resulted in a higher stimulatory effect on the mares than the conventional teasing method and seemed to initiate the oestrous behaviour, with most of the OVX mares showing oestrous signs during conventional teasing only after ‘paddock teasing’ had been performed at least once. It has been shown that groups of mares teased in a paddock with a stallion show a broader repertoire of receptive behaviour and fewer negative signs compared with mares teased using more traditional methods (Asa, Goldfoot & Ginther, 1979). Since it was noted that proceptive behaviour in OVX and anovulatory mares involved more kicking and avoidance reactions compared with intact mares (Asa et al., 1980a), more intense teasing methods may be necessary to elicit sexual behaviour in such mares. In this context, owners have reported that the oestrous signs in mares after ovariectomy are of a lower intensity than prior to the operation (Hooper et al., 1993). Thus, although ovariectomy in the mare as a method to treat unwanted sexual behaviour is not reliable and may in fact increase the number of days a mare shows oestrous signs, some mares with strong sexual behaviour may benefit from ovariectomy since the intensity of the oestrous signs appears to be attenuated in some OVX mares.

Adrenal sex steroid hormones

ACTH treatment in several species, including the horse, has shown the adrenal gland to be a significant source of sex steroid hormones (Watson & Munro, 1984; Watson & Hinrichs, 1989; Jopson, Fisher & Suttie, 1990; Bolaños, Molina & Forsberg, 1997; Tsuma et al., 1998; Van Lier et al., 1998; Båge et al., 2000; Brandt et al., 2006). The fact that OVX mares display sexual signs has been attributed to adrenal sex steroid hormones (Asa et al., 1980b). In the present study (papers II and III), a single intravenous injection of ACTH was used to stimulate adrenal gland hormone production in intact normal mares, OVX mares and mares that according to their owners suffered from oestrous-related behavioural abnormalities. The purpose of the injection was to cause maximal stimulation of the adrenal gland and the treatment was not used as a model to simulate stress. A significant production of progesterone, androstenedione and testosterone was demonstrated in all three mare groups. Cortisol levels rose significantly and within the same time-frame, showing the dose was sufficient to stimulate the adrenal gland and demonstrating an adrenal source for all hormones quantified.

Diurnal rhythm

A diurnal rhythm of cortisol was shown in both intact (paper II) and OVX mares (paper III), with peak levels in the morning and a nadir in the evening, which is in accordance with previous studies in horses (James et al., 1970; Larsson et al., 1979; Irvine & Alexander, 1994). The presence of a diurnal rhythm indicates all mares (both department- and client-owned) were accustomed to their environment at the time of the experimental studies, since it was demonstrated that the stress of novel surroundings is sufficient to disturb the cortisol rhythm in horses (Irvine & Alexander, 1994). Moreover, the regular management routine at the department may have aided in entraining a diurnal cortisol rhythm (Irvine & Alexander, 1994).

Although levels of both progesterone and androstenedione changed significantly over the 26-h period in intact mares (paper II), no such observation was made in OVX mares (paper III). The changes in plasma steroid levels observed in the intact mares were most likely due to alterations in ovarian levels of androstenedione and progesterone and no apparent diurnal rhythm in either hormone could be demonstrated.

Ovarian versus adrenal source

Mean basal progesterone levels were low in intact mares at oestrus (≤ 0.4 nmol/l) (paper II), but ovariectomy decreased levels even further (paper III), indicating that both the ovaries and adrenal gland contribute to basal plasma progesterone concentrations in the mare at oestrus. Similarly, androstenedione and testosterone levels were significantly higher in intact mares at oestrus as compared with OVX mares, showing that, as for progesterone, both the ovaries and adrenal glands secrete these androgens during non-stressful conditions at oestrus (paper III). The significant increase in androgen levels after ACTH treatment in the present study (papers I and II) lends further support that C19 steroid hormones (i.e. androgens) are produced by the mare adrenal gland, as in e.g. primates, hamsters and guinea pigs [Tremblay et al., 1994; Cloutier et al., 1995; Arlt et al., 2002; Conley, Pattison & Bird, 2004 (review)]. As stated in the introduction, adrenal androgen production has previously been demonstrated *in vitro* in mare adrenal cortical tissue (Silberzahn et al., 1984) and *in vivo* in OVX mares after ACTH stimulation (Watson & Hinrichs, 1989). In the present study, since both androstenedione and testosterone increased within the same time-frame after the ACTH treatment, a direct testosterone secretion by the adrenal gland, rather than a peripheral conversion from androstenedione, is most probable. The enzyme required for this conversion, 17-β-hydroxysteroid dehydrogenase (17β-HSD), was in the rhesus monkey found to be present in several tissues examined, including the adrenal gland (Martel et al., 1994).

Oestradiol was not detected after ovariectomy in the present study (paper III), which indicates an exclusively ovarian source for this hormone in the mare. This is in accordance with the study of Watson & Hinrichs (1989), where no oestradiol of adrenal origin in OVX mares was observed. Also, no adrenal source of oestradiol was found in the pig (Mwanza et al., 2000). However, to detect low hormone levels, other methods may be necessary. For example, immunisation of OVX ewes against oestrone and oestradiol resulted in lower uterine weights and uterine oestradiol concentrations, indicating a non-ovarian oestrogen source (Adams et al., 1990). This source was proposed to be the adrenal gland, since an earlier study by the same group showed decreased uterine oestradiol levels after adrenalectomy (Atkinson & Adams, 1988). However, the authors were unable to conclude if there was a direct secretion of adrenal oestrogens or if the oestrogens were a result of adrenal androgen aromatisation in peripheral tissues (Adams et al., 1990). In the present study, aromatisation of adrenal androgens to oestrogens in peripheral tissues cannot be excluded and may have caused the unexpected uterine oedema observed in the OVX mares, even though peripheral oestradiol concentrations remained below the assay's detection level after ACTH treatment (paper III).

Thus, the possible effect of adrenal steroid hormones on oestrous behaviour in the OVX mares could either be due to oestrogens aromatised from adrenal androgens in peripheral tissues or be due to a direct effect of androgens. Androgens can induce sexual behaviour in mares, e.g. via exogenous testosterone propionate treatment (Thompson, Godke & Squires, 1983). Moreover, testosterone producing granulosa-theca cell tumours can, in some mares, lead to nymphomania-like behaviour (Meagher et al., 1978). In humans and primates, studies indicate that androgens act in the brain both directly via androgen receptors and, after local aromatisation, via oestradiol receptors [Bancroft, 2005 (review)].

Gonadal-adrenal interactions

In the present study (paper III), ovariectomy affected the diurnal rhythm of cortisol, with significantly lower levels in the evening; that is, during the rhythm's nadir. Cortisol levels during the night and early morning hours were, however, higher in the OVX mares, resulting in a more pronounced rhythm in these mares as compared with when intact. The decrease in nadir levels contrasts with other species, such as the rat and rhesus monkey, where instead decreases in peak corticosteroid levels have been observed after ovariectomy (Smith & Norman, 1987; Atkinson & Waddell, 1997). Nevertheless, the effect of ovariectomy on the diurnal rhythm indicates ovarian substances can affect adrenal function in the mare. Seasonal variation in the diurnal rhythm was not a cause, since time of year was the same for both mare groups. In the present study (paper IV), positive staining for ERα was found in the adrenal cortex. Therefore, oestradiol appears, at least in part, to act at the adrenal level. Moreover, ERα staining was also observed in the cell cytoplasm of the adrenal medulla in mares after ovariectomy, indicating oestradiol may have an effect on catecholamine secretion. However, the cytoplasmic location of the receptors is an uncommon finding and may indicate degraded receptor proteins (Sukjumlong et al., 2004) or newly synthesised ERα (Yamashita et al., 1990).

A direct stimulatory role of oestradiol on the adrenal gland, as was observed in the rat (Atkinson & Waddell, 1997; Lo, Chang & Wang, 2000) and sheep (Van Lier, Perez-Clariget & Forsberg, 2003), could not be found in the present study. We found that the high endogenous oestradiol levels in intact mares during oestrus (paper II) had no significant effect on the overall ACTH-induced cortisol response, as compared with when mares were ovariectomised with very low plasma oestradiol levels (paper III). There is evidence that the mare may differ from other species studied regarding gonadal-adrenal interactions. Asa, Robinson & Ginther (1983) observed higher cortisol levels in mares during dioestrus than at oestrus, i.e. at low endogenous oestradiol concentrations. In addition, the mean cortisol levels during the oestrous cycle correlated negatively to maximum follicular size. This is in contrast to other species, e.g. the sow, where elevated cortisol levels were observed at oestrus (Ash & Heap, 1975; Dalin, Nyberg & Eliasson, 1988). Also, in women, cortisol levels were found to be higher during the follicular phase as compared with the luteal phase, with a positive coupling between the LH surge and maximal cortisol levels (Kerdelhue et al., 2002). Contrary, in the mare it was suggested that low cortisol levels at oestrus might be necessary for normal follicle growth and LH release, since dexamethasone treatment reduced both follicle size and inhibited ovulation (Asa & Ginther, 1982). However, although endogenous cortisol levels in sows were higher at oestrus (Ash & Heap, 1975; Dalin, Nyberg & Eliasson, 1988), both hydrocortisone acetate and ACTH treatment prevented the pre-ovulatory LH surge and ovulation in gilts (Barb et al., 1982). In sheep, continuous infusion of cortisol inhibited or delayed the pre-ovulatory LH surge (Daley et al., 1999). These somewhat contradictory findings may be due to species differences in oestrous cycle endocrinology (e.g. the long LH peak in the mare), qualitative differences between endogenous and synthetic corticosteroids, as well as differences in exposure lengths.

In contrast to oestradiol, an effect of progesterone on adrenal function is much less studied. Progesterone treatment did not have any effect on basal or stress-induced HPA activity in OVX rats (Carey et al., 1995). However, *in vitro*, progesterone was found to affect mineralcorticoid receptors, suggesting progesterone may have a modulating role in the enhanced HPA activity seen in the rat at pro-oestrus (Carey et al., 1995). In the present study (paper IV), a positive but weak staining (few cells and low intensity) for PR was found in the cell nuclei of the adrenal cortex, indicating that a direct effect of progesterone on the mare adrenal gland may be possible. This is in accordance with some studies performed in other species, where, as stated in the introduction, adrenal PR was found in the rat (Uotinen et al., 1999), adrenal PR mRNA in sheep (Van Lier et al., 2003) and an adrenal progesterone binding protein in the guinea pig (Demura, Driscoll & Strott, 1989). However, other studies have failed to demonstrate adrenal PR, such as in the rhesus monkey (Hirst et al., 1992). Similarly, no adrenal bindning protein could be detetected in adrenocortical nuclei from rat, dog, pig and chinchilla (Demura, Driscoll and Strott, 1989).

In the present study (paper IV), PR staining, in addition to ERα, was found in the cytoplasm of cells in the adrenal medulla. To our knowledge, progesterone receptors in the adrenal medulla have not previously been described. However, both progesterone and oestradiol were demonstrated to alter catecholamine metabolism in the adrenal medulla of the rat (Fernández-Ruiz et al., 1988). Progesterone had an inhibitory effect on bovine adrenal medullary catecholamine secretion, which has raised the interesting question as to whether there is an intra-adrenal regulation of adrenal medullary function by adrenocortical progesterone during stress (Armstrong & Stuenkel, 2005).

An unexpected finding in the present study (papers II and III) was that ACTH appeared to have a stimulatory effect on ovarian androstenedione secretion. In accordance, in bovine ovary tissue, physiological concentrations of cortisol directly increased the thecal cell production of androstenedione (Spicer & Chamberlain, 1998). The mechanism for this interesting interaction requires further study.

'Problem' mares

Although no differences in basal cortisol levels were observed in the present study (paper II), a difference in adrenocortical reactivity was found between intact 'problem' mares and intact controls, with 'problem' mares showing a significantly lower cortisol response to ACTH stimulation. The reason for this is unclear. In several species, chronic stress has been shown to affect adrenocortical responsiveness to ACTH or to an acute stressor. For example, bulls subjected to the stress of a novel housing environment showed a reduced response to ACTH treatment, despite the basic cortisol secretion having returned to control values (Ladewig & Smidt, 1989). This reduced adrenocortical reactivity was suggested to be caused by a desensitisation of adrenal cells due to prolonged elevation of endogenous ACTH levels. Such ACTH induced refractoriness of adrenal cells has been demonstrated *in vitro* in cultured adrenal cells of the mouse (Morera, Cathiard & Saez, 1978) and the rat (Rani, Keri & Ramachandran, 1983), which was suggested to be caused by a defect in the conversion of cholesterol to pregnenolone (Rani, Keri & Ramachandran, 1983). Although no differences in emotionality was observed in the behavioural tests in the present study (paper I), the 'problem' mares may have experienced a greater endogenous stress level during oestrus compared with the controls, which resulted in a blunted cortisol response to exogenous ACTH, but was not reflected in the mares' basal cortisol levels or diurnal cortisol rhythm. Moreover, in horses, other factors, such as exercise, can affect adrenocortical reactivity. Training was found to induce elevations in endogenous ACTH and more trained horses had a lower cortisol response to exogenous ACTH (Marc et al., 2000). In the present study (paper II), six of the seven 'problem' mares had been ridden and/or driven regularly prior to the study period, whereas most of the control mares had not been exercised recently. However, at the time of the study, all mares were managed the same (that is, received no exercise), which makes such an explanation for the difference in adrenocortical reactivity unlikely.

In contrast to a diminished cortisol response after a period of chronic stress, as described above, some other studies have demonstrated an enhanced response to exogenous ACTH following a period of stress, e.g. in pigs (Von Borell & Ladewig, 1989), lactating cattle (Friend, Gwazdauskas & Polan, 1979) and veal calves (Dantzer et al., 1983). This augmentation of the cortisol response to stimulation may be due to chronic endogenous ACTH exposure resulting in an increased synthesis of steroidogenic hormones [Simpson & Waterman, 1983 (review)]. In the pig, adrenocortical response to ACTH has also been shown to be an individual characteristic, regardless of influences from the environment (Von Borell & Ladewig, 1989). It is, however, difficult to draw conclusions regarding the results of the present study from studies in other species. The low number of animals and the heterogenic history of the 'problem' mares further make interpretation difficult. It was not possible to match mare groups for age and breed, which may have affected the results. Nonetheless, it is of interest to note that the groups did differ significantly in spite of the few mares included in the study.

The 'problem' mares also differed in progesterone response, with a significantly greater increase in progesterone levels during the first three hours after the ACTH injection, as compared with control mares (paper II). The reason for this difference is also difficult to explain, but may indicate a difference in adrenal function, especially since the 'problem' mares also showed a lower increase in cortisol during the same time period, as compared with the controls. Adrenal enzyme defects are found in humans, such as a defect in the gene encoding the P450 21-hydroxylase enzyme (P450c21), which leads to a deficient cortisol production and an elevation in 17α-OH-P4, 17α-OH-P5, DHEA, androstenedione and testosterone (Glatt, Garzon & Popovic, 2005). Mild defects in this gene cause few clinical signs, but may be diagnosed using ACTH-stimulation tests, where high levels of 17α-OH-P4 are diagnostic (Carlson et al., 1999). Adrenal enzyme defects have been suggested as one possible cause of aggressive behaviour in mares, attributed to the high endogenous androgen levels observed in some aggressive mares (Beaver & Amoss, 1982; Roberts & Beaver, 1987). However, the 'problem' mares in the present study did not show greater levels of adrenal androgens (androstenedione and testosterone) after ACTH stimulation as compared with control mares. This finding makes an enzyme defect, as described in humans, less probable and the differences in progesterone response may simply have been due to individual variation. Regardless of the observed differences, the progesterone levels after ACTH stimulation in both normal and 'problem' mares were well below normal luteal phase levels (Ginther, 1992c) and likely to be without biological significance.

In the present study (paper II), progesterone, oestradiol and two androgens (androstenedione and testosterone) were measured in mares after ACTH treatment. However, other steroid hormones may also have been secreted by the mares' adrenal glands. It is generally accepted that humans and non-human primates are the main species producing adrenal DHEA [Adams, 1985; Labrie, Dupont & Bélanger, 1985; Conley, Pattison & Bird, 2004 (review)]. However, in a study by Rance et al. (1976), mares were found to have a pre-ovulatory DHEA peak, the source of which was suggested to be the adrenal gland, since dexamethasone treatment of one of the mares reduced the DHEA levels.

In women, allopregnanolone is secreted by both the ovary and adrenal gland (Genazzani et al., 1998; Bernardi et al., 2000). When samples from one of the OVX mares in the present study were analysed for allopregnanolone (Hedberg et al., 2006), no increase in response to ACTH treatment was found. This neurosteroid is nonetheless interesting and deserves further study in the mare, especially when considering its anxiolytic properties in laboratory animals (Wieland et al., 1991; Brot et al., 1997; Gomez et al., 2002) and its probable involvement in premenstrual syndrome in women (Bicikova et al., 1998; Monteleone et al., 2000).

Induction of dioestrous ovulation

Although few mares with true behavioural abnormalities were found in the present study, this, as stated earlier, may be an environmental effect and many mare owners seem to experience behavioural problems in their mares exclusively when competing (Christensson, 1991). Although speculative, the difference in adrenocortical reactivity found in the 'problem' mares may indicate that they have high endogenous stress levels and represent mares that are more 'easily stressed' and perturbed by stressful situations, such as a competition. There currently is no satisfactory treatment of strong oestrous signs that can be used in competing mares. The present, as well as earlier, studies show that ovariectomy is unlikely to abolish oestrous signs, although the intensity of sexual behaviour might be reduced (Asa et al., 1980a; Hooper et al., 1993). Whether other behaviours associated with the oestrous cycle, such as decreased cooperation and increased aggression, would be attenuated by ovariectomy is not clear. For example, aggression in stallions is not always reduced by gonadectomy, i.e. castration. In one study, only 40 % of aggressive stallions improved their behaviour towards other horses, although between 60 to 70 % became less aggressive towards people (Line, Hart & Sanders, 1985).

In paper V, hCG was used to induce late dioestrous ovulations in order to prolong the luteal phase and cause elevated endogenous progesterone levels for an increased period of time. Progesterone is not only a potent inhibitor of sexual signs in mares, but was as an exogenous treatment claimed by owners to improve behaviour in 85 % of mares with oestrous-related behavioural deviations (Christensson, 1991). In the present study, the prolonged luteal phases after the hCG-induced ovulations appeared to be caused by the developed corpora lutea being refractory at the time of prostaglandin $F_{2\alpha}$ release (Douglas & Ginther, 1975). In accordance with other studies, the prolonged luteal phases in the present study lasted approximately 2-3 months, which seems to be the intrinsic life span of equine corpora lutea (Hughes, Stabenfeldt & Evans, 1975; King & Evans, 1988). The reason that luteolysis does not occur during a prolonged luteal phase might be due to a decreased sensitivity of the uterus to oxytocin or a diminished uterine ability to secrete prostaglandin $F_{2\alpha}$, as was suggested by Kindahl et al. (2000). It was further proposed that for the uterus to regain its ability to secrete prostaglandin $F_{2\alpha}$, a period of low progesterone levels (oestrus) is necessary.

The reason for the prolonged luteal phases observed in the control mares in the present study was not investigated. Spontaneous prolongation of the luteal phase, due to an idiopathic failure of the uterus to secrete adequate amounts of prostaglandin $F_{2\alpha}$ to induce luteolysis, has been described (Henry et al., 1981). Unfortunately, measurement of the prostaglandin metabolite was not possible in the present study. However, it has been suggested that spontaneous luteal phase prolongations in mares with no apparent uterine pathologies are in fact due to dioestrous ovulations that, for various reasons, are not detected (Ginther, 1992b). Since the mares in the present study were only followed with rectal palpation (including ultrasound) for up to three days post-treatment, some spontaneous dioestrous ovulations may have gone undetected.

The mares in the present study (paper V) showed a large inter- and intra-variability in dioestrous follicle size, with one mare never developing large follicles during the luteal phase. Two follicular waves have been reported in some mares, whereas others appear to have only one primary follicular wave (Ginther, 1990; Sirois, Ball & Fortune, 1989). Breed differences seem to exist and large dioestrous follicles (> 30 mm) that lead to ovulation appear to occur frequently in e.g. Thoroughbreds (Hughes, Marcelo & Stabenfeldt, 1985), but more rarely in e.g. Standardbred trotters (Henry et al., 1981), which was the numerically dominating breed in the present study. Thus, using other breeds may have produced different results, with more mares being able to be treated earlier. It may also be possible to modify the method by using repeated injections of hCG in smaller doses, since ovulation was successfully induced in pony mares with follicles as small as 20 mm using daily hCG injections of 200 IU (Bour, Palmer &

Driancourt, 1985). At present, however, additional studies are required to assess the method's potential use in practice.

Concluding remarks and future perspectives

The present study brought forward several difficulties with studying oestrous-related behavioural problems in the mare. Environmental aspects, handling factors and owner misinterpretation of their mare's behaviour all have to be considered. Mares may need to be extensively studied in their home environment in order to find behavioural problems that are clearly oestrous-related. Interestingly, when the owners of the 'problem' mares in the present study were contacted at least one year after the study, many of them reported an improvement in their mare's behaviour after having implemented a change in their mare's environment, indicating, as in the present study, that the environment plays a major role in mare behaviour.

By studying the normal mare, the interactions between oestrous behaviour, hormonal status and external environment can be more fully understood. This in turn will result in a better comprehension of mares that appear to behave in a deviant manner. In today's society where the horse often is expected to perform optimally at all times, we need to not only ensure athletic competence, but become more aware of what may affect the horse's behaviour and disposition.

The objective tests used in the present study showed a tendency to an effect of oestrous cycle stage on exploratory behaviour. Further studies, using a larger number of mares, are needed to confirm whether oestrous-cycle stage affects other behaviours than sexual in the mare. Using young mares of the same age and breed, where previous experiences and handling factors can be avoided, may show more clearly temperamental changes across the oestrous cycle. Moreover, considering that many owners appear to experience a decreased cooperation in their mares during oestrus, a handling test would be of specific interest.

The present study demonstrated that the adrenal and ovarian functions in the mare are inter-related. Further studies focusing on these interactions are needed, directed also at other levels of the HPA and HPG axes, e.g. the pituitary and hypothalamus. Immunisation of OVX mares against various sex steroid hormones, such as oestrogens and androgens, enabling detection of low levels of hormone, and evaluating what effect this might have on oestrous behaviour and uterine oedema, would be of particular interest.

Conclusions

Conclusions regarding mare behaviour and adrenal gland function from the present study were as follows.

I. Behaviour

- When examining mares with suspected oestrous-related behavioural deviations, owner misinterpretation of mare behaviour and/or environmental factors have to be considered.

- Mares in dioestrus appeared more receptive to a novel object as compared with mares in oestrus, as shown by the mares' tendencies to explore a novel object earlier when tested in the luteal phase first.

- Mares in oestrus did not react to isolation with greater emotionality than when in dioestrus, as assessed by heart rate measurements.

- Mares showed oestrous signs after ovariectomy and in general displayed more days of oestrus after ovariectomy as compared with when intact. However, oestrous signs in OVX mares did not follow a normal cyclic pattern. This shows that the ovary is needed for regular cyclic sexual behaviour in the mare.

II. Adrenal gland function and interactions with the ovary

- Ovariectomy affected the diurnal cortisol rhythm, with a decrease in the nadir (evening) levels, but an increase in the cortisol levels during the night and early morning, resulting in a more pronounced rhythm after ovariectomy, as compared with when the mares were intact.

- Androstenedione, testosterone and progesterone were secreted by both the ovary and adrenal gland in intact mares at oestrus.

- Endogenous oestradiol had no clear stimulatory effect on mare adrenal gland function.

- Ovarian androstenedione was stimulated by exogenous ACTH.

- Mares with a history of deviant oestrous behaviour had a reduced adrenocortical reactivity as compared with control mares, with a blunted cortisol response to adrenal stimulation, which may indicate a different pre-treatment stress level in these mares as compared with normal controls.

III. Adrenal steroid receptors

- Clear positive staining for ERα, but also weak staining for PR, was found in cell nuclei of the adrenal cortex, indicative of a direct effect of ovarian steroids on the mare's adrenal gland. Ovariectomy increased the staining intensity of ERα in the adrenal cortex, as compared with intact mares.

- In some mare groups, positive staining for both ERα and PR was observed in the cytoplasm of adrenal medullary cells, indicating that ovarian steroids may have an effect on catecholamine secretion.

IV. Method to suppress unwanted cyclicity

- In mares, dioestrous ovulations can be induced by hCG and cause a prolonged luteal phase when treatment is performed in the late luteal phase. However, further studies are required to assess the method's effectiveness and practical implications.

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Populärvetenskaplig sammanfattning

Starka brunstsymptom hos sto kan påverka stoets användbarhet och hanterbarhet, i synnerhet om stoet är avsett för tävling. Då diagnosen brunstbeteendestörning ofta ställs av stoägaren själv, och få ston undersöks av veterinärer, är orsaken till beteendet i många fall oklar. Att behandla tävlingshästar med långtidsverkande progesteronpreparat (progesteron = gulkroppshormon) för att trycka ned oönskat brunstbeteende är inte längre tillåtet i de flesta europeiska länder. Istället har andra metoder för att hämma brunstbeteende hos sto utvecklats, som t.ex. att behandla med örtpreparat.

Det är vanligt att brunstrelaterade störningar yttrar sig som en minskad samarbetsvilja och i vissa fall en ökad aggressivitet hos stoet, snarare än ett starkt brunstbeteende. Det är därför viktigt att stoets beteende undersöks med andra medel än traditionell brunstpassning med hingst. Objektiva beteendetester för att beskriva hästens temperament har på senare år utvecklats. Ett test, det så kallade ”novel object” testet, undersöker individens reaktion på ett främmande föremål och mäter hos unga hästar graden av rädsla men hos äldre hästar, graden av nervositet. Ett annat test är att separera ett flockdjur från andra individer i flocken (s.k. isoleringstest), vilket är stressande och mäter individens reaktion på separation. Då stoets brunstbeteende kan tänkas ha en flocksammanhållande effekt, är det möjligt att ston med starka brunster även reagerar starkt på isolering.

Stoet är unikt bland icke-primater eftersom det kan visa brunstbeteende trots inaktiva äggstockar eller efter det att äggstockarna opererats bort (ovariektomi). Man vet inte varför stoet visar brunst utan att vara befruktningsduglig, men det har spekulerats att beteendet är ett sätt att stärka bandet mellan hingst och sto. Vad som ger upphov till brunstbeteende hos ston med låg eller obefintlig hormonproduktion från äggstockarna är ännu inte klarlagt, men det finns en teori om att beteendet orsakas av könshormoner från binjuren. Hos ett antal djurslag har man sett att binjuren vid stimulering utsöndrar könshormon som t.ex. progesteron och testosteron (hanligt könshormon). Det har även föreslagits att ett aggressivt beteende hos vissa ston kan bero på en förändrad produktion av binjurehormoner, men detta har aldrig blivit ordentligt undersökt. Dessutom förekommer det en interaktion mellan hypotalamus-hypofys-binjureaxeln och hypotalamus-hypofys-äggstocksaxeln hos vissa djurslag, som t.ex. får, råtta och primater, men en liknande interaktion hos häst har ännu inte studerats.

För att närmare undersöka stoets beteende och binjurefunktion, utfördes en serie försök på ston som enligt sina ägare visade brunstbeteendestörningar (”problem” ston) (n = 7), på ston med normalt brunstbeteende (kontrollston) (n = 5) samt på ovariektomerade ston (n = 5). Då det visade sig vara svårt att få tag på s.k. ”problem” ston, utformades försöken så att även det normala stoets beteende och binjurefunktion undersöktes. Utöver konventionell brunstpassning gjordes två olika beteendetester, ett ”novel object” test och ett isoleringstest, på de intakta stona (”problem”- och kontrollston) och dessa ston testades både i brunst och i lutealfas. De ovariektomerade stona brunstpassades för hingst på konventionellt sätt (från juni till september) samt genom att sto och hingst släpptes lösa i en paddock under en tio minuters period (juli och november), en metod avsedd att ge ökad kontakt mellan stoet och hingsten. För att undersöka stoets binjurefunktion, studerades binjurens könshormonproduktion genom att samtliga ovanstående stogrupper behandlades med adrenokortikotropt hormon (ACTH; Synachten®, 0.5 mg i.v.), ett hormon som stimulerar binjuren, och därefter analyserades plasmakoncentrationerna av kortisol, progesteron, testosteron och androstenedion (ett hormon som kan omvandlas till testosteron). Vidare användes immunohistokemi för att studera förekomsten av progesteron- och östradiolreceptorer i binjurar från intakta och ovariektomerade ston, de senare med eller utan föregående östrogenbehandling.

Innan försöken påbörjades, genomfördes en noggrann klinisk undersökning av samtliga intakta ston. Inga patologiska fynd i livmoder, äggstockar, urinvägar eller rörelseapparaten kunde upptäckas hos något av stona. Under vistelsen vid institutionen (minst två brunstcykler per sto) visade fem av de sju s.k. ”problem” stona ett avvikande beteende under brunsten. Ett av stona tryckte sig mot skötaren, visade en stark ovilja att röra sig framåt samt visade ofta brunst mot skötaren. Ett annat sto slog med bakdelen mot boxväggen och skrek och/eller pep. Ytterligare ett sto visade mycket starka brunstsymptom. Två av ”problem” stona visade svaga eller inga yttre tecken på brunst vid brunstpassning (s.k. tyst brunst). Att inte alla ston visade tydliga beteendestörningar under studietiden beror sannolikt att den förändrade miljön och hanteringen, med fasta rutiner och uteblivna prestationskrav (d.v.s. ingen träning eller tävling), påverkade stonas beteenden.

Det förekom inga skillnader i hur ”problem”- och kontrollston reagerade i beteendetesterna. Stona reagerade dessutom lika i testen oberoende av vilken fas av brunstcykeln de testades i. I ”novel object” testet påverkades dock resultatet av vilken fas (brunst eller lutealfas) som stoet först testades i. De var framförallt stona som först testades i lutealfas som undersökte föremålet tidigare vid det första testtillfället jämfört med det andra tillfället. Detta kan tyda på att ston i lutealfas är mer mottagliga för förändringar i närmiljön. Skillnaderna mellan testtillfällena tyder också på en inlärningseffekt, där stona snabbt vande sig vid testsituationen. I likhet med tidigare studier på vuxna hästar (> 3 år) tycktes stona reagera med en ökad nervositet (förhöjd hjärtfrekvens), men inte med rädsla (utforskade föremålet).

Alla ovariektomerade ston visade brunst vid konventionell brunstpassning (från två till 78 dagar under perioden juli-september). Vid den mer intensiva brunstpassningen var det tre ston som visade brunst i juli, varav ett sto tillät hingsten att betäcka henne. Ett sto visade brunst i november. Generellt visade de ovariektomerade stona fler dagar brunst än då de var intakta, året innan (152 dagar jämfört med 44 dagar), och med oregelbundna intervall.

Plasmanivåerna av samtliga analyserade hormon ökade efter ACTH injektionen. Intakta ston i brunst hade högre nivåer av testosteron och androstenedion vid alla provtagningstillfällen, både kontrollprover och prover efter ACTH behandling, jämfört med efter ovariektomi. Detta visar att hos intakta ston i brunst producerar både äggstockar och binjurar dessa könshormoner. Inga skillnader förekom mellan ”problem”- och kontrollston i ökningen av androstenedion eller testosteron efter ACTH behandlingen. De höga östradiolnivåerna under brunsten hade dessutom ingen stimulerande inverkan på binjurens kortisolproduktion, så som visats hos andra djurslag. Dock hade ”problem” stona ett lägre kortisolsvar vid ACTH behandling jämfört med kontroller. Orsaken till detta är oklar, men det kan tyda på en förhöjd endogen stress hos ”problem” stona under brunsten, vilket medför till ett minskat svar på exogent tillfört ACTH.

Både östradiol- och progesteronreceptorer kunde observeras i binjurebarkens cellkärnor hos undersökta ston. Detta tyder på att det hos sto finns en direkt påverkan av östradiol och progesteron på binjurens funktion. I binjurens märg kunde även en infärgning observeras i cellernas cytoplasma vilket tyder på att progesteron och östradiol också kan påverka stobinjurens katekolaminproduktion.

Då det idag inte är tillåtet att använda progesteronpreparat för att hämma oönskat brunstbeteende hos tävlingsston, gjordes dessutom en preliminär studie för att utvärdera effekten av att framkalla ägglossning under stoets lutealfas. Hos sto kan ägglossning under denna fas orsaka en förlängning av densamma, om den bildade gulkroppen är omogen vid luteolysen. Gulkroppen kvarstår då som regel i två till tre månader och under denna tid visar stoet inte brunst på grund av progesteronets hämmande inverkan. Fyra ston behandlades under lutealfasen då de hade en follikel på minst 3 cm med humant gonadotropin (hCG; 3000 IE i.m.), ett ämne som framkallar ägglossning hos sto. Ett sto fick aldrig tillräckligt stor follikel och kunde därför inte behandlas. Fyra kontrollston behandlades med koksalt. Tre av de fyra hCG-behandlade stona fick ägglossning under lutealfasen och därefter förlängda lutealfaser på 58, 68 och 82 dagar. Inget av kontrollstona fick ägglossning efter koksaltsbehandlingen. Metoden bör upprepas på ett större antal djur för att klargöra dess praktiska användbarhet, men resultaten tyder på att metoden kan fungera om stoet får en tillräckligt stor follikel och hCG-behandlingen leder till ägglossning.

Sammanfattande visade studien att det hos sto finns en koppling mellan äggstock och binjure. Vidare visade studien att äggstocken, genom att producera gulkroppshormon, är nödvändig för att stoet ska visa brunstbeteende med cykliskt intervall. Dessutom tyder resultaten på att ston i lutealfas är mer mottagliga för miljöförändringar jämfört med ston i brunst. Objektiva beteendetester kunde inte påvisa skillnader i beteende mellan ”problem”- och kontrollston.