The relationship between steroid hormones and performance in warmblood sport horses

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Summary

Successful performance in dressage horses and show jumpers depends on the physical ability and fitness of the horse, as well as its willingness to perform the tasks that are requested by the rider. The horse's ability to respond appropriately to the rider and the environment directly influences the horse's usefulness and monetary value to humans. Many breeding organizations emphasize the importance of temperament in their breeding goals by defining desirable behavioral traits in the breed description. Few studies in horses have investigated the relationship between steroid hormones and performance at competition, or between steroid hormones and fearfulness. However, increased levels of steroid hormones such as cortisol and testosterone have been shown to affect the performance of human athletes.

Therefore, the aim of this PhD thesis was to investigate the relationship between steroid hormone concentrations in young sports horses and competition scores (Paper I and Paper II) and steroid hormone concentrations and fearfulness (Paper II). Further, the aim was to compare the fear response in two handling tests to competition scores in young horses (Paper I). An additional aim was to determine the relationship between testosterone concentrations in equine serum and saliva for further use in sports horses (Paper III)

The results presented in this thesis are based on three separate studies.

In Study 1 the temperament trait "fearfulness" was measured on 141 warmblood horses as the reactivity response in two different handling tests (a moving object test and a bridge test). A blood sample was collected before the handling tests for serum testosterone and estradiol determination and heart rate was measured throughout the tests. The horses participated in competitions 2-4 weeks after the handling tests. Performance was recorded as the official competition scores in the individual competitions. Of the 141 horses, 50 also participated in saliva sampling at competition. These horses had saliva samples taken three times daily on 3 consecutive days at competition. The results in study 1 indicated that a higher testosterone concentration in mares and geldings was associated with reduced fear reactions and a lower peak heart rate. However, in stallions, higher testosterone concentrations only affected the latency to resume feeding after a successive exposure to a moving object. High estradiol concentrations were also related to a reduction in some fear responses in mares. Testosterone levels were unrelated to competition scores, but a high competition score was related to a shorter latency to touch the Bridge for 3 years old dressage mares. In show jumpers, an increased reaction and heart rate in the Moving object test and increased latency time to cross the Bridge were related to higher baseline cortisol concentrations at competition.

Study 2 was designed as a study over time including data from a total of 126 horses between 3-6 years of age. Data was collected on-site at home and at three specific events counting competitions in dressage and showjumping, to enable an assessment of cortisol concentrations in different environments and the relation between the blood cortisol levels and the performance at a competition. The results in Study 2 demonstrated that competition scores correlated positively to baseline cortisol concentrations in one out of three events, whereas there was no significant relationship at the other two events. Salivary cortisol concentrations followed a diurnal rhythm with the highest concentrations measured in the morning and the lowest in the evening at home and in the competition environment. Salivary cortisol concentrations were higher during the competitions than at home except for one single event where there was no difference in saliva levels of cortisol for showjumpers between home and the competition. Dressage horses had a higher baseline cortisol concentration at competition than the show jumpers. Exercise in general caused cortisol concentrations to increase in both showjumpers and dressage horses.

Study 3 was designed to investigate the correlation between testosterone in equine saliva and serum, and to obtain additional knowledge on the physiology (diurnal rhythm and concentration levels) of testosterone in geldings and mares. Blood and saliva samples were collected from 67 horses in the morning, at midday and in the evening. The results in Study 3 demonstrated a weak correlation between saliva and serum testosterone concentrations. Stallions had higher serum testosterone concentrations than mares and geldings, but there was no significant effect of sex on salivary testosterone concentrations. The time of day did not affect the concentration of testosterone in either saliva or serum.

In conclusion, this thesis provides new knowledge regarding the steroid hormones testosterone, estradiol and cortisol in young warmblood horses and their relation to competition performance and responses in handling tests. Baseline cortisol levels were higher in a competition environment than at home, however, the diurnal rhythm was maintained. There was no difference between sexes in responses in handling tests but within sex, increased testosterone and estradiol levels were associated with less pronounced fear responses in handling tests. Salivary testosterone had a weak correlation with serum testosterone, and it cannot be recommended to use saliva as a proxy for serum when measuring testosterone levels.

Dansk resumé

For at præstere på topplan ved dressur- og springkonkurrencer skal sportsheste have de fysiske forudsætninger for at udføre opgaven, de skal være i god fysisk form og have viljen til at udføre de opgaver, som rytteren kræver af dem. Evnen til at lære og til at respondere korrekt på forskellige stimuli har ofte direkte indflydelse på hestens anvendelighed og værdi. Mange avlsforbund har specifikke karaktertræk inkluderet i deres avlsmål og et karaktertræk som frygtsomhed har været forbundet med nedsat præstation. Studier af humane atleter har vist, at et øget niveau af både kortisol og testosteron kan påvirke præstation, både fysisk og mentalt. Der findes dog meget få studier om forholdet mellem steroid hormoner, temperament og præstation hos sportsheste.

Formålet med dette studie har derfor været at undersøge forholdet mellem steroidhormon - koncentrationen hos unge sportsheste og de resultater, hestene opnåede i konkurrence (artikel I og artikel II) og forholdet mellem steroidhormoner og frygtsomhed (artikel II). Desuden at sammenligne frygtresponset i to håndteringstests med resultater opnået ved konkurrencer for unge sportsheste (artikel I). Endnu et formål har været at bestemme forholdet mellem testosteron-koncentrationen i hesteserum og -spyt, for eventuelt fremover at kunne anvende spyt-tests til sportsheste (artikel III).

Der er gennemført tre forskellige studier:

I Studie 1 fik 141 varmblodsheste målt deres frygtrespons i to forskellige håndteringstests (Moving object test og Bridge test). En blodprøve til bestemmelse af serumkoncentrationen af testosteron (hopper, vallakker og hingste) og serum østradiol (hopper) blev udtaget før testen blev gennemført, og hestenes puls blev målt under testen. To til fire uger senere deltog hestene i udvalgte konkurrencer. Under konkurrencerne fik 50 af de 141 heste udtaget spytprøver tre gange dagligt tre dage i træk til bestemmelse af spyttets kortisolkoncentration. Resultaterne i artikel I peger på, at en højere koncentration af testosteron hos hopper og vallakker er forbundet med lavere puls og mindre frygtreaktion ved håndteringstests. For hingstenes vedkommende var der en hurtigere tilvænning til en pludselig bevægelse af en genstand (Moving object test) hos hingste med et højere testosteronniveau. Det var dog ikke muligt at vise en klar sammenhængen mellem hormonniveauer og præstation.

I Studie nummer 2 blev der indsamlet spyt- prøver fra 126 heste mellem tre og seks år i hestenes hjemme-miljø samt ved tre forskellige stævner, og sammenhængen med konkurrence-resultaterne blev undersøgt. Det viste sig, at der var sammenhæng mellem spyt-kortisolniveauet og konkurrence-resultatet ved ét af de tre stævner, men ikke ved de to andre. Spyttets kortisolniveau fulgte en døgnrytme med den højeste koncentration om morgenen og den laveste om aftenen. Dette gjaldt for prøverne fra hjemme-miljøet såvel som fra konkurrence-dagene. Kortisol-koncentrationen i spyt var højere ved konkurrencer end hjemme, og dressurheste havde et højere

basalt kortisolniveau ved konkurrencer end springheste. Fysisk udfoldelse forårsagede en stigning i kortisolkoncentrationen hos både spring- og dressurheste.

Studie nummer 3 havde til formål at undersøge sammenhængen mellem testosteron-koncentrationen i henholdsvis spyt og serum hos heste, samt at indhente yderligere information om testosterons fysiologi (døgnrytme og koncentrationsniveau) hos vallakker og hopper. Blod- og spytprøver blev udtaget morgen, middag og aften fra 67 heste. Resultaterne i studie 3 viste en svag sammenhæng mellem testosteronkoncentration i spyt og serum. Hingste havde en højere serumkoncentration af testosteron end hopper og vallakker, mens der ikke var signifikant effekt af køn på testosteronkoncentrationen i spyt. Tidspunktet på dagen var ikke korreleret med koncentrationen af testosteron i hverken spyt eller serum.

Konklusionen er at denne afhandling bidrager med ny viden om sammenhængen mellem præstation under konkurrence, frygtsomhed og koncentrationen af testosteron, østradiol og kortisol i unge varmblodsheste. Et konkurrencemiljø kan forårsage et øget basis-kortisolniveau, specielt i mere frygtsomme heste, men ikke nok til at ændre kortisols døgnrytme. Sammenhængen mellem hormonniveau og præstation er ikke klar, men en øget koncentration af testosteron og østradiol kan nedsætte hestes frygtrespons under håndteringstests. Yderligere studier af sammenhængen mellem frygtrespons og de hormonelle ændringer der sker i hoppens reproduktionscyklus vil bidrage med viden så træning og præstation under konkurrencer kan optimeres. Testosteronniveauet i spyt korrelerede ikke med serumkoncentrationen af testosteron, og det kan ikke anbefales at bruge spyt som substitut for serum i målinger af testosteronniveauer hos heste.

Preface

The industrial PhD program is an industry-focused research project and PhD education that is carried out by a PhD candidate in collaboration with a company and a university.

This industrial PhD thesis was requested by Højgård Equine Hospital and the Warmblood Studs Stutteri Ask and Blue Hors. Højgård Equine Hospital is a highly specialized hospital, serving clients with horses performing at the highest level. Stutteri Ask and Blue Hors are commercial stud farms that produce, educate, compete, and sell horses for international show jumping and grand prix dressage. Successful performance in show jumpers and dressage horses requires that the horses are healthy and trainable, and Stutteri Ask and Blue Hors work closely with Højgård Equine Hospital to achieve this. Højgård Equine Hospital has gained access to new and valuable knowledge by integrating an industrial PhD student in the team, and this has supported the company's business activities in terms of providing the newest evidence-based guidance on how to manage sport horses.

All studies in his thesis were conducted on show jumpers and dressage horses in the field, and measurements were performed on horses in their home environment and at competition locations. Laboratory work and additional analyses were provided by the Unit of Physiology, Pathophysiology and Experimental Endocrinology, University of Veterinary Medicine, Austria, and the Department of Animal Science, Aarhus University.

The industrial PhD program was supervised by principal supervisor Professor Lene Munksgaard and co-supervisors associate professor Janne Winther Christensen, Department of Animal Science, Aarhus University, and postdoc Rasmus Bovbjerg Jensen, Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Professor Per Aagaard, Department of Sports Science and Clinical Biomechanics, University of Southern Denmark and DVM Lars Christian Nielsen, Højgaard Equine Hospital.

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Contents

Abbreviations

ACTH	Adrenocorticotropic hormone
BT	Bridge test
CBG	Corticosteroid binding-globulin
DW	Danish Warmblood association
DCY	Danish championship for 5 yr-old horses
DH	Dressage horse
DRF	Danish riding federation
ELISA	Enzyme-linked immunosorbent assay
EIA	Enzyme immunoassay
EXDIF	Exercise-induced increase in saliva cortisol concentrations
HR	Heart rate
HPA axis	Hypothalamic–pituitary–adrenal axis
LH	Luteinizing hormone
MT	Mare testing
MO	Moving object
RIA	Radioimmunoassay
SHGB	Sex hormone-binding globulin
SJ	Showjumper
SH13	Danish national stallion show in 2013
SH14	Danish national stallion show in 2014
SS	Stallion selection
T/C ratio	Testosterone/cortisol ratio
VO2 max	Maximum rate of oxygen consumption
YHC	Young horse championship

List of original manuscripts

Paper I

The relationship between steroid hormone concentrations, responses in two handling tests and competition scores in warmblood sports horses - a field study (Manuscript ready for submission).

Paper II

An exploratory study of competition scores and salivary cortisol concentrations in Warmblood horses (Published in Domestic Animal Endocrinology 2017)

Paper III

The effect of sex and time of day on testosterone concentrations in equine saliva and serum (Published in Comparative Exercise Physiology 2016).

1. Introduction

Fitness and the physical ability of the horse are important for successful competition performance in dressage horses and showjumpers (referred to as sport horses), in addition the temperament of the individual horse might influence performance. Many breeding organizations emphasize the importance of temperament in their breeding goals by defining desirable temperamental traits in the breed description, and riders request horses that are sensitive, willing to perform and have the ability to concentrate despite environmental disturbances (Koenen et al., 2004). The horse's ability to respond appropriately to the rider and the environment directly influences the safety of the rider as well as the usefulness of the horse in competitions. In addition, successful competition performance will significantly increase the value of sport horses.

In humans, there is evidence to support that steroid hormone levels are related to both physiological and psychological competition performance. Sports competitions in humans are often divided into competitions for males and females, due to the superior performance of male athletes. However, this is not the case in competitions for sport horses. A few equine studies have investigated the relationship between competition performance and gender and found stallions to have superior performance to mares and geldings (Whitaker et al., 2008; Marsalek et al., 2005). Opinions on gender-related behavior and performance also exists amongst riders (Whitaker et al., 2003, 2008; Hedberg 2006) and behavioral problems in mares have been related to increased levels of reproductive hormones (Meagher et al., 1977; McCue et al., 2006).

In human sports, it has been suggested that a higher level of testosterone – between athletes of the same sex as well as between the sexes – is advantageous (ACSM 2006), and it has been shown that elite female athletes have a consistently higher level of free testosterone compared to non-athletes (Cook et al. 2012; Healy et al., 2014). Increased testosterone levels around the time of competition is considered to have a beneficial impact on performance in many sport disciplines in human athlete's due to increased risk taking, reduced fear and anxiety as well as increased motivation to compete (Booth et al., 1989; Frye and Seliga, 2001; Herman et al., 2006; Castro and Edwards 2016). In addition, risk taking, motivation and competitiveness has been linked to extrovert personality types who performed better in sports (Egloff and Gruhn, 1996) in opposition to introvert human athletes where a high level of anxiety interfered with concentration and was negatively related to sports performance (Eysenck et al., 1982). Like testosterone, estradiol has been associated with regulation of fear and risk-taking in females; i.e. low estrogen is associated with increased risk of anxiety disorders (Vermeersch et al., 2008; Glover et al., 2013) and basal estradiol concentration was positively correlated with motivation in women (Stanton and Schultheiss, 2007; Stanton and Edelstein, 2009).

In sports horses only a few studies have investigated the relationship between temperament and competition performance. Fearfulness is one of the most studied temperamental traits and it has been shown that more fearful and highly reactive horses perform inferior to less fearful horses in ridden competitions and free jumping tests (Visser et al., 2008; Rothman et al., 2014a). Another study found that more fearful horses knocked down fewer bars in show jumping competitions (Lansade et al., 2016). However, no studies have investigated the relationship between testosterone and estradiol concentration and fear reactions in horses. Through awareness of the fearfulness of the individual horse, the handler or rider can better predict behavioral reactions in different situations (Lansade et al., 2008) and the horse can be habituated to and prepared for potentially frightening and stressful situations like competitions.

This thesis aims to extend the knowledge of what affects competition performance in sport horses and will focus on the association between steroid hormones and performance at competition and in temperament tests. To be able to increase performance and at the same time justify the continued use of horses in sports, it is central to investigate the actual influence of competition on stress levels in horses, as well as the relationship between steroid hormones, fearfulness and performance in competition situations.

2. Background (State-of-the-Art)

This state-of-the art section contains a review about steroid hormones and the association between steroid hormones and performance in humans and horses. Focus will be on changes in hormone concentrations related to physical exercise during acute exercise and long-term training as well as in stressful situations related to competition. Furthermore, the association between steroid hormones and the temperamental trait fearfulness, and the association to performance in competition are discussed. Finally, the temperament tests and measured parameters used to estimate fearfulness, and the method used for sampling and detection of steroid hormone concentration related to the experiments used in this thesis are reviewed.

2.1 Steroid hormones and performance

In equestrian competitions mares, geldings and stallions compete on equal terms contrary to the racing industry, where race programs are constructed with the assumption that sex differences exist (Gaffney and Cunningham, 1988; Mukai et al., 2003). Analysis of Thoroughbred races on tracks in the USA, France and Australia showed that intact males were on average significantly faster than females and geldings, but the sexual dimorphism was only 1.007 in shorter races and 1.014 in longer races (Van Damme et al., 2008). A study by Harkins et al. (1992) found that mares and stallions performed equally during training runs, but during competitive runs, the stallions outran the mares. One study demonstrated the superior performance of elite eventing stallions when measured by points over the animal's entire career, compared to mares and geldings (Whitaker, 2003). The same author found no differences between eventing mares and geldings when comparing penalty scores, final ranks or lifetime points. The author did, however, notice a steady drop in the percentage of mares in competition as the level of competition increased (Whitaker, 2008). Marsalek et al. (2005) found that stallions reached a higher percentage of clear rounds in show jumping than mares and geldings. Furthermore, they found that mares would more frequently refuse to jump compared to stallions or geldings. Thus, there is some evidence that stallions can outperform females and geldings in performance, which may be linked to steroid hormone levels. There seems to be a higher percentage of stallions compared to mares and geldings in high level professional show jumping than at lower levels, but it has not been investigated whether this is caused by superior athletic performance influenced by testosterone concentration, the result of breeding selection or the fact that stallions are deselected by amateurs and ridden by professional riders because of their more difficult temperament.

In human athletes, it has been suggested that a higher level of testosterone – between athletes of the same sex as well as between the sexes – is advantageous (ACSM, 2006; Casto and Edwards, 2016). It appears to be irrelevant whether the higher level of testosterone is of endogenous or exogenous origin (Wood and Stanton, 2012). In addition, it has been shown that elite female athletes have a consistently higher level of free testosterone compared to non-athletes (Cook et al., 2012). One study showed that the mean testosterone level was doubled in professional female athletes compared to non-professional female athletes (Healy et al., 2014). However, the results included some very high outliers, generating a skewed distribution. In humans, testosterone may also have an impact on performance in many sport disciplines through its effect on the brain, i.e. by increasing motivation and aggressiveness (Hartgens and Kuipers, 2005; Archer 2006; Castro and Edwards 2016). Only one study has focused on the association between testosterone and competition performance in horses. Knobbe et al. (2015) sampled horses from races for mares and found that two winning mares had higher levels of endogenous testosterone than non-winners, yet these were later identified as having an intersex condition, producing higher levels of testosterone. It would be of interest to further investigate the relationship between testosterone concentration both between sex and in horses of the same sex in relation to performance in different equestrian disciplines. If the relationship is confirmed professional riders could select for show jumpers with a high testosterone level whom respond better to physical training and have a greater chance of winning a competition. For amateur riders, the link between steroid hormone concentration and behaviour might be more important.

Another hormone that has been related to performance is cortisol. Cortisol has an indirect impact on behavioural patterns important in competitions, including aggression, arousal and mobilization of physiological resources to deal with impending threats or challenges (Bateup, 2002). In humans, a positive correlation was found between the post-exercise salivary cortisol concentrations and weight lifting and wrestling performance during a competition (Passerlergue et al., 1995, Passerlergue and Lac, 1999). Furthermore, an anticipatory increase in cortisol prior to competition was associated with improved performance in judo athletes (Salvador et al., 2003) and rowers (Snegovskaya and Viru, 1993). The state of readiness to make behavioural responses is referred to as arousal and cortisol concentration is associated with the mechanisms controlling arousal. Arousal is defined as a physiological and psychological activation that leads to a state of wakefulness and attention, and is strongly connected with motivation (Starling, 2013). Thus, a certain level of arousal appears beneficial for performance. The Ideal Performance State in sports or Peak Performance for athletes is the optimal physiological and psychological level of arousal measured by muscular tension, heart rate, blood pressure, brain wave patterns and breathing composure that results in peak performance (Harmison 2006). The Yerkes–Dodson law has been used to describe the relationship between stress and performance, and refers to an empirical association between arousal and performance (Salehi et al., 2010; Chaby et al., 2015). According to the Yerkes–Dodson law, performance increases with psychological and physiological arousal, but only to a certain extent, and performance is impaired under conditions above or below the optimal stress level. For example, this relationship was demonstrated in a study in which rats trained in water with temperatures of 16 °C, 19 °C and 25 °C; the highest concentrations of cortisol were reached at 16 °C, and the lowest concentrations at 25 °C. The best performance and the fastest learning skills were observed at a temperature of 19 °C (Salehi et al., 2010).

In horses, there are no reported studies on the relations between performance at dressage competitions and cortisol concentration and only one study has reviewed the relationship between cortisol concentration and show jumping performance. This study found that a higher post exercise salivary cortisol response to a show jumping competition was associated with fewer faults compared to horses with a lower cortisol response (Peeters et al., 2013). The results should however be interpreted with caution since a clear definition of sampling time for the post exercise cortisol concentration is missing and there are no measures of exercise intensity, therefore it cannot be determined if the increased cortisol concentration in high performing horses were caused by an increase in physical exercise or caused by arousal. In another sports discipline (trotting) two studies reported that decreased performance to standardized maximal- or near-maximal-velocity exercise was associated with a lower plasma cortisol response to exercise (Standardbred trotters; Golland et al., 1999; Hamlin et al., 2002). However, since cortisol concentrations reflect both psychological and exercise-induced physiological factors it is necessary to include further measures for physiological parameters such as heart rate and lactate to be able to separate the cortisol response into cortisol release caused by an increased physiological demand and arousal. To date no such data on the relationship between cortisol concentration and performance exist. Testosterone also has a physiological and a psychological component that might influence performance in horses. Therefore, this thesis investigates the relationship between testosterone and cortisol concentration and competition performance (Paper I and II). Baseline and post-exercise steroid hormone concentrations are influenced by both acute and chronic physical exercise and therefore this information is provided in the next part.

2.2 Steroid hormones and physical performance

A normal "healthy training" induces a physiological response involving steroid hormones. Testosterone has an anabolic effect and plays an important role in the growth and maintenance of skeletal muscle, bone and red blood cells (Zitzmann and Nieschlag, 2001). Increases in muscular strength with training can be attributed to increases in resting testosterone levels and strength training can positively increase testosterone level (Kramer, 1992). Physiologically, sex differences related to testosterone concentration is found in terms of muscle fiber composition. An increased basal level of testosterone and type IIA fast-twitch muscle fibers is found in human athletes with higher explosive power and sprint-running performance (Bosko et al., 1996). A lower proportion of type IIA muscle fibers is found in mares and geldings compared to stallions (Roneus et al., 1991) and the lower proportion of type IIA muscle fibers may result in more rapid fatigue and less power. There are also individual susceptibility to testosterone concentration that influences the physiological and psychological response to a given testosterone level (Kraemer, 1992; Zitzmann and Nieschlag, 2001).

Cortisol is a life-sustaining adrenal hormone essential to the maintenance of homeostasis, and healthy exercise levels induce a physiological increase in cortisol levels. One of the main functions of cortisol is to make energy available. During this process, cortisol increases the production of glucose from fat or amino acids via gluconeogenesis. Cortisol is considered a catabolic hormone due to this breakdown of protein (Thornton, 1985). The appropriate release of cortisol allows an individual to tolerate the challenges to homeostasis that occur in everyday life (Thornton, 1985).

The limiting factor of performance in most human athletes and horses is the inability to train due to health issues associated with the musculoskeletal system (Murray et al., 2010; Vetter and Symonds, 2010). The effect of training relies to an extent on the mechanism of overcompensation, and as a consequence there is a narrow margin between healthy training and overtraining leading to a drop in competition performance. Excessive training can produce an undesirable neuroendocrine exercise-stress response, and overtraining is a result of periods with high-intensity training combined with inadequate recovery periods (Adlercreutz et al., 1986; Hackney, 2006). During overtraining, an increase in cortisol concentration will cause elevations in glucose concentration to provide the energy necessary for the individual to cope with various stressors. However, as a result of glucose conversion from mobilized endogenous amino acids the body may suffer from a reduction of muscle mass after long periods of elevated cortisol concentrations (Matteri et al., 2000). An unexpected drop in performance despite an increased training load can be used as an indication of overtraining however to prevent over training it is more important to athletes to be able to measure the actual training status before overtraining occurs. Symptoms of depression, irritability and anxiety, a psychological tests and hormone analysis, can be used to identify an imbalance in the anabolic/catabolic balance, or chronic fatigue in human athletes (Maso et al., 2004; Meeusen et al., 2006). Similarly, in horses, measurable indications of training status are important (de Graaf-Roelfsema et al., 2007). The physiological signs associated with overtraining is an increased heart rate, an increase in muscle enzymes and a loss of body weight (McGowan and Whitworth, 2008). Experimental overtraining studies have been conducted in horses with emphasis on loss of performance, changes of mood and changes in hormone concentrations. However, there are difficulties related to identification of loss of performance, as well as ability to test the psychological state of the horse. The behavioural signs associated with overtraining is nervousness (Persson et al., 1980, 1983), irritability and unwillingness (Golland et al., 1999a). In addition, there is a discrepancy between studies in regards to hormone response to long term training (Bruin et al., 1994; Tyler et al., 1996, 1998; Golland et al., 1999a,b, 2003; Tyler-McGowan et al., 1999; Hamlin et al., 2002; McGowan et al., 2002; de Graaf-Roelfsema et al., 2007; Rivero et al., 2008), this is further discussed in section 2.2.3.1 (Response to long-term exercise).

There are conflicting results regarding the role of testosterone and cortisol in relation to overtraining in racehorses and no studies have been conducted in dressage horses and show jumpers. Thus, further studies investigating steroid hormone changes related to training and competition in sports horses are needed to improve surveillance of training to prevent overtraining. Non-invasive measurements of salivary testosterone might have potential for monitoring testosterone concentrations in horses in relation to overtraining. However, this requires robust and reliable methods for correct interpretation of results and this has not been investigated in horses.

2.2.1 Testosterone

Testosterone is secreted episodic from the Leydig cells in stallions, and a diurnal rhythm can be detected in secretion patterns (Sharma, 1976). The production of testosterone starts to increase at 80 weeks of age (Naden et al., 1990), and will increase with age as well as during the breeding season (Johnson et al., 1983; McDonnell and Murray, 1995; Khalil et al., 2009; Aurich et al., 2015).

There are currently only a small number of studies investigating testosterone concentration, variation and cyclicity in mares. The vast majority of testosterone is produced in the theca cells of the developing follicles in mares, and a limited amount is produced in the adrenal cortex controlled by the release of adrenocorticotrophic hormone (ACTH) from the hypothalamus (Watson and Hinrichs, 1988; Ginther et al., 2007a). The concentration of testosterone increases during the follicular-phase as the dominant follicle grows (Ginther et al., 2007a; Meinecke et al., 1987) and a positive correlation has been found between an increase in follicular diameter and androgen concentration (Meinecke et al., 1987). However, there are inconsistencies between studies since another study showed an increase in testosterone around the time of ovulation and on days 9, 10 or 11 after ovulation in some mares, and an increase only during estrus (with a peak just prior to ovulation) in other mares (Silberzahn et al., 1978). Testosterone levels twice as high as the basal levels can cause stallion-like behaviour or aggressive behaviour as seen in mares with testosterone producing ovarian tumours (Meager et al., 1977; McCue et al., 2006). In women, 25–30% of circulating testosterone is of ovarian origin, with the remaining being produced by the adrenal cortex (Piltonen et al., 2002). The adrenal glands is also involved in steroid hormone production in horses and treatment of ovariectomized mares with ACTH lead to an increase in plasma levels of testosterone, but not progesterone or estradiol (Hedberg et al., 2007a,b; Watson and Hinrichs, 1988). In geldings, the only source of testosterone is the adrenal glands (Morganti, 2010). In stressful situations, the adrenal gland can increase the production of both testosterone and cortisol which can induce aggression or stallion-like behaviour in geldings as well as in mares (Morganti, 2010; Beaver and Amoss, 1982). Treatment with ACTH in mares with normal as well as abnormal estrous behavior significantly increased levels of both cortisol and testosterone (Hedberg et al., 2007a,b). The increased levels of testosterone in response to ACTH varied markedly between individuals, with some mares showing a much greater increase than others (Hedberg et al., 2007a,b)

In humans, stress can affect levels of testosterone. Acute psychosocial stress can result in increased concentrations of testosterone in both men and women (Booth et al., 1989; Mazur et al., 1992; Josephs et al., 2006). Pain has also been shown to increase the levels of testosterone in humans (Craft et al., 2004), and a significant increase in testosterone has been observed in horses with chronic disease (Ayala and Martos, 2013). Other authors have found conflicting results, for example, Fenster et al. (1999) observed that a decreasing level of serum testosterone was one of the first signs of chronic stress in man. Likewise, Baker et al. (1982) found significantly lower concentrations of testosterone in stressed racehorses compared to non-stressed racehorses, where stressed racehorses were defined as those “not performing to the trainers’ expectations” without any obvious health-related explanation. However, the time of measuring testosterone concentration in relation to stressful events as well as the definition of acute or chronic stress varies between studies and at this point it is not possible to make a conclusion regarding stress related testosterone response.

As previously mentioned, serum testosterone concentration in stallions is considered to be episodic and has been found to have a diurnal pattern with peak testosterone concentrations in the morning and a nadir in the evening or at night (Kirkpatrick et al., 1976; Sharma, 1976). However, another study (Bono et al., 1982) did not find a diurnal rhythm in blood testosterone concentrations in stallions and further studies are needed to be able to investigate the relationship between testosterone concentration and performance as well as behaviour in temperament tests. As there are no published studies regarding the diurnal rhythm of testosterone concentration in mares and geldings, this also needs further investigation, and is addressed in this thesis (Paper III).

2.2.1.1 Testosterone and exercise

Human athletes with higher testosterone levels achieve greater muscle strength and power, despite doing the same volume and intensity of strength training as athletes with lower levels of testosterone (Ahtiainen et al., 2004; Cadore et al., 2010; Häkkinen et al., 1993; Häkkinen and Pakarinen, 1995). Other authors have also suggested an association between trainability and testosterone (Cook et al., 2012; Hansen and Kjaer, 2016). There a very few studies regarding testosterone concentration and exercise in horses. In relation to acute exercise Golland et al. (1989a) found that an acute bout of exercise at maximal or sub-maximal intensity resulted in an elevated testosterone concentration for 32 hours after exercise in Standardbred trotters. Elevated levels of cortisol but decreased levels of plasma testosterone after an acute bout of exercise were found in studies of horses exposed to chronic training (Baker et al., 1982; Wiest et al., 1988; Rabb et al., 1989), whereas others have failed to show an effect on testosterone concentration after 32 weeks of training in young Standardbreds (Leleu and Haentjens, 2010). Months of racetrack training and an ongoing competition schedule (1-2 per month) resulted in lower plasma testosterone concentrations in stallion racehorses (Soma et al., 2012; Baker et al., 1989). It seems like acute high intensity exercise will increase testosterone concentration but the level of previous training and overtraining might influence the response to exercise. Possibly the testosterone response to training is dependent on the type of training i.e. intensity and duration. More information is needed to be able to use the testosterone as a tool to monitor training.

2.2.2 Estradiol

Estradiol is important in the regulation of the female reproductive cycle, and has important effects in many tissues including tendons, bone, fat, skin, liver, and the brain (Hansen and Kjaer, 2016). Estradiol is mainly produced within the follicles in the female ovaries, but the conversion (aromatization) of precursor molecules also occurs in fat, liver, adrenal, breast, and neural tissues. Expressions of oestrus behaviour in mares are related to estradiol, and if estradiol is administered to ovariectomized mares, the mares will show signs of oestrus (Asa et al., 1980, 1984). Estrous behaviour occurs in ovariectomized mares as a result of the absence of progesterone and may be stimulated by estrogen produced in extragonadal tissues. Estradiol concentration is fluctuating throughout the reproductive cycle in both women (Glover et al., 2013) and mares (Medan et al., 2004;

Nett et al., 1973). A diurnal rhythm of free estradiol has been detected in women (Bau et al., 2003), but this pattern has not been established in mares.

2.2.2.1 Estradiol and exercise

The wide fluctuations in sex hormone concentrations might affect athletic performance in women. The impairment of physical performance in women in the late luteal and menstrual phases is generally attributed to the symptoms of premenstrual and menstrual syndrome: fatigue, fluid retention, weight gain and mood changes including irritability, depression, and loss of motivation (Bale and Davies, 1983). In terms of sports performance, estradiol concentration has an influence on collagen structural proteins, which are important elements in both tendons and ligaments (Liu et al., 1997) and estradiol plays a pivotal role in maintaining the homeostasis of female connective tissue (Hansen and Kjaer 2016). Studies on estradiol and performance in female athletes suggest that aerobic capacity is influenced by the menstrual cycle, and therefore by estradiol concentration (Lebrun et al., 1995), and that exercise performance and muscle glycogen content are enhanced during the luteal phase of the menstrual cycle (Nicklas and Hackney, 1989). However, other studies did not find the menstrual cycle to have an effect on anaerobic performance, aerobic endurance performance, isokinetic muscle strength, maximal intensity whole body sprinting, or the metabolic responses to such exercise (Lebrun et al., 1995; Tsampoukos et al., 2010). In conclusion, the actual relationship between estradiol and exercise in women is not clear and has not been studied in horses.

2.2.3 Cortisol

In horses, cortisol is often referred to as the stress hormone, and the event that induces an increase in cortisol response is referred to as a stressor (Möstl and Palme, 2002). Cortisol is released during the neuroendocrine response and is responsible for initiating specific physiological changes in response to acute and chronic stressors (Möstl and Palme, 2002). Cortisol is considered a catabolic hormone due to its role in breakdown of protein and the appropriate release of cortisol allows an individual to tolerate the challenges to homeostasis that occur in everyday life (Thornton, 1985). A chronically elevated concentration of cortisol will cause a constant breakdown of tissue, inadequate recovery and subsequently increase the risk of injury (Galambos et al., 2005; Nippert and Schmidt, 2008). Furthermore, prolonged elevated concentrations of cortisol can cause immunosuppression and decreased reproductive performance (Möstl and Palme, 2002).

The same stressor may cause different responses in different horses, and the perception of a stressor depends on the nature and experience of the individual. Horses appear to fear the unfamiliar, and novel situations are often perceived as stressful. At competitions, the horses are introduced to a new environment; they are surrounded by unfamiliar horses and stay in unfamiliar stables. Becker-Birck et al. (2013) even found that dressage horses and show jumpers competing in their home environment had a higher baseline cortisol concentration during competition days than pre-competition days. This could be induced by environmental changes, spectators, a change in the behaviour of the rider and it is also possible that the horse can anticipate that something unusual is happening. This anticipatory increase in salivary cortisol concentration was also found in horses 30 minutes prior to transportation in studies by Schmidt et al. (2010b,c). Several authors have studied stressful situations such as the association between cortisol release and transportation. Medica et al. (2010) reported a significant increase in cortisol in horses with various levels of experience after transportation to a long-distance trekking event, and the same was found in dressage horses after transportation to a show (Cayado et al., 2006). In contrast, Fazio et al. (2008b) did not find an increase in cortisol after the transportation of experienced show jumpers. Schmidt et al. (2010d) examined the cortisol response in Warmblood horses between 5-22 years of age to 3-8 hours of transportation. The study showed an increase in cortisol concentration irrespective of the journey duration, and the cortisol concentration remained elevated throughout the transportation. In another study by Schmidt et al. (2010c) it was shown that naiive horses habituated to transport and cortisol concentration decreased over 4 repeated exposures. Decreased cortisol concentration as a result of habituation was also found in experienced show horses after long-term transportation (Schmidt et al., 2010b). Level of experience or habituation to competition also causes a decrease in cortisol response to competition in both dressage and show jumping horses (Covalesky et al., 1992; Cayado et al., 2006). Cayado et al. (2006) also found a discipline related difference in cortisol response to a new competition environment, with dressage horses having higher cortisol response than show jumpers. The previous exposure to competitions for the individual horses in Cayado et al. (2006) were not recorded therefore it can only be speculated if the results could be a reflection of difference in competition experience (habituation) between dressage horses and show jumpers, or if dressage horses were more sensitive to the environment. Von Borstel et al. (2010) measured fearfulness in warmblood horses and found that show jumpers were less fearful than other horses but fearfulness decreased with level of training in all horses, however, cortisol concentrations were not measured. In the studies by von Borstel et al. (2010) and Cayado et al. (2006) the study population consisted of horses between 3-20 years of age with various previous experience and the discipline related difference in cortisol response to a competition environment might be different for a group of younger and more inexperienced dressage horses and show jumpers.

Factors thought to influence cortisol level include the occurrence of short-term fluctuations and the time of day (Irvine and Alexander, 1994; Aurich et al., 2015). Cortisol follows a diurnal pattern in horses, with a peak in the morning, observed between 06:00 and 09:00 (Irvine and Alexander, 1994; Golland, 1999; Tortonese and Short, 2012; Becker-Birck, 2013; Bohak, 2013; Aurich, 2015) and a nadir at night, reported between 16:00 and 23:00 (Toutain et al., 1988; Bohak, 2013). Diurnal rhythm can be affected by several factors. However, despite being exposed to environmental changes, a diurnal rhythm persisted in trained racehorses both before and after exposure to jetlag (Tortonese and Short, 2012). This was also observed in horses of different age groups and training status, both in the field and in different housing conditions (Irvine and Alexander, 1994; Aurich et al., 2015). Other studies observed a disrupted diurnal rhythm of cortisol when young mares were moved from the field to an isolated stable in a new environment (Harewood and McGowan, 2005), or horses moved from one stable to another stable in a new environment (Irvine and Alexander, 1994).

Erber et al. (2013) transferred 3-year-old Warmblood mares that were in training from group housing to individual boxes. Salivary cortisol was measured at time 0, 5, 15 and 30 minutes post transfer. The change in housing caused cortisol concentration to double within 30 minutes and it remained at an increased level for 6 hours. The next day, the diurnal rhythm was re-established, but the basal cortisol level was maintained at an increased level over 5 days. When considering cortisol concentration in response to environmental changes and training, it is important to be able to separate the physical parameter. It can be a complex situation, however cortisol release in response to physical training is temporary, and baseline values are restored within an hour after the rider dismounts and the horse is returned to the stable (Schmidt et al., 2010a). There is agreement between studies on the diurnal rhythm of cortisol in horses when they are undisturbed in their home environment; however, there are inconsistent results regarding the maintenance of a diurnal rhythm when exposed to various stressful situations. For example, the influence of several days of competition in an unfamiliar environment on the diurnal rhythm of cortisol remains to be explored and will be addressed in this thesis (Paper II)

Age does not seem to have a significant effect on cortisol concentration in horses between 1 and 24 years of age (Aurich et al., 2015; Janczarek et al., 2013; Kang and Lee, 2016) and it is likely that experience and habituation to procedures and environment play a larger role. Similarly, sex does not seem to influence cortisol concentration except in sexually active breeding stallions, who have higher concentrations during the breeding season than adult geldings (Aurich et al., 2015). In healthy horses, there is no effect of season (Place et al., 2010) and the increase in the breeding season found in the study by Aurich et al. (2015) could be influenced by the housing system of the stallions, where sexually active stallions were housed in adjacent boxes. The influence of interaction amongst stallions was also shown in other studies where cortisol concentration increased in breeding stallions upon hearing the vocalization of other stallions being sexually stimulated (Villani et al., 2006; Rabb et al., 1989). One study found a higher cortisol level in mares than in geldings participating in long competitive endurance rides (Janczarek et al., 2013), however, there was no information on other parameters such as the difference in training level, which could potentially have influenced the cortisol concentration. In order to determine the influence of age and sex on cortisol concentration, it is important to avoid other confounding factors. There is a possibility that age is sometimes confused with habituation after repeated exposures.

2.2.3.1 Cortisol and exercise

A stress-activated response from both the autonomic nervous system and the hypothalamic–pituitary–adrenal axis (HPA) axis is induced by acute exercise (Hyppä, 2005), and the response in a trained, competing individual reflects the response to exercise and the stress of the competition, in addition it reflects the actual physical condition created by long term training (Healy et al., 2014). It has been suggested that acute changes in cortisol may be used to evaluate the metabolic demands of alternate resistance exercise programs, or to monitor training strain (Nunes et al., 2011), a detailed description of which will follow.

Response to acute bouts of exercise

The magnitude of the cortisol response in humans to an acute bout of exercise is associated with the maximum rate of oxygen consumption (VO2 max), and exercise at intensities below 50% of the VO2 max do not cause increased levels of cortisol (McMurray and Hackney, 2005). Strength training will also trigger a significant acute response that is dependent on the intensity and volume of training (Kraemer, 1992). In horses, cortisol also increases after an acute bout of exercise in standardized training sessions on treadmills or by riding (Covalesky et al., 1992; Marc et al., 2000; Malinowski et al., 2006). This response also depends on the duration, intensity and type of training (Desmecht et al., 1996; Lindner et al., 2000). A linear relationship between cortisol concentration in racehorses and their increased speed on a treadmill has been shown to exist (Church et al., 1987; Jimenez et al., 1998) and Ferlazzo et al. (2009) found an association between raising the height of show jumping fences and increasing serum cortisol concentrations. The cortisol response to competition, measured in saliva or serum, in different equestrian disciplines has been investigated in various studies. The studies report that the horses are sampled post exercise however there is a variation in the time defining post exercise. The following responses were found; cortisol increase (compared to the baseline) 150-360% when show jumping (Peeters et al., 2013; Lekeux, 1991; Becker-Birk et al., 2013). Measurements taken at dressage competitions found cortisol levels increased by 200% (Becker-Birk et al., 2013). Based on these studies, it is likely that both high-intensity, short-term exercise and low-intensity, longer-lasting exercise induce a cortisol response, however most studies do not have an exact measure of exercise intensity or a standardized time and frequency of post exercise cortisol measurements, therefore making interpretation difficult.

Other factors than exercise intensity plays a role in the magnitude of cortisol response therefore making the exact measure of physical performance very important. For example; One study found significantly higher cortisol concentrations following jumping exercise at competitions compared to identical exercise at home (Covalesky et al., 1992), indicating that the environment was affecting cortisol concentration. However, another study, in which a dressage test was performed in a familiar arena both with and without spectators, found no association between cortisol concentration and the presence of spectators (von Lewinsky et al., 2013). As previously discussed habituation to competition significantly decreases the cortisol response to acute exercise (Covalesky et al., 1992; Cayado et al., 2006; Mircean et al. 2007), and similarly an increased training status will also reduce the cortisol response to an acute bout of exercise (Marc et al., 2000). In conclusion, it is important to have information on level of training and competition experience when measuring the cortisol response to exercise. Even then, the actual physiologically induced increase can be difficult to measure since the anticipation of exercise in itself, can induce an increase in the cortisol concentration in some horses (Irvine and Alexander, 1994).

Response to long-term exercise

In human athletes, regular exercise patterns will cause the neuroendocrine stress response to exercise to become attenuated when the exercise session is performed at the same absolute workload, leading to a reduction in resting, basal cortisol levels in many situations (Hackney, 2006). However, an increase in the resting level of cortisol was observed in athletes with a prolonged decrease in performance following training of excessive intensity and volume (Fry and Kraemer, 1997). A higher resting cortisol level was also found in elite athletes compared to non-elite athletes (Cook et al., 2012) but in contrast to the athletes in the study by Fry and Kraemer (1997), the elite athletes did not have an indication of decreased performance (Cook et al., 2012).

As in humans, studies investigating the relationship between long-term training and cortisol concentration in horses, are not consistent. The training status of the horse can influence both resting baseline cortisol concentration and, as previously discussed, the response to an acute bout of exercise. In riding horses, long-term training decreases the baseline plasma cortisol level (Fazio et al., 2008b), and the cortisol response to riding events was subsequently influenced by previous training (Fazio et al., 2008b). Brandenberger and Follenius (1974) and Marc et al. (2000) also confirmed a decrease in post exercise cortisol concentrations compared to baseline following acute exercise in horses with a high level of fitness compared to less fit horses. In some studies, there were no change in baseline cortisol concentration in trained versus non-trained horses (Irvine and Alexander, 1994; Kelley et al., 2011; Schmidt et al., 2010a). Others have detected an increase in baseline cortisol concentration both in trained western horses during a 6-week training program (Medica et al., 2011) and in Thoroughbred racehorses (Persson et al., 1980; Freestone et al., 1991).

Despite the conflicting results an increased baseline cortisol concentration has been investigated and used as an indication of overtraining in both humans and horses as mentioned in section 2.2. Golland et al. (1999a) studied the effects of training and overtraining on plasma cortisol concentrations at rest and after standardized exercise tests in Standardbred horses and found that peak cortisol concentrations after exercise decreased significantly in the over-trained group, as did mean cortisol concentrations over a 120-minute period after exercise. However, mean and total cortisol concentrations in resting horses were not significantly different in the over-trained group. In addition, peak cortisol concentrations after administration of ACTH were not significantly different in the over-trained group. Other studies also investigated the effect of overtraining and reported a decrease in basal cortisol level in over-trained Standardbreds (Hamlin et al., 2002) or no change in basal cortisol during short-term overtraining (Bruin et al., 1994). Leleu and Haentjens (2010) investigated the occurrence of maladaptation to training under real training conditions. In a longitudinal study in the field, they compared morphological, haematological and endocrine changes in a group of 14 2-year-old Standardbreds presenting signs of maladaptation to training to a control group of 40 individuals. They were unable to detect any difference in testosterone and cortisol concentration between the groups, but they found that weight loss could be used to identify the horses suspected of maladaptation to training. It is difficult to interpret the results from overtraining studies since the parameters used to identify the occurrence of overtraining (as well as the training methods and training schedules of the horses) vary among studies. The presented studies were all performed on racehorses, and there is no existing literature on overtraining in dressage horses or showjumpers.

These divergent results in both horses and humans indicate that there are factors and relationships influencing cortisol concentration that are not fully understood. The interpretation of a single measurement of baseline cortisol concentration should therefore be done with caution. It is of great importance to know the exact training status of the individual and to have repeated measures over time to be able to interpret the origin of the cortisol response.

2.3 Steroid hormones and temperament

Kilgour (1975) presented the following definition "Temperament is the behavioural characteristics resulting from the individual's physical, hormonal and nervous organization" (cited by Seamann, 2002) which fits into the scope of this thesis. Opinions on gender-related temperament, behaviour and performance exists amongst riders. A general perception is that stallions are more aggressive, less sensitive to the rider and more distracted by the environment than mares and geldings. It is also a common perception that mares can have rideability issues related to the estrous cycle and there is evidence to support that behavioral problems in mares are related to increased levels of reproductive hormones (Hedberg et al., 2006; Meagher, 1977). It is well known that the intensity of oestrous symptoms in the intact mare varies, with some animals showing very strong and/or exaggerated symptoms (sometimes referred to as nymphomania). Such disturbed estrous behavior may lead to difficulties in the training and handling of the mare, particularly for those who wish to use the animal for competition purposes (Hedberg, 2005). Studies involving steroid hormones (except cortisol) and temperament in horses have focused on the influence of testosterone on breeding behaviour in stallions and on oestrous behaviour related to the oestrous cycle in mares. Differences in an animal's reaction in various test situations have been found to depend on gender, as well as oestrous-cycle stage in experimental studies in rats (Diaz-Veliz et al., 1989; Mora et al., 1996). Sex differences have been studied as possible factors affecting the response in behavioural or temperament tests in various mammals, such as rats, chimpanzee and sheep, with males showing less fear reactions than females (Archer, 1974, 1975; Buirski et al., 1978; Vandenheede and Bouissou, 1993a). The effect of hormonal treatments on response in behavioural and temperament tests has also been studied and a prolonged androgen treatment reduces fearfulness in heifers and ewes (Bouissou 1982: Bussy and Bouissou, 1994; Vandenheede and Bouissou, 1993b). In hunting dogs, decreasing the level of steroid hormones by gonadectomy increased the odds for behavioral problems such as separation anxiety and fear of noises compared to sexually intact dogs (Zink et al., 2014).

To my knowledge, only one study has been performed in the horse, where oestrous-cycle stage has been considered in relation to reactions in standardised temperament tests. This was done by Hedberg (2005) who tested 7 mares with owner reported behavioural problems and 5 control mares in a novel object test and an isolation test. The mares were tested initially during oestrus (defined by the presence of a follicle of minimum 30 mm and uterine oedema indicating oestrus), and again in dioestrous 5-12 days post-ovulation. In this study, there was no relationship between behavioural variables or heart rate scores and the stage of the oestrous cycle. More studies are needed to exclude or confirm the relationship between oestrous cycle and behaviour in healthy mares. Tests might be repeated at other stages of the oestrous cycle and a higher number of mares should be tested.

2.3.1 Testosterone and temperament

Research on the relationship between testosterone concentration and temperament in horses has focused on the relationship between testosterone concentration and behaviour related to breeding as well as the social interaction amongst individuals in herds (McDonnel et al., 1992, 1995a,b) and not on behaviour related to competition or temperament tests. However, in humans the relationship between testosterone and behaviour that can influence performance in competitive situations has been investigated and it is well-described that anticipation to an impending competition will increase testosterone concentration in both male and female athletes (Castro and Edwards 2016; Booth et al., 1989; Mazur et al., 1992; Mazur and Booth 1998; Oliveira et al., 2009; Salvador et al., 2003; Suay et al., 1999). An important function of testosterone in humans is its role in motivation, and animal models have shown a link between testosterone and the dopaminergic system, which modulates reward processing and motivational drive (Nyby 2008). Likewise, human patients with low testosterone levels (hypogonadism) show apathy and lack of motivation, whereas testosterone administration in healthy individuals induces the motivation to act (Eisenegger et al., 2010).

In general, an increase in testosterone concentration can reduce anxiety and fear as well as enhance cognition and elevate moods in humans (Chichinadze et al., 2012; Frye, 2001). Herman et al., (2006) found that a single dose of testosterone in humans would reduce subconscious fear and fear-potentiated startle reactions. Reduced fear increases novelty seeking which is a personality trait associated with explorative activity in response to novel stimulation. Like other personality traits, novelty seeking has been found to be hereditable and is associated with free testosterone concentration (Maattanen et al., 2013; Tsuchimine, 2015). Studies in rodents have shown that administration of testosterone reduces anxiety-like behaviour, fear responses and enhances motivation for specific exploration of novel situations and unknown objects (Diaz-Veliz et al., 1991; Frye and Seliga, 2001; Carmen Arenas et al., 2015)

In stallions, an increase in testosterone concentration increased both aggressiveness and sexual behaviour (McDonnel, 1995a,b) and elevated levels of testosterone may cause aggressive and stallion-like behaviour in mares (Meagher et al., 1977; Busamente, 1998; Morganti et al., 2010). However, there are no studies on the relationship between testosterone concentration and behaviour such as anxiety, fear or novelty seeking that could potentially affect performance in horses. Based on the human literature concerning the relationship between testosterone concentration and temperament, fearfulness in horses could potentially be reduced with increased testosterone concentrations. Thus, it seems reasonable to pursue further information with regard to horses.

2.3.2 Estradiol and temperament

Estradiol has an influence on neural processes that regulate fear inhibition in adult women; it was suggested that low estrogen may be associated with increased risk for anxiety disorders through dysregulated fear responses (Glover et al., 2013). In young women, a high estradiol concentration is associated with increased risk-taking measured as aggressiveness in computer tests (Vermeersch et al., 2008) and basal estradiol concentration is positively correlated with power motivation (a personality measure of dominance motivation) in women (Stanton and Schultheiss, 2007; Stanton and Edelstein, 2009). Experimentally increased levels of estradiol have been shown to reduce anxiety, increase aggressiveness and positively influence reward processing and motivation in female rodents (Monaghan and Glickman, 1992; Frye and Seliga, 2001; Aikey et al., 2002; Nyby 2008; Walf et al., 2009). The relationship between estradiol and behaviour such as fear or anxiety in horses has not yet been studied, but a study on the effect on reproductive behaviour in mares showed that an increase in estradiol concentration caused an increased oestrous behaviour (Asa et al., 1984). Currently there are no research on the relationship between stage of the mares' reproductive cycle and athletic performance or behaviour other than mating behaviour.

2.3.3 Cortisol and temperament

The relationships between behaviour or temperamental traits and cortisol concentrations have been investigated in horses and I therefore focus on these studies rather than the human literature in this section. Reactivity score and cortisol response to sudden stimuli and novel objects, as well as temperamental traits scored by riding instructors was assessed by Anderson et al. (1999) in 100 horses used for therapeutic riding programs. No significant correlations were found between subjectively scored temperamental traits and cortisol concentrations, but there was a tendency towards a higher reactivity score being related to a higher cortisol concentrations. This relationship was also investigated by Young et al. (2012) during four different husbandry procedures lasting 10 minutes (sound of clippers, social isolation, grooming procedure and sound of fireworks) where a significant correlation was found between mean behavioural score and peak cortisol concentration, where cortisol was measured from the end of procedure and in 10 minutes intervals for 40 minutes. The behavioural score and percentage change in cortisol concentration was combined and the horses were divided into four stress groups (no, low, medium or high stress). Horses characterized as agitated, anxious, active and aggressive by professional handlers were all in the high stress group. Hall et al. (2014) investigated the relationship between ridden behaviour and baseline or post-exercise cortisol concentration in a group of ridden horses in a familiar environment without any stressful happening. They did not to find a relationship between ridden behaviour and baseline or post-exercise cortisol concentration. Neither did they find a relationship between cortisol concentration at the time of sampling and the scored behaviour during cortisol sampling. There was however, a positive correlation between age of horse and handling score, with younger horses showing greater anxiety than older horses. A significant effect of current role on handling score was also found, with competition horses scored as being significantly more anxious than leisure horses or riding school horses. Malmkvist et al. (2012) approached the subject differently; they separated 60 Warmblood horses living under the same conditions into two groups depending on the occurrence of gastric ulcers. The two groups were exposed to a novel object test in individual pens in their home environment, and cortisol concentration was measured before and after exposure. The results showed that horses in the ulcer group spent more time away from the novel object and had a higher cortisol response to it, and thus were more sensitive to stress. Results regarding cortisol concentration and temperamental traits are inconclusive but there are indications towards an existing relationship between increased reactivity and anxious behaviour and an increased cortisol concentration in response to stressors.

There are human studies investigating the relationship between personality or behaviour and the concentration of both testosterone and cortisol and one study suggested that the effect of testosterone on human aggression is moderated by cortisol (Mehta et al., 2008). A high testosterone concentration is associated with increased aggression only when cortisol concentrations are low. High levels of cortisol play a critical role in blocking the influence of testosterone on competition and dominance. When cortisol increases, the body is mobilized to escape danger rather than to respond to any influence that testosterone has on other types of behaviour (Mehta et al., 2008). However, this relationship remains to be investigated in horses but could provide us with useful information in regard to future training of sports horses.

2.4 Performance and temperament

There are human studies exploring the association between steroid hormones and temperamental traits related to performance, however there is limited information on this subject in sports horses.
In humans, extroverted athletes perform better in competition than introverted athletes. Introvert athletes are more anxious and anxiety can interfere with concentration and has been shown to negatively affect sports performance. In contrast extrovert personality types are more competitive and take greater risks at competitions than introverts (Egloff and Gruhn, 1996). In horses, the type of competition influence which temperamental traits might be most advantageous but there are also individual preferences amongst riders in terms of desired temperamental traits. In a recent study involving 800 polish horse owners (both professionals and amateurs), low aggression and a willingness to seek human contact were desired temperamental traits (Suwala et al., 2016). Visser et al. (2008) found that professional riders preferred to ride horses ranked as very attentive to the riders aids, however attentiveness was not related to any measures of temperament obtained in behavioural tests. In another study Visser et al. (2003b) investigated the relationship between responses in behavioural tests and temperament assessed by riders. Responsiveness to the environment and attention to the rider were the temperament measures mostly agreed on, however they were not able to find any relationship to behavioural parameters in a novel object test and only the parameter patience measured in the Bridge test correlated to responsiveness to the environment.

A trait like ‘flightiness’ may be generally advantageous to racehorse performance (Hausberger, 2011), however this is not regarded as a desired trait in dressage horses or showjumpers. Warmblood riding horses categorized as flighty in behavioural tests also showed more evasive behaviour when ridden, and the same horses were very sensitive to environmental challenges when ridden by unfamiliar professional riders (Visser et al., 2008). A few studies have investigated the relationship between a behavioral measure and the actual performance at competition. Rothmann et al. (2014)

investigated how reactivity measured during an official conformation evaluation was associated with rideability score and performance in 3-year-old Danish Warmblood mares. They found that highly reactive horses received lower rideability scores in show jumping and free jumping, but no association with performance traits in dressage was found. Other studies have reported a relationship between behavioral response and different measures of performance. Von Borstel et al. (2011) assessed 3- and 4-year-old Warmblood stallions during a 4-week performance test and found that stumbling and head tossing were negatively related to the temperament score given by the judges, whereas the head posture of the horses was positively related to the temperament score, with a high score given to horses with their nose behind vertical. In a study of 3- to 17-year-old Warmblood horses, Von Borstel et al. (2010) found that an attenuated reaction and decreased time to resume feeding occurred during a moving object test in horses with higher show-jumping index points (as calculated from the pedigree and not the actual physical performance of the individual). In contrast, Visser et al. (2003a) were unable to find an association between show jumping performance in young horses and variables measured in novel object and bridge tests earlier in life. However, they found that a decreased latency time to touch the novel object in a test was positively associated with learning in other tests.

There is great variation in behavioral measures and performance goals across the studies already mentioned and also in the time period from behavioral measures to performance score. However, there seems to be an association between a low performance score in both dressage and show jumping and horses that are very reactive and shows signs of aversive behavior when handled or ridden. It is important for riders to identify whether specific temperamental traits are related to superior performance in a given discipline, the latter question being addressed in the present thesis (Paper I)

2.4.1 The temperamental trait fearfulness

As indicated in the previous section, performance in dressage horses and showjumpers might be related to individual behavioural characteristics also known as temperament. Equine temperamental traits are often included in the breeding goals for warmblood sport horses (Koenen et al., 2004; Gerber Olsson et al., 2000) and horse's performance, management and value are influenced by learning ability, motivation and stress reactions to different stressors (Olczak et al., 2015).

Fearfulness is considered to be one of the basic traits of an individual's temperament. Fear reactions have an obvious survival value in wild animals, and horses typically avoid – or flee from – novel stimuli. While fear is an emotional state induced by the perception of actual or potential danger, fearfulness can be defined as a predisposition to react in a similar manner to a variety of fear-provoking events (Lansade et al., 2008). For survival in the wild fear might be beneficial, however, high levels of fear can have a negative impact on performance, health, reproduction and welfare in animals (Boissy, 1995) therefore, it is of importance to be able to identify fearful individuals. In a number of studies fearfulness has been referred to as the most important and stable temperament trait (Anderson, 1999; McCann et al., 1988; Lansade et al., 2008; McCall et al., 2006; Von Borstel, 2011) and the same studies have used reactivity to a specific stressor, as a measure of fearfulness.

Specific temperamental traits have been found to be heritable. Fearfulness (as tested in an arena test, a novel object test and a bridge test) was dependent on sires in French Saddlebreds (Wollf et al., 1997), and neophobic reactions were more pronounced in Arabians than French Saddlebreds (Hausberger et al., 2004). High levels of reactivity in a novel situation were found to be heritable in 3-year-old Danish Warmblood mares (Rothmann et al., 2014b). In a study on Thoroughbred racehorses, Oki et al. (2007) estimated the heritability of behavioral responses at three different veterinarian inspections and found that heritability ranged from 0.23 to 0.28. The heritability of temperament traits was also supported by Momozawa et al. (2005), who found a variation in the Dopamine D4 gene that was responsible for curiosity and vigilance traits in Thoroughbred horses.

One study by Hausberger et al. (2004) did not find any effect of age on fear reactions or reactivity in horses. However, Visser et al. (2001) found that as horses got older (8-33 months), they tended to spend more time exploring novel objects or new environments instead of running away, yet older horses also need more attempts to cross in a bridge test. In contrast to this, Von Borstel et al. (2010) found that reactions in handling tests were weaker with increasing age in mares and geldings between 3 and 17 years, and Visser et al. (2003b) investigated the parameters ‘flightiness’ and ‘sensitiveness’ in mature riding school horses, finding that the older horses were less prone to run than young horses. However, this effect of age might be a reflection of experience and habituation due to repeated exposure to changes in environment. The influence of age and experience on performance in temperament tests was also shown by Visser et al. (2002) who found that heart rate decreased with age in horses between 9-22 months but also that untrained horses had a higher heart rate than trained horses. Overall, it is likely that age is often confounded by the previous experience of the horse, and information on its history is therefore of great value. Discipline related differences in behaviour have been investigated and one study found that show jumping horses reacted significantly less than other groups of horses in a moving object test (Von Borstel et al., 2010). Furthermore, Hausberger et al. (2011) showed that show jumpers were also more prone to touch a novel object than dressage horses. They also found that dressage horses showed high locomotor and excited behavioral patterns such as snorting during a novel-object test without a handler.

Desensitization is an effective and gentle training method to reduce fearfulness in horses faced with frightening situations and knowledge about habituation greatly affects the way in which horses may be trained to react calmly towards frightening objects (Christensen et al., 2006). Habituation is defined as a behavioral response decrement that results from repeated stimulation and that does not involve sensory adaptation/sensory fatigue or motor fatigue (Rankin et al., 2009). Habituation is often considered to be the simplest form of learning, as individuals learn that it is not necessary to respond to a particular stimulus after repeated stimulation has shown it to be harmless (Seaman et al., 2002). Habituation is relatively stimulus-specific; there is some evidence to support that horses generalize between different objects of varying shape if the colour is constant, but not between objects of varying shape and colour (Christensen et al., 2008a). However, object generalization in horses can be increased by habituating them to a range of colours and shapes simultaneously (Christensen et al., 2011). Bearing in mind that testosterone and estradiol concentration can be related to fearfulness in humans and other animals, it is of interest to investigate the relationship between testosterone and estradiol concentration and habituation in horses.

2.5 Measuring steroid hormones

There are several different methods for collecting samples to analyze steroid hormone concentrations and measuring steroid hormones in equine serum or plasma has been extensively validated (Mormède et al., 2007; Peeters et al., 2011). However, non-invasive procedures for analyzing saliva, urine and feces samples have also provided accessible methods for measuring cortisol, testosterone and other hormones (Palme and Möstl 1997; Möstl and Palme, 2002). Saliva sampling provides a non-invasive alternative to blood sampling for measuring hormone levels. Blood sampling requires certified personnel and could potentially be a stress factor for the horse, whereas saliva sampling is believed to be less stressful and easy to obtain (Malamud and Tabak, 1993). Saliva sample collection procedures make it possible to conduct reduced-stress non-invasive repeated sampling over the course of minutes, hours, days or longer, and can be used in large populations or in situations where blood sampling is prohibited. However, the usability is dependent on the reliability of measuring the specific salivary hormone concentration. Therefore, existing literature on the subject is shortly reviewed in the following.

The physiology of steroid hormones in relationship to saliva is of importance when measuring concentrations in saliva. Steroid hormones enter the salivary glands by passive diffusion. The speed at which hormones can be transferred from blood to saliva is controlled as they pass through the lipophilic layers of the capillaries and glandular epithelial cells. Steroids are lipophilic molecules, so they transfer through lipophilic barriers more rapidly than hydrophilic molecules such as peptides (Groschl, 2008). Human salivary production is continuous, but in horses, the mechanical effect of chewing is the main stimulus for activating salivary gland secretion (Burgen and Emmelin, 1961; Alexander, 1966). The range of salivary pH in horses is 7.4 - 7.9 (Alexander, 1966), and in humans it is 5.5 - 6.0 (Horner, 1976). The rate of salivary flow has an influence on the concentration of electrolytes measured, but it does not influence the concentration of urea (Alexander, 1966). There is no existing literature on the influence of salivary flow on hormone concentrations in horses.

2.5.1 Cortisol concentration in saliva and in plasma

Cortisol is considered to be in the inactive state when bound to its specific binding protein, corticosteroid binding-globulin (CBG), which allows for the distribution and availability of cortisol throughout the body (Siiteri et al., 1981). Several factors can alter the levels of CBG (including stress from housing or social status), and hence influence the amount of cortisol bound and the amount that reaches the cells (Alexander and Irvine, 1998). As well as the amount of CBG, its binding capacity can also affect the equilibrium between free and bound cortisol (Alexander and Irvine, 1998), and only the free cortisol can be measured in saliva.

It has been shown that salivary cortisol is an accurate detector of plasma cortisol concentrations in horses. Cortisol diffuses rapidly into saliva, and the salivary cortisol concentrations reliably mirror cortisol concentrations in blood, providing a less-invasive method for sampling (Peeters et al., 2011). This has been confirmed in other studies where they found correlation coefficients of 0.83-0.93 between plasma and salivary cortisol concentrations in horses, and concluded that salivary cortisol can be used to examine the function of the HPA axis (Van der Kolk et al., 2001; Lebelt et al., 1996).

Kedziersky (2014) found that salivary cortisol samples taken 30 minutes after acute exercise showed a high correlation with serum samples obtained immediately after exercise. With regard to free and total cortisol concentration, Peeters et al. (2011) used an ACTH stimulation test and found a positive and significant saliva-to-serum cortisol correlation (r=0.9), and a strong but nonlinear association between salivary and total serum cortisol concentrations (r=0.8). However, Bohak et al. (2013) found salivary cortisol levels were more likely to be correlated with free plasma cortisol than with the total plasma cortisol concentration. The time that salivary cortisol concentration peaked after exposure to a stressor was determined by Christensen et al. (2014), who found the highest salivary cortisol concentration between 0 and 5 minutes after a 10-minute ridden test. In conclusion, a large variation can be found in the correlation between serum cortisol and saliva cortisol among studies, however, it seems like salivary cortisol concentration reflects serum cortisol concentration and it is presumed that saliva sampling is less stressful to the horse than blood sampling.

2.5.2 Testosterone concentration in saliva and in plasma

In humans, about 97–98% of testosterone in serum is protein-bound, with high affinity for sex hormone-binding globulin (SHBG). In females, 60-70% of serum testosterone is bound to SHBG, whereas only 50-60% is bound to SHBG in males (Fiers et al., 2014). The remaining part is bound with much lower affinity to albumin, or is available for biological action as the free testosterone fraction. Between 1% and 15% of testosterone in serum is in its unbound or biologically active form. The bioavailable testosterone accounts for the free testosterone fraction of around 2-3%, as well as some of the testosterone that is loosely bound to albumin (Wang et al., 1981; Fiers et al., 2014). Literature relating to the properties of testosterone in the equine body is scarce, but it is believed to have the same properties as in humans.

Salivary testosterone reflects the free fraction of plasma testosterone that diffuses through the salivary glands, and a correlation with free testosterone in serum has been reported in humans (Vittek et al., 1985). Fiers et al. (2014) found that testosterone binding to saliva proteins was dependent on the pH level, with testosterone peaks at pH 5.3 and 8.4. Most experimental studies of post-competition changes in testosterone have used serum or saliva sampling, and in older studies it is well established that serum and salivary testosterone levels are highly correlated in humans (Smith et al., 1979; Riad-

Fahmy et al., 1982; Ellison, 1988). High correlations between salivary and serum free testosterone concentrations have been observed in humans, regardless of the immunoassay method (Wang et al., 1982; Granger et al., 2004). However, Johnson et al. (1987) found the concentrations of salivary testosterone to be higher than unbound testosterone in serum, reflecting a non-linear transfer from serum to saliva. This implies a metabolic transformation of steroid hormones by the salivary gland, or the transfer of testosterone bound to protein from the circulation into saliva.

There seems to be a difference between the sexes, since the serum-saliva correlation for testosterone is very high for men, but only modest for women. Salivary testosterone levels in women are correlated with serum total levels, though not with free testosterone levels in serum, perhaps because the values for women often fall near the lower end of the measurable range for both serum and saliva immunoassay kits (Vermeulen et al., 1999; Granger et al., 2004). Despite the conflicting evidence on the relationship between testosterone in serum and saliva, salivary testosterone samples from humans are extensively used in research concerning acute and chronic exercise (Budde et al., 2010; Crewther, 2010; Sedghroohi et al., 2011; Nunes et al., 2011), the effect on the neuromuscular system and psychological parameters (Hasagawa et al., 2008), and in fertility research for the treatment of male hypogonadism (Arregger et al., 2007). A positive relationship between testosterone concentration in saliva and plasma has been found in other species, such as chimpanzees (Kutsukake et al., 2009) and two other breeds of monkey (Arslan et al., 1984). Kutsukake et al. (2009) also found a clear pattern of diurnal fluctuation for salivary testosterone. However, salivary testosterone concentration has not been shown to correlate with plasma values in guinea pigs (Fenske, 1996). To date, only one published paper has examined testosterone measurements in equine saliva. Boudene et al. (1977) concluded that the concentration of testosterone measured in equine saliva by radioimmunoassay (RIA) was too high due to mucopolysaccharids interfering with the measurement.

In conclusion, there is some evidence that saliva can be used for measurements of testosterone concentrations, though the only study involving horses questions the reliability of saliva samples. However, if saliva samples can be used for measuring testosterone in horses, it would have a great potential as non-invasive method for monitoring changes in testosterone concentrations in sports horses.

3. Aims and Hypothesis

Based on human literature and a limited number of equine studies it seems relevant to pursue more information regarding the relationship between steroid hormones, fearfulness and competition performance. Therefore, the main objectives of this PhD thesis are;

- To investigate the relationship between steroid hormone concentration and competition score in both dressage horses and showjumpers
- To investigate the relationship between steroid hormones and fear reactions in handling tests.
- To investigate the relationship between fear reactions in handling tests and competition score
- To investigate the cortisol response to several days of competition and the diurnal variation both in the home environment and at competitions in relation to age, sex and discipline.
- To investigate the correlation between testosterone concentrations in serum and saliva
- To investigate the diurnal variation of testosterone in horses

The following hypotheses are tested:

- Baseline serum testosterone concentration is positively correlated with scores at dressage and show jumping competitions (Paper I)
- Baseline and post exercise salivary cortisol levels measured at competition are positively correlated with scores at dressage and show jumping competitions (Paper II).
- Stallions will have lower fear responses in handling tests compared to mares and geldings (Paper I).
- Horses with high concentrations of serum testosterone and estradiol show reduced fear responses in handling tests and faster habituation with repeated testing compared to horses with low concentrations (Paper I)
- Horses with lower fear reactions in handling tests in the home environment receive higher competition scores than horses with higher fear reactions (Paper I)
- Horses with lower fear reactions in the handling tests in the home environment have a lower salivary cortisol response in a competition environment than horses with more pronounced fear reactions (Paper I)
- Environmental changes due to competition will disturb the diurnal rhythm in baseline salivary cortisol concentration (Paper II).
- Cortisol concentration is related to discipline but not related to age and sex at home or in a competition environment (Paper II).
- There is a diurnal rhythm in the secretion of salivary testosterone as well as in serum testosterone concentrations in stallions, mares and geldings (Paper III).
- There is a positive correlation between serum and salivary testosterone concentrations (Paper III)

4. Study design and justification of methodology

The studies in this thesis were conducted on privately owned sport horses, and the acceptance, commitment, confidence and patience of the owners, riders and grooms was therefore crucial to the practical performance of the studies and their continuing participation in parts of the data collection. Data were collected both in the home environment of the horses and at four different events with specific competitions. Regulations from the Danish Warmblood organization (DW) and the Danish Riding Federation (DRF) regarding interference with the horses had to be followed during sampling at the events.

4.1 Study design

The study designs are briefly described in the following, and detailed descriptions of each study can be found in the respective papers. The experimental work was conducted in three studies.

Study 1 included data obtained during competitions as well data from the home environment (results from two handling tests and data on hormone levels) and could therefore be split into two parts (Figure 1). The first part included the results of two handling tests and blood sampling of 141 horses in their home environment. In this part, we measured the responses in handling tests (Moving Object test and Bridge test), including heart rate. Blood samples were collected for analysis of estradiol and testosterone levels.

The 141 horses tested in part 1 participated in specific young-horse dressage and show jumping competitions for 3- to 6-year-old horses (Stallion Show (SS), Danish Young Horse Championship (DYC) and Mare Testing (MT)), 2-4 weeks after the handling test. Performance was recorded as official competition scores in the individual competitions. The relationship between hormone levels and fear responses in handling tests, and the relationship between fear responses in handling tests and scores at competition are presented in Paper I.

Of the 141 horses that participated in part 1 of the study, 50 also participated in part 2. These horses had saliva samples taken in their home environment three times daily, prior to the competitions, and were also sampled three times daily on 3 consecutive days at competition. The salivary cortisol data from home and competition and their relationship with competition score is described in Paper II, whereas the relationship between saliva cortisol and responses in handling tests is described in Paper I.

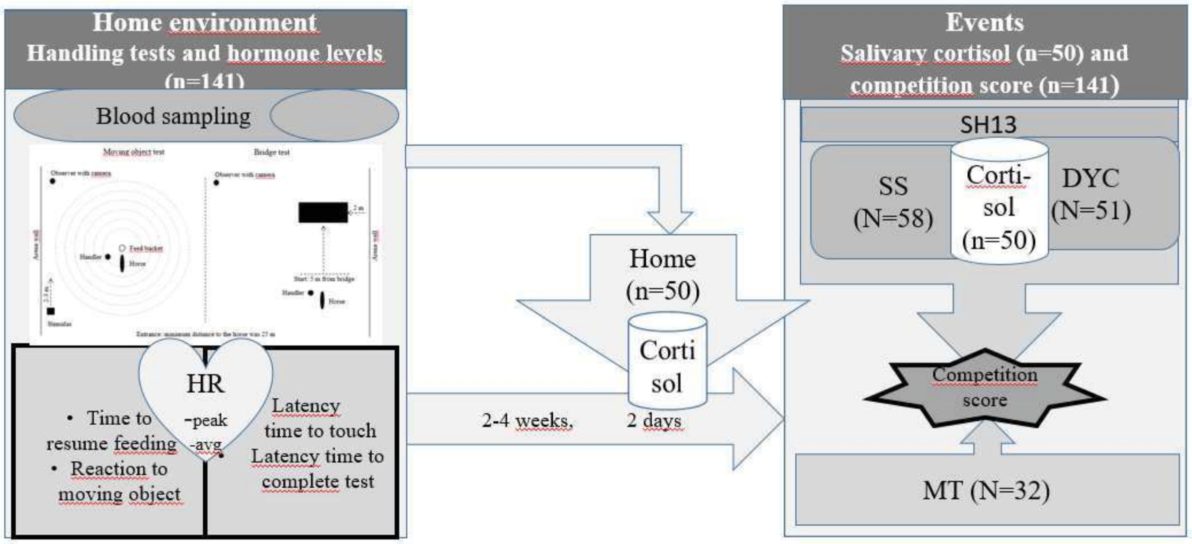

Figure 1. An illustration of study 1, SH13 event including competition Stallion Show (SS) and Danish Young Horse Championship (DYC), and Mare Testing (MT).

Study 2 was designed as a descriptive study over time. Data were collected on-site at home and at events to enable an assessment of cortisol concentration in different environments, and its relationship with performance at competition (Figure 2). In total, 126 horses aged between 3 and 6 years were included. The horses were trained on a daily basis by professional riders and were selected to participate in the upcoming events SH13 (SS and DYC), Young Horse Championship (YHC) and Stallion Show 2014 (SH14). Salivary cortisol concentration was measured in the morning, midday and evening during event SH13 and YHC. At event SH14, saliva was only sampled once daily, at midday, at the request of the owners.

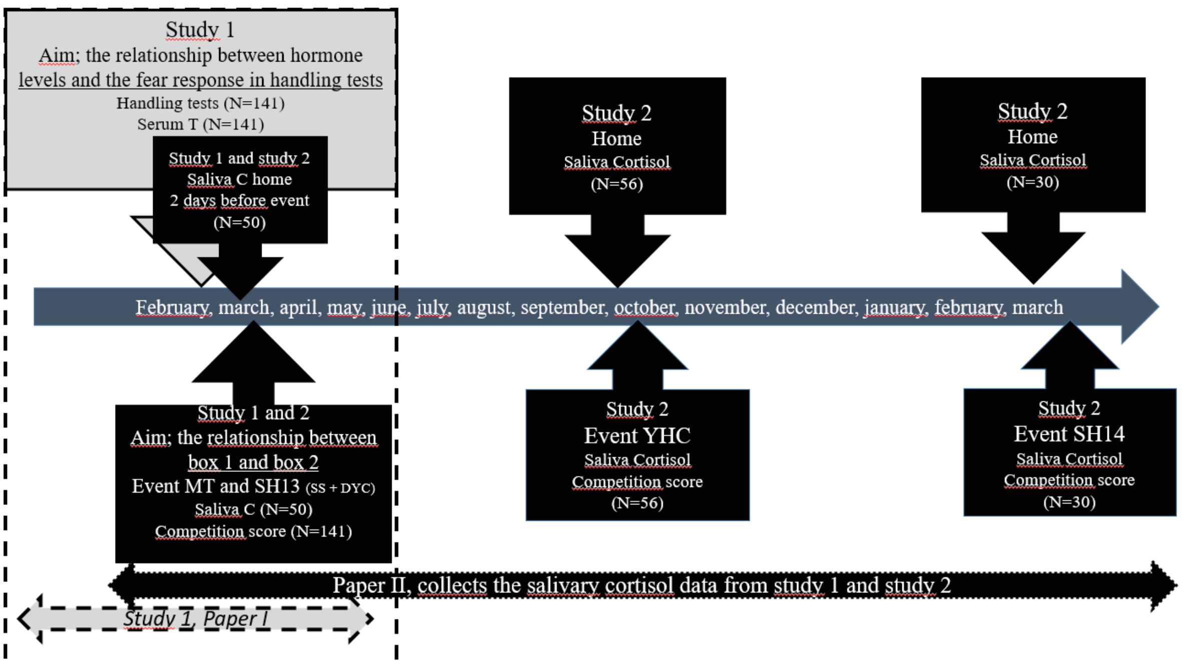

Figure 2. The study design of studies 1 and 2. Study 1 was separated into two parts, the gray box illustrates the time of handling tests and blood sampling, the black boxes inside the dashed line illustrate the time of salivary cortisol sampling, the data are described in Paper I. The black boxes illustrate the time of sampling in study 2, as described in Paper II. Serum testosterone concentration (serum T), Mare Testing (MT), Stallion Show 13 (SH13), Young Horse Championship (YHC), Stallion Show 14 (SH14).

In study 3 we investigated the relationship between serum and salivary testosterone levels and the diurnal variation of testosterone concentration. Saliva samples obtained in study 1 and study 2 were analyzed for both salivary cortisol concentration and salivary testosterone concentration. However, preliminary data analysis failed to show a difference between salivary testosterone concentration in stallions and mares. Therefore, study 3 was designed to investigate the correlation between testosterone in equine saliva and serum and to obtain additional knowledge on the physiology (diurnal rhythm and concentration levels) of testosterone in geldings and mares (Figure 3). Blood samples were collected from the horses before salivary sampling; this was repeated three times – in the morning, midday and evening. A total of 67 horses were used for this study, and the data are presented in Paper III. The outlines of studies 1, 2 and 3 are illustrated in Table 1.

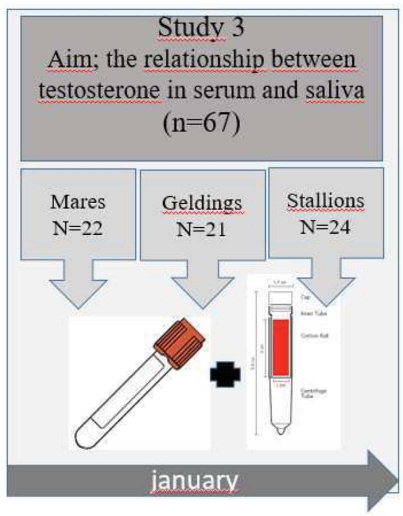

Figure 3. Blood and saliva were sampled simultaneously to compare the relationship between serum and saliva testosterone levels in mares, geldings and stallions (Paper III).

Table 1. Overview of the measurements performed in study 1 to 3 at the events Stallion Show 2013 (SH13), Danish Young Horse Championship (DYC), Mare Testing (MT), Young Horse Championship (YHC), Stallion Show 2014 (SH14) and the correlation study.

Study	**1**			**2**			**3**
Data collection events	**SH13**		**MT**	**SH13 (SS and DYC)**	**YHC**	**SH14**	**Correlation Study**
	SS	**DYC**					
Year **Month**	2013 Feb-Mar	2013 Feb-Mar	2013 Mar	2013 Oct	2013 Oct	2014 Mar	2016 Jan
Numbers	58	51	32	40	56	30	67
Sex	Stallions	Mixed	Mares	Mixed	Mixed	Stallions	Mixed
Age	3-6	5	3	4-6	4-6	3	3-24
Discipline	D+SJ	D+SJ	D+SJ	D+SJ	D+SJ	D+SJ	D+SJ
Cortisol (C)							
Times per day	3	3	-	3	3	1	3
Time of day	mo,mi,ev	mo,mi,ev	-	mo,mi,ev	Mo,mi,ev	Mi	Mo,mi,ev
Days collected	4	4	-	2	2	5	1
Home/Event	H/E	H/E	-	H/E	H/E	H/E	H
Serum/Saliva	Saliva	Saliva	-	Saliva	Saliva	Saliva	-
Testosterone	**(T)**						
Times per day	1	1	1				3
Time of day	Ev	Ev	Ev				Mo,mi,ev
Days collected	1	1	1				1
Home/Event	H	H	H				H
Serum/Saliva	Se	Se	Se				Serum / Saliva
Exercise							
Home/Event	H/E	H/E	-	S	E	E	-
Serum/Saliva	Saliva	Saliva	-	Saliva	Saliva	Saliva	-
Competition score	Yes	Yes	Yes	Yes	Yes	Yes	No
Handling test	Yes	Yes	Yes	No	No	No	No
Parameter	Cortisol (C) -Diurnal rhythm (DR) -Day (D) -Exercise (Ex) Testosterone (T) Estradiol (Es) Handling test Performance (P)		T Es P	C DR D Ex P	C DR D Ex P	C D Ex P	Testosterone-correlation Serum / Saliva Diurnal rhythm
Publication	Paper I Paper II	Paper I Paper II	Paper I	Paper II	Paper II	Paper II	Paper III

4.2 Brief presentation and justification of applied methodology

4.2.1 General considerations across experiments

The study population in this thesis consisted of privately owned horses, and the studies were therefore performed mostly as observational studies. Written consent was given from both the owner and the trainer before the horses were included in the study and a controlled distribution (i.e. sex, age and discipline) was therefore not possible. However, the results provide information based on a group of young, high-level show jumpers and dressage horses sampled on-site, which is therefore valuable in terms of reflecting real-life situations.

The horses in this study were categorized into only one breed – Danish Warmbloods (DW). However, this is a rough definition of breed, since DW consists of a variety of European breeds (Hanoverian, Oldenburger, Holstein, Danish or Dutch Warmblood etc.). Previous studies have shown effects of breed on reactions in novel object tests and bridge tests (Hausberger et al., 2004, 2011). In addition, a within-breed genetic component from two sire lines has been shown to have an effect on fearfulness in French Saddlebreds (Wolff et al., 1997) and in Thoroughbred racehorses, where curiosity and lower vigilance was associated with a specific allele on the Dopamine D4 receptor (Momozawa et al., 2005). An effect of breed in the population of horses used in this study cannot be excluded, but this was not further investigated.

Details on age distribution, sex and discipline are summarized in Table 1 and presented in the corresponding papers. The horses were handled for at least 1 month and were broken in to ride before sampling. The horses were trained and ridden on a daily basis by professional riders who were familiar with the horses. In the different home environments, the horses were stabled in individual pens and management routines could not be controlled (e.g. feeding, bedding, housing, paddock time).

To the best of my knowledge, and as advised by the owners, the horses selected for the studies in this thesis were trained and competing (if applicable) solely in either dressage or show jumping. In addition, a horse was considered to belong to one genetic line (D or SJ) if its sire had a minimum breeding value of 120 index points in the respective discipline, and at least 10 points less with no more than a total of 119 in the other discipline. Where genetic evaluation was unavailable for the sires, the requirement was that both parents originated from a genetic line of the same discipline. A pedigree breeding value was then calculated based on the breeding values of the sire (and dam, when available), and the above rules were applied.

4.2.2 Definition of Competition scores used in this thesis

Competition score is used as a measure of performance in dressage and show jumping competitions for young horses. However, the scores are given by judges based on their impression of parameters such as conformation, movement, and rideability. Dressage performance is scored by judges at all competition levels, but the individual parameters can change from level to level, consequently involving scores for rideability and gait. In the young-horse show jumping competitions included in this thesis, jumping scores were based on judges' opinions on rideability, canter and jumping technique (Table 2). In other show jumping competitions, scores are based on actual faults, which is a more objective measure. Throughout this thesis, the focus is on performance scores in young-horse competitions, where scores are dependent on judges in both disciplines. The level of competition and the specific events selected for the performance evaluation in this thesis were chosen based on the importance placed on these events by the breeders and owners of the young horses. The Stallion Selection (SS) and Mare Testing (MT) for 3-year-old horses, the Danish Young Horse Championship (DYC) for 5-year-old horses, and the Young Horses Championship for horses aged 4-6 years are initial important performance goals for young Warmblood horses from Stutteri Ask and Blue Horse stud, as well as other Danish breeders. The Stallion Selection (SH13 and SH14), Mare Testing (MT) for the 3-year-old mares, and the Danish Championship for 5-year-old horses (DYC) are competitions arranged by the Danish Warmblood association (DW). The DW score for dressage horse rideability is based on: attention and confidence; acceptance of the bridle and lightness of the forehand; lightness and ease of movements; impulsion; desire to move forward, and learning ability. The DW score for show jumper rideability is based on the parameters: level of carefulness, power of the take-off and rationality of the jumps.

The YHC for horses aged 4, 5 and 6 years are regulated and judged by the guidelines from the DRF. In addition to the scores for gait, they also receive scores for rideability and capacity. The DRF score given for rideability in dressage championships is based on: lightness and ease of presentation, impulsion and contact (acceptance of the bridle), temper, willingness to work, trainability, attention and submission. The DRF score given for capacity in YHC for dressage horses is based on the general impression of gait (including a natural way of showing them), the potential and ability to be a dressage horse, balance and activity of the hindquarter, positivity and trainability, impulsion and self-carriage. There is no existing description of the DRF score for rideability and capacity for show jumping horses in YHC.

To summarize across young-horse competitions, description of the parameter rideability fits into three categories: temperamental traits, physical ability and the perception of the rider. The preferred temperamental traits are: attentive, confident, careful, rational, and positive. The physical ability is expressed by the ease of movement, impulsion and powerful take-off. Acceptance of the bridle and lightness of the forehand, desire to move forward, willingness to work, and submission describe the way rideability should feel to the rider. In addition, the rideability score includes the trainability and learning ability of the horse.

Table 2 Overview of the evaluation parameters, judges and scores at the events Stallion Show 2013 (SH13), Danish Young Horse Championship (DYC), Mare Testing (MT), Young Horse Championship (YHC), Stallion Show 2014 (SH14).

Competition	Age	Comp days	Parameters	Judges	Maximum scores
SH13 Stallion Test Dressage horses	3	3	*Conformation, walk, trot, canter, rideability, and overall impression	Two judges on ground	100, 10 marks are given from 0-10
MT Dressage horses	3	1	*Conformation, walk, trot, canter, rideability, and overall impression	A judge on ground and a test rider	100, 10 marks are given from 0-10
MT Show jumpers	3	1	*Conformation, canter, technique, rideability, capacity and overall impression	A judge on ground and a test rider	100, 10 marks are given from 0-10
SH13 Stallion Test Show jumpers	3	3	*Conformation, canter, technique, rideability, capacity and overall impression	Two judges on ground	100, 10 marks are given from 0-10
DYC Young Horse Dressage horses	5	2	Walk, trot, canter, rideability, capacity overall impression	Two judges on ground and a test rider	100, 10 marks are given from 0-10
DYC Young Horse Show jumpers	5	2	*Conformation, canter, technique, rideability, capacity and overall impression	Two judges on ground and a test rider	100, 10 marks are given from 0-10
SH14 Stallion Test Dressage horses	3	3	*Conformation, walk, trot, canter, rideability, and overall impression	Two judges on ground	100, 10 marks are given from 0-10
SH14 Stallion Test Show jumpers	3	3	*Conformation, canter, technique, rideability, capacity and overall impression	Two judges on ground	100, 10 marks are given from 0-10
YHC Dressage horses	4, 5, 6	1	Walk, trot, canter, rideability, capacity → overall impression	Two judges on ground	100, 10 marks are given from 0-10
YHC Show jumpers	5	1	Walk, trot, canter, rideability, capacity → overall impression	Two judges on ground	100, 10 marks are given from 0-10
***Conformation**	Including following parameters: Descriptions and marks are given for each parameter. Marks are given from A-I, where the ***maximum "letter" (the most optimal conformation)*** is given for each parameter. 1) <u>Type of stallion and frame:</u> Large vs. short frame (***A***), long-legged vs. short-legged (***C***), harmonious vs. non-hamonious (***A***), refined vs. heavy (***C***), strong vs. weak expression of gender (***A***) 2) <u>Conformation of head and neck:</u> Small vs. big head (***C***), strong vs. weak expression (***A***), light vs. heavy connection (***C***), long vs. short neck (***C***), thin vs. thick neck (***E***), arched vs. straight neck (***C***) 3) <u>Conformation of shoulder and withers:</u> Long vs. short shoulder (***C***), sloping vs. upright shoulder (***B***), long vs. short withers (***B***), high vs. low withers (***C***) 4) <u>Conformation of topline and hindquarter</u>: Long vs. short topline (***D***), long vs. short croup (***B***), sloping vs. upright croup (***C***), long vs. short gaskins (***C***) 5) <u>Conformation of limbs</u>: Substantial vs. poorly defined joints (***A***), over at knee vs. back at knee (***D***), weak vs. upright pastern (***D***), big vs. small hooves (***B***), toing out vs. toing in (***D***)				

The competition for stallions aged 3 years in SS, SH13 and SH14 events included 3 days of scoring. The stallions were shown free in the arena on day 2 and in hand on days 1 and 3, and were not ridden at any time. Scores for conformation were included, but no rideability or other behavioral scores were included. The competition for 3-year-old mares (MT) only lasted 1 day and the horses were ridden and scored for rideability by their normal rider and by a test rider, and conformation was part of the competition score. The 5-year-old horses in DYC competed over 2 days. On day 1, they performed a fixed dressage test or jumping course with their normal rider and on day 2 they were ridden by a test rider for a fixed amount of time. Rideability was scored by the test rider and judges on the ground for both tests. The YHC for both dressage horses and show jumpers lasted for 1 day, and the competition design depended on the age of the horse. The 4-year-old dressage horses entered the competition arena two horses at a time and the judges asked to see the three gaits in both a natural balance and extended. They spent a total of 15 minutes in the arena. The 5- and 6-year-old dressage horses performed a fixed dressage test of different levels of complexity in the arena. The show jumpers aged 4, 5 and 6 years performed a fixed course, and the height of the fences and the technical difficulty increased with age. The different competitions and performance score cannot be compared directly because of the large variation in the parameters that determine performance score, as presented above. This should be kept in mind when discussing the results in this thesis.

4.2.3 Selection of handling tests for determination of fearfulness

A variety of tests (familiar/non-familiar test arenas, novel objects, novel surfaces, sudden movements and questionnaires) have been used to estimate fearfulness in horses (Anderson et al., 1999; Visser et al., 2001, 2008; Lansade, 2008; von Borstel et al., 2010; Lansade et al., 2016). Through temperament tests, horses are challenged to show their fearfulness. The advantage of using temperament tests is that these can be carried out in a more standardized way than subjective assessments and questionnaires (Anderson et al., 1999; Visser et al., 2008). The ability to identify horses that are fearful and reactive to stressful situations provides valuable information on which horses could benefit from specific training and habituation to situations like competition and new environments. The level of fearfulness of an individual can be determined by observing specific reactions to novel objects during short tests performed as early as 6-8 weeks of age (Lansade et al., 2008; Christensen et al., 2016).

Whether temperament tests should have the horses running free or involve a human handler or rider is debatable. Fear test without a handler is considered as the most reliable way to measure fear as horses have been shown to react differently to frightening situations in the presence of a handler. Von Borstel et al. (2011a) found that a rider or handler influences, but not completely masks, the horses' intrinsic behaviour in a temperament test, and this influence appeared to be stronger on behavioural variables and heart rate variability than on the horses' heart rates. The handler is a confounding factor and the response measured might be a response to the handler in a frightening situation (Keeling et al., 2009); Von Borstel et al., 2011a). The previous handling experience may affect the behavioural responses in a test involving a familiar handler but not an unfamiliar handler (Marsbøll et al., 2013).

In studies by Visser et al. (2008) and Von Borstel et al. (2011a), novel objects were introduced in the presence of a rider. Von Borstel et al. (2011a) showed that when horses were ridden they had a higher response to stimuli than when handled and reactions in ridden tests and temperament tests involving a handler were moderately correlated to the reactions measured in the absence of a human handler or rider, which was also confirmed by Lansade et al. (2016).

In practice, tests that most closely resemble the practical circumstances should be selected and as performance in dressage and show jumping was of interest in this thesis, a ridden test to evaluate fearfulness would have been highly relevant. However, it was not possible to use a ridden test, because the horses were young and unexperienced, and the level of training could not be controlled. For practical reasons and constrains, it was then decided to use a test involving a handler in study 1. To standardize the test procedures, it was decided to use an unfamiliar handler in all the tests (the handler was then the same person in all tests).

The selection of the specific handling tests to assess reactivity and fearfulness in this study were based on the literature where fearfulness toward novel objects, novel surfaces and suddenly moving objects have been measured (Visser et al., 2001; Lansade, 2008; von Borstel et al., 2010; Lansade et al., 2016). The moving object test was selected based on the studies by Lansade et al. (2016) and von Borstel et al. (2010). Reactivity measured by parameters such as flight distance and latency to touch a novel object or to resume eating after exposure to moving or sudden objects, has been used as a measure for fearfulness in recent studies in Warmblood sport horses (Von Borstel, 2010; Lansade et al., 2016; Christensen et al., 2008a; McCall et al., 2006). The bridge test is very well described in literature and it was also used in this thesis (Wolff et al., 1997; Visser et al., 2001, 2002, 2003, 2008; Hausberger et al., 2011). In the bridge test a mattress, plate or other substance is placed on the ground and the horse is required to pass the "bridge". Often a handler leads the horse and tries to make the horse cross the bridge with as little interference as possible with the animal. Lansade et al. (2016) used a feed bucket placed on a novel surface to measure latency time to touch the novel surface as a handling test to detect fear in show jumpers. Based on the results in these studies and the study population in this thesis the Moving Object test and the Bridge test with a handler were selected.

In the bridge test, the latency time to touch the bridge either with a hoof or the muzzle was measured, as well as the total time for completion of the test. It is possible that the latency to touch the bridge should have differentiated between hoof and muzzle, since these may express differing emotions. A comparison of the bridge test outcome between studies might be difficult since there is a large variation in the design of the bridge. In Paper I, a 1,5 x 4 m white tablecloth was used, whereas other studies used a brown floor mat measuring 1.2 x 9 m (von Borstel et al., 2011) or a plywood plate measuring 2 x 4 m (Visser et al., 2008).

In the moving object test, the latency to resume feeding has been used as a response parameter and has been evaluated in tests without a handler as a valid indicator of fearfulness in young warmblood horses (Christensen et al., 2008a,b; Lansade, 2008). Von Borstel et al. (2010) also used the latency to resume feeding as a response parameter in horses with a handler. Holding the horse in a lunge line causes some restriction on the range of movement away from the fixed point (the feed bucket), however, this modification was made to prevent the horse from running around in the arena and risking becoming excited or aggravated.

A detailed description of the handling tests (Moving Object and Bridge test) can be found in Paper I. A modified moving object test was deemed to be appropriate for the study population in study 2, as the horses in the study by von Borstel et al. (2010) were of similar breed and discipline. As opposed to von Borstel et al. (2010), the horses in study 1 were only exposed to the Moving Object three times (rather than five times as in the original study) to reduce the total time of testing.

Heart rate (HR) was also measured during both tests since HR variables are useful in differentiating between individuals and quantifying aspects of temperament (Visser et al., 2002). Behaviour does not always correspond with HR measurements because some horses do not exhibit stress through visible (evasive) behavior, therefore HR measurements provides an additional tool to assess stress in horses (Munsters et al., 2013). In study 1 the average HR during each test was calculated and the peak HR was measured at each exposure to the Moving object, and at the peak HR in the Bridge test.

4.2.4 Sampling methods and handling

There are several different methods for collecting samples to analyze steroid hormone concentrations. Measuring steroid hormones in equine serum or plasma has been extensively validated (Singh et al., 1997; Mormède et al., 2007; Peeters et al., 2011) and blood sampling is easy to perform. However, it requires certified personnel and could potentially be a stress factor for the horse. Non-invasive procedures as sampling of saliva is also an accessible method for measuring cortisol, testosterone and other hormones (Möstl and Palme, 2002; Koren et al., 2002) and saliva sample collection procedures make it possible to conduct reduced-stress non-invasive repeated sampling over the course of minutes, hours, days or longer, and can be use in large populations (Malamud and Tabak, 1993). For Study 1, serum was needed for measuring testosterone and estradiol concentration, due to the small amount of information on measuring these hormones in saliva from horses. Measuring cortisol concentration in saliva is an accepted method in equine studies and for Study 2 and part of Study 1, where repeated sampling from privately owned horses was requested on-site (both in the home environment and at competition), saliva sampling was chosen to avoid any side effects of sample collection and to follow the competition regulations set by the organizing committee. For Study 3 both blood and saliva sampling were needed to fulfill the aim of the paper.

Blood sampling for Study 1 was done immediately before performing the handling tests. When sampling saliva, the following consideration's were made; Baseline saliva samples were obtained at least 2 h after exercise when the horses were resting in their boxes, either prior to feeding or at least 1 h after feeding. Since the experiments involved sampling privately owned horses at competitions, complete control of feeding and grooming management etc. could not be guaranteed. Despite precautions being taken in terms of sampling and feeding times, it is possible that the horses were given a treat shortly before sampling, or that they would have had access to small amounts of feed or left-over hay from the previous feeding. Christensen et al., (2014) demonstrated that cortisol concentration remained increased within 5 minutes after exercise therefore post-exercise sample was collected 5-10 minutes after an acute bout of exercise for Study 2.

4.2.4.1 Collection devices

Passive drooling is often used to provide non-stimulated saliva, and the method is thought to give the most reliable results in humans (Fiers et al., 2014). The analytical results achieved with passive drooling have been reliable for most peptide hormones and steroids (Groschl et al., 2008). However, even in humans it can be difficult to obtain enough saliva from passive drooling, and citric acid is therefore often applied to the tongue to increase the flow of saliva in order to ensure maximal secretion. The drawback of using citric acid is that it has been reported to interfere with immunoassay analysis by decreasing the pH of the sample (Fiers et al., 2014). To the authors' knowledge, only one study in animals have used passive drooling to collect saliva and that was in anesthetized monkeys (Arslan et al., 1983) and does not seem to be an option in horses.

The most commonly used sampling device in equine studies measuring salivary cortisol concentration is the synthetic swabs by Salivette® (Nümbrecht-Rommelsdorf, Germany) therefore these were used for saliva sampling throughout the studies in this thesis. The swabs were placed under and over the tongue with the help of an arterial clamp for approximately 1 min, until they were soaked with saliva as described in previous equine studies (Aurich et al., 2015; Becker-Birck et al., 2013).

4.2.4.2 Sample handling

There are some divergent results regarding salivary sample collection and handling. A study by Granger et al., (2008) found that salivary testosterone measurements were sensitive to storage conditions, whereas Chen et al. (1992) found that glucocorticoids and androgens had a high stability in saliva and could be stored in untreated saliva for a number of days at room temperature. Blood contamination, saliva stimulation and the collection device itself have been found to influence results, and it is recommended that samples should be frozen at -80°C if long-term storage is required prior to analysis (Groschl et al., 2008; Kivlighan et al., 2004). In this thesis, swabs were placed in a polypropylene tube and stored at -18 °C within 20 min of sampling. Saliva samples were further processed by thawing and centrifugation (2,500×g). Saliva was then transferred to new tubes and refrozen until analysis. Blood samples were kept below 15 degrees after collection and centrifuged within 2 hours and serum was frozen immediately after centrifugation.

4.2.5 Method of steroid hormone analysis

For all salivary samples, cortisol analysis was performed by Unit of Physiology, Patophysiology and Experimental Endocrinology, University of Veterinary Medicine, Vienna. The method for analyzing cortisol in saliva has been extensively validated, and there are studies with salivary cortisol results published from this laboratory (Palme and Möstl, 1997; Aurich et al., 2015; Becker-Birck et al., 2013). A description of methodology can be found in Paper II. There are currently no published studies on successful salivary testosterone analysis in horses, but in personal communication (July 2013), Prof. Palme confirmed the possibility of analyzing salivary testosterone in his laboratory. Saliva samples for testosterone measurements were further processed by thawing and centrifugation (2,500×*g* for 15 min at 4 degrees) since human studies have found that a freeze-thaw cycle followed by centrifugation removes mucopolysaccharides from the saliva sample (Arregger et al., 2007). Samples were then transferred to new tubes and re-frozen until analysis and a description of methodology can be found in Paper III. Serum testosterone analysis for Paper III was performed by Rupert Palme's laboratory in a testosterone enzyme immunoassay (EIA). For paper I, serum testosterone and estradiol were analyzed by commercial ELISA assays (Neogen Corp. Product #402510 and #402110, Lexington KY, 40511-1205 USA, respectively). The instructions recommended by the manufacturer were followed.

The relationship between steroid hormone concentrations, responses in two handling tests and competition scores in warmblood sports horses - a field study

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Abstract

Little is known about the relationship between behavior, competition performance and the hormones testosterone and estradiol in sport horses. In this study hormone concentrations in serum (testosterone and estradiol) and saliva (cortisol) were compared to reactions in behavioral tests and competition performance in horses. Warmblood sports horses (141 in total, age 3-5 years) were assessed in a Moving object (MO) and a Bridge (B) test prior to a competition where performance was scored. Immediately prior to the handling tests, a blood sample was collected for analysis of serum testosterone and estradiol concentrations. During competition, salivary samples were collected from 50 horses for analysis of cortisol concentrations. Testosterone concentrations only correlated to one parameter in the MO test in mares and geldings (flight reaction: $r = -0.41$, $p = 0.006$), i.e. horses with less flight reaction had higher testosterone levels. Estradiol concentrations in mares were negatively correlated to some variables in the MO test (e.g. latency to resume feeding: $r = -0.35$, $p = 0.02$ and peak heart rate (HR): $r = -0.43$, $p < 0.001$) and B test (average HR: $r = -0.30$, $p = 0.04$). There were no correlations between competition scores and hormone concentrations. Finally, showjumpers with an increased fear response (e.g. peak HR: $r = 0.50$, $p = 0.04$) in the MO test also had higher baseline cortisol concentrations at competition.

In conclusion, the results in this study suggest that there is no clear relationship between performance, hormones concentration and behavioral responses in the two tests. However, there could be an association between fear reactions in a MO test and the level of testosterone and estradiol concentrations in horses.

Keywords: competition performance, handling test, temperament, dressage horse, testosterone, estradiol, salivary cortisol, showjumping

1. Introduction

In horses, not only superior physical performance but also an appropriate temperament is needed to achieve optimal performance in dressage or showjumping. Identification of horse temperament are therefore important for the use of the horse, performance in sports, the monetary value to humans as well as welfare and safety of the horse and rider (McCall, 1990; Cooper, 1998; Murphy and Arkins, 2007). Awareness of the temperamental traits of the individual horse can help the handler or rider predict behavioral reactions in different situations (Lansade et al., 2008a,b), safety precautions can be made and the horse can be habituated to and prepared for stressful situations.

In humans, links between personality and performance in sports have been demonstrated (Allen et al., 2013). In introvert human athletes, anxiety interfered with concentration and was negatively related to sports performance (Eysenck et al., 1982) whereas extrovert personality types took greater risks and were more competitive (Egloff and Gruhn, 1996). Testosterone concentrations may have an important role since high testosterone levels were associated with decreased fear and anxiety as well as increased aggressiveness (Herman et al., 2006; Frye and Seliga., 2001; Mazur and Booth 1998; Oliveira et al., 2009; Salvador et al., 2003; Suay et al., 1999). Increased testosterone levels around the time of competition is considered to have a beneficial impact on performance in many sport disciplines in human athletes due to increased risk taking and motivation to compete (for review, Castro and Edwards 2016; Booth et al., 1989). In human sports, it has been suggested that a higher level of testosterone – between athletes of the same sex as well as between the sexes – is advantageous (ACSM 2006; Wood and Stanton 2012), and it has been shown that elite female athletes have a consistently higher level of free testosterone compared to non-athletes (Cook et al. 2012). One study showed the mean testosterone level was twice as high in professional female athletes than in non-professional female athletes (Healy et al., 2014).

Like testosterone, estradiol has been associated with risk-taking in young females (Vermeersch et al., 2008) and basal estradiol concentration was positively correlated with motivation in women (Stanton & Edelstein, 2009; Stanton & Schultheiss, 2007).

A study by Glover et al. (2013) found that the level of estradiol influences neural processes that regulate fear inhibition in adult women; it was suggested that low estrogen may be associated with increased risk of anxiety disorders through dysregulated fear responses.

In rodents, experimentally increased levels of testosterone have been shown to reduce anxiety, increase aggressiveness and positively influence reward processing and motivational drive (Nyby 2008; Aikey et al., 2002; Frye and Seliga 2001; Monaghan and Glickman 1992) and in female rats, a high estradiol concentration was associated with reduced anxiety (Walf et al., 2009). Zink et al. (2014) investigated behavior in gonadectomized hunting dogs and found that the odds for behavioral problems (such as separation anxiety, fear of noises, aggression and hyperactivity) were significantly higher than the odds for sexually intact dogs. In line with that there is evidence to support that behavioral problems in mares are related to altered levels of reproductive hormones (McCue et al., 2006; Meagher et al., 1977), however the link between testosterone, estradiol, behavioral traits and performance in horses has not been studied.

Fearfulness are regarded as the most important and stable component of temperament in horses (Anderson et al., 1999; McCann et al., 1988; Lansade et al., 2008a,b; McCall et al., 2006; Von Borstel et al., 2011) and can be estimated by observation of a few specific reactions towards novel objects during short tests (Lansade et al., 2008a,b; Christensen et al., 2006) and parameters such as flight distance and latency to resume eating in surprise tests (e.g. involving moving objects) have been reported to be reliable behavioral indicators of the trait fearfulness in horses (Lansade et al., 2008a,b).

Studies suggest that horses selected for specific disciplines may respond differently in fear tests; e.g. dressage horses reacted more fearfully than showjumpers in a novel object test (Hausberger et al., 2011), and showjumpers had an attenuated response compared to non-showjumpers in a surprise fear test (Von Borstel et al., 2010). It has further been demonstrated that fearful horses had more refusals in a show jumping competition (Lansade et al., 2016), and highly reactive horses received lower grades in rideability and free jumping in another study (Rothmann et al., 2014).

The aim of the present study was to compare hormone concentrations in serum (testosterone and estradiol) to reactions in behavioral tests and competition performance. In addition, the relationship between reactions in behavioral tests, the salivary cortisol response to competition as well as competition scores in young dressage horses and showjumpers was explored. The hypotheses were 1) horses with high serum concentrations of testosterone and estradiol show reduced fear responses in handling tests and faster habituation with repeated testing compared to horses with low concentrations, 2) horses with lower fear reactions in handling tests in the home environment receive higher competition scores than horses with higher fear reactions, 3) horses with lower fear reactions in the handling tests in the home environment have a lower salivary cortisol response in a competition environment than horses with more pronounced fear reactions.

2. Materials and Methods

2.1. Horses

Owners of a total of 274 horses signed up for three competitions for young dressage horses and showjumpers were offered to participate in the study. The three competitions were: Stallion Show for 3 year old stallions (SS), Mare Testing for 3 year old mares (MT) and the Danish Young horse Championship in dressage and showjumping for 5 year old horses (DYC). The competitions SS and

DYC were held at the same time and in the same environment, whereas MT was held separately at a different location. The horses were Danish, Dutch or German warmbloods defined as either dressage horses or showjumpers by pedigree and the entrance into the competitions. Out of the 274 horses signed up for the competitions, 162 horses entered this study and an agreement form was signed by the owner.

The 3 year old horses had been in training for the purpose of the competition for 10 to 20 weeks, but the amount of handling before this training was variable. Training included lunging, free jumping and mounting of a rider including basic riding. The 5 year old horses were all ridden for a period of at least one year.

The horses were tested in their home environment 2-4 weeks before the different competitions at 45 different locations with one to 15 horses at each location. Of the 162 horses included in the study, data collection was successful for 141 horses (Table 1), whereas 21 horses were excluded due to missing data (e.g. video file or heart rate recordings).

Table 1. Distribution of age, sex and discipline (dressage (D) or showjumping (SJ)) of horses at the three competitions: Stallion show for 3 year old stallions (SS), Mare testing for 3 year old mares (MT) and the Danish young horse championship in dressage and showjumping for 5 year old horses (DYC).

Event (n = 141 horses)	SS		MT		DYC					
Age	3 (n = 58)		3 (n = 32)		5 (n = 51)					
Sex	Stallions		Mares		Stallions		Geldings		Mares	
Discipline	D	SJ	D	SJ	D	SJ	D	SJ	D	SJ
	36	22	26	6	2	5	19	6	10	9

2.2. Ethics

All procedures were acceptable according to the Danish Animal Experiments Inspectorate under the Ministry of Justice in pursuance of the Animal Experimentation Act (LBK No. 1306).

2.3. Experimental design

All horses were exposed to two different handling tests: a surprise test consisting of a suddenly moving object in the test arena (**MO)** and bridge test (**B**). The two tests were conducted in a set order in an environment familiar to the horse. The tests took place in the usual working arenas, with the ground covered with sand or synthetic footing. After finishing the MO test, the horse left the arena for approximately 3 minutes while the B test was prepared. The same person, who was unfamiliar to the test horses, handled the horses in both tests. The MO and B tests were recorded on video for later analysis of the reactions and the position of the horses throughout the tests.

In the stable, before performing the handling tests, a 10 ml blood sample was drawn from the jugular vein using a 20 gauge needle in to plain tubes. The samples were obtained between 10:00-18:00 and stored at 5°C for a maximum of 5 hours before centrifuging (2500 x g) and freezing at -18°C.

Heart rate (HR) monitoring equipment (Polar 800, Polar Electro OY, Kemple, Finland) was fitted on the horse in the stable prior to testing. The HR equipment consisted of an electrode belt with a built-in transmitter and a wristwatch receiver. Water and exploratory gel were used to optimize the contact between electrode and skin and the receiver stored data from the transmitter (RR).

A group of 50 horses (30 stallions, 10 mares, 10 geldings, aged 3 and 5 years) selected for the events SS and DYC were subjected to salivary cortisol sampling at the events. The horses were selected based on owner commitment. Saliva was collected with specifically designed cotton rolls (Salivette, Nümbrecht-Rommelsdorf, Germany). The cotton rolls were placed under and over the tongue with the help of an arterial clamp for approximately 1 min, until they were soaked with saliva. The cotton roll was then placed in a polypropylene tube and stored at -18°C until analysis. Sampling of saliva was performed in the morning (06:00-08:00), at midday (11:00-13:00) and in the evening (17:00-19:00) over 3 days at the event. Mean baseline cortisol concentration per day and across days was calculated. Saliva was sampled either before feeding or a minimum of 1 h after feeding, and at least 2 h after exercise when the horses were resting in their boxes.

2.4. Description of handling tests

*2.4.1. Moving object in the arena (**MO**)*

The horse was taken to a familiar indoor arena without the company of other horses. The horse was handled with a head collar and a 7 meter long rope. The moving object was placed to the side of the entrance so that the horse could see it only after entering the arena. A test design as described by von Borstel et al. (2010) was used (Figure 1). The moving object consisted of a white plastic bag (50 x 50 x 50 cm) containing 3 kg of straw, connected to a 30 meter rope. With a bucket placed in the center, 2, 4, and 7 meter circles were marked in the surface of the arena to make a marker for measuring reaction to the moving object (**MO_flight reaction**).

Feed familiar to the horse was placed in the bucket. When the horse was eating from the bucket, the object was moved 1 meter forward in a quick sudden movement by the helper. If the horse reacted, the handler extended the lunging line as needed, following the horse if necessary, but otherwise remained as passive as possible to minimize disturbance of the horse's movement and behavior. Latency for the horse to resume feeding from the bucket was measured (**MO_latency**), if the horse did not return within 60 seconds the test was disrupted and the handler led the horse back to the bucket. The handler offered the bucket to the horse to encourage it to eat and when it resumed feeding, the procedure was repeated an additional two times **(MO_flight reaction2, MO_flight reaction3 and MO_latency2, MO_latency3**). Data were recorded as described in Table 2.

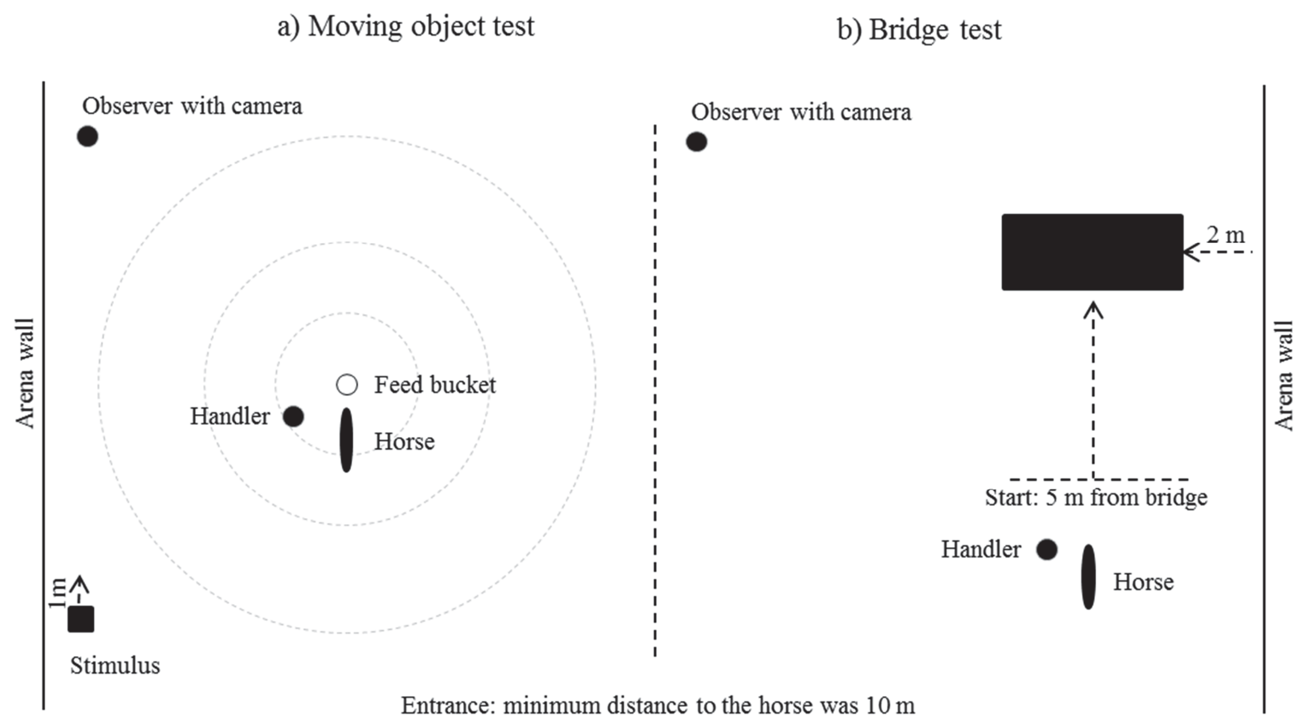

Figure 1. Overview of experimental set-up; (a) the moving object (MO) set-up showing the horse's position in relation to stimulus, feed bucket, handler and observer. (b) the Bridge (B) test set-up showing the horse's position in relation to the bridge.

*2.4.2 Bridge test (**B**)*

The bridge consisted of a 2 meter wide and 5 meter long white and green wax tablecloth that was placed on the long side of the arena. The horse had to move away from the arena entrance. The handler walked the horse with a slight contact on the lunge and applied pressure through the rope and head collar if the horse stopped. Pressure was released as the horse moved forward (negative reinforcement). The starting point of the test was defined as the turn towards the bridge 5 meters in front of the bridge (Figure 1).

If the horse avoided to step onto the bridge and passed by on one side, it was led back to the front of the bridge. The latency to the first contact with the bridge with either the muzzle or a hoof was measured as (**B_latency touch**). The test was stopped either when the horse had crossed the bridge or after 5 min, which was the maximum time allowed for this test. The total time to completion of the test was measured as (**B_latency cross**). Data were recorded as described in Table 2.

Table 2. Description of the response parameters measured during the Moving Object (MO) and Bridge (B) tests.

Response Parameters	**Description**
Moving Object test	
MO_latency (exposure 1,2,3)	Time (s) from the horse is exposed to the moving object until the horse resumes feeding. The time limit is 60 s, after which the horse is led back to the bucket
MO_latency total	The sum of the latencies above. Maximum total time 180 sec
MO_ flight reaction (exposure 1,2,3)	The flight reaction when the horse moves away from the feeding bucket after exposure to the moving object. The flight reaction is given the scores: 0 if the horse do not move the front hoofs, 1 if the horse moves less than 2 meters away, 2 if the horse moves 2-4 meters away and 3 if the horse extends the lunge. The horse is restricted in a 7 meter lunge
MO_ flight reaction total	The sum of the distances above. Maximum total flight reaction 9.
MO_HRavg	Average heart rate during all three exposures
MO_HRpeak (exposure 1,2,3)	The peak heart rate at each of the exposures to the moving object
Bridge test	
B_latency touch	Time (s) from start of the test until the horse touches the bridge with a hoof or the muzzle
B_latency cross	Time (s) from start of the test until the horse has crossed the bridge with all four legs. Maximum time to completion 300 seconds
B_HRavg	Average heart rate during the test
B_HRpeak	The peak heart rate during the test

2.5. Hormones

2.5.1. Analysis of serum testosterone and estradiol

Serum testosterone and estradiol were analyzed by commercial ELISA assays (Neogen Corp. Product #402510 and #402110, Lexington KY, 40511-1205 USA, respectively). The instructions recommended by the manufacturer were followed. The intra-assay coefficients of variation were 7.4% and 4.5% for 0.041 and 0.190 ng/ml testosterone control materials, and 1.6% and 1.3% for 0.041 and 0.388 ng/ml for estradiol control materials, respectively.

2.5.2 Analysis of salivary cortisol

The saliva (after a 1:10 dilution with assay buffer) was analyzed using a cortisol enzyme immunoassay without extraction, validated for equine saliva (Schmidt et al., 2009). Values should be interpreted as cortisol immunoreactivity, because antiserum cross-reacts with cortisone and some cortisone metabolites. The intra-assay coefficient of variation was 5.0%, the inter-assay variation was 6.7%, and the minimum detectable concentration was 0.1 ng/ml.

2.6. Scores at competition

Horses in this study were competing at three different events (SS, MT and DYC) in dressage or showjumping. The scores at the respective competitions were based on the judges' evaluation of different parameters as described in Table 3. At events MT and DYC the individual competition score could vary between 0-100, and the actual competition score was used for correlation analysis. However, in event SS there was a fixed score of 59 for poor performance (9/48 stallions), a fixed score of 69 for medium performance (17/48 stallions) and an individual score for horses above 69 for high performers (22/48 stallions). In the 3 year old dressage and showjumping stallions, competition performance is therefore evaluated in three performance groups instead of actual scores. Group 1 is horses with a score from 70 to 100, Group 2 is horses with the set score of 69 and Group 3 is horses with the set score of 59.

Table 3. Evaluation parameters and scores for dressage or showjumping horses at the three events: Stallion show for 3 year old stallions (SS), Mare testing for 3 year old mares (MT) and the Danish young horse championship in dressage and showjumping for 5 year old horses (DYC).

Competition	**Age**	**Comp days**	**Parameters**	**Judges**	**Maximum scores**
Stallion test dressage horses (SS) (n = 36)	3	3	Conformation (5 marks), walk, trot, canter and rideability, overall impression	Two judges on ground	100 Ten marks are given from 0-10
Stallion test show jumpers (SS) (n = 22)	3	3	Conformation (5 marks), canter, technique, rideability, capacity and overall impression	Two judges on ground	100 Ten marks are given from 0-10
Mare test dressage horses (MT) (n = 26)	3	1	Conformation (5 marks), walk, trot, canter, rideability and overall impression	A judge on ground and a test rider.	100 Ten marks are given from 0-10
Mare test show jumpers (MT) (n = 6)	3	1	Conformation (5 marks) canter, technique, rideability, capacity and overall impression	A judge on ground and a test rider.	100 Ten marks are given from 0-10
Young Horse, dressage (DYC) (n = 34)	5	2	Walk, trot, canter, rideability, capacity overall impression, rideability, score by test rider (3 marks)	Two judges on ground and a test rider	100 Ten marks are given from 0-10
Young Horse, showjumping (DYC) (n = 20)	5	2	Canter, technique, rideability, capacity and overall impression, rideability score by test rider (4 marks).	Two judges on ground and a test rider	100 Ten marks are given from 0-10

2.7. Data handling and statistics

2.7.1. Response parameters in tests

In the MO test, the parameters MO_latency, MO_flight reaction and MO_HRpeak were measured at the three repetitive exposures to the sudden object movement. Based on the development of the measured response parameters between exposure 1, 2 and 3, the horses were categorized into 3 groups for each parameter:

Group 1: horses that responded with a decrease in MO_latency, MO_flight reaction or MO_HRpeak from exposure 1 to exposure 2 to exposure 3 (i.e., horses showing responses indicative of *habituation*). Group 2: horses with an inconsistent response (decrease or increase) from exposure 1 to exposure 2 to exposure 3.

Group 3: horses responding with an increase in MO_latency, MO_flight reaction or MO_HRpeak from exposure 1 to exposure 2 to exposure 3 (i.e., horses showing behavior indicative of *sensitisation*).

2.7.2. Statistical analysis

All statistical analysis was performed using the SigmaPlot statistics package, version 13.0 (Systat Software Inc., Chicago, USA). Normality of data was assessed using the Shapiro-Wilk test, and variance homogeneity was assessed via the Brown-Forsythe test. Post hoc testing was performed using a pairwise comparison procedure (Holm-Sidak test) for parametric data. Due to the skewed distribution on age, sex and discipline across events we were unable to perform a multivariable analysis including all these factors. Therefore, the obtained data were analyzed in a number of separate analyses as described below. Effects were considered to be significant at $p < 0.05$ and showing a tendency at $0.05 < p < 0.10$. Values are presented as the mean ± standard error (SEM).

The effect of sex on testosterone concentrations was analyzed in two separate one-way ANOVA tests for 3 and 5-yr old horses, and since there was no differences between 5 year old mares and geldings these were merged into one mixed group (stallions n = 7, Mixed n = 44) before further analysis. The effects of sex (stallions and mixed), age (3 and 5 year old) and discipline (dressage and showjumping) on testosterone and handling test parameters were then analyzed in a three-way ANOVA. Estradiol data from mares were analyzed in a two-way ANOVA to test for the effect of age (3 and 5 year old) and discipline (dressage and showjumping) on estradiol concentrations.

The effect of sex (stallion and mixed group) and response group (1, 2 and 3) on testosterone concentrations were analysed in a two-way ANOVA. The response variables testosterone, cortisol, MO_latency total, MO_flight reaction total, B_latency touch and B_latency cross were logarithm transformed to fit normality throughout the analysis.

Spearman's rank correlation coefficient was used to test for correlations between responses in handling tests and testosterone concentrations (the mixed group and stallions were analyzed separately) and estradiol (mares).

Spearman's rank correlation coefficient was also used to test for correlations between responses in the handling tests and competition scores. Since the competition scores originated from different competitions, the correlation analysis was run separately for (i) 3 year old dressage mares (showjumpers were not tested due to low numbers), (ii) 5 year old dressage horses and (iii) 5 year old showjumpers. Correlations between testosterone and competition score were also run separately for (i) 3 year old dressage mares and geldings (showjumpers were not tested due to low numbers), (ii) 5 year old dressage mares and geldings and (iii) 5 year old mare and gelding showjumpers. Due to the low number of individuals, the 5 year old stallions were not tested.

For the 3 year old stallions, competition score group was treated as a class variable and the effect testosterone and on responses in handling tests were analyzed in a one-way ANOVA.

Finally, the correlation between responses in handling tests and the mean baseline cortisol concentration at show was tested in a Spearman's rank correlation coefficient.

3. Results

3.1. The effect of sex, age and discipline on testosterone and estradiol and responses in handling tests

The 3 year old stallions had higher testosterone concentrations than 3 year old mares (stallions: 0.79 ± 0.06 ng/ml and mares: 0.48 ± 0.11 ng/ml, p = 0.014) and the 5 year old stallions had higher testosterone concentrations than mares and geldings of the same age (stallions: 0.73 ± 0.18 ng/ml, mares: 0.26 ± 0.04 ng/ml, geldings: 0.29 ± 0.04 ng/ml, $p < 0.001$). Since the 5 year old mares and geldings did not differ, they were merged to a mixed group in the remaining analyses for testosterone. There were no significant interactions and no effect of age or discipline on testosterone concentrations, nor on any of the response parameters in the handlings tests (Table 4). The effect of sex was only significant for testosterone concentrations. However, dressage horses tended to have higher average heart rates than showjumpers in both handling tests (Table 4). Estradiol was only measured in the mares and tended to be higher in the 3 year old (0.72 ± 0.11 ng/ml) than the 5 year old mares (0.44 ± 0.12 ng/ml, $p = 0.08$), whereas there was no effect of discipline (dressage: 0.52 ± 0.09 ng/ml and showjumping: 0.64 ± 0.13 ng/ml, $p = 0.46$).

Dressage horses tended to have higher average heart rates than showjumpers in both handling tests (Table 4). None of the behavioural responses were affected by age, gender or discipline.

Table 4. The effects of sex (stallions and mixed), age (3 and 5 year old) and discipline (dressage and showjumping) on testosterone concentrations (ng/ml) and responses measured in two handling tests for all 141 horses. Seconds (s), meters (m). Values are presented as mean ± SEM.

N = 141	Sex		Age		Discipline		*p*-value		
Parameters	Stallions	Mix	3	5	Dressage	Show jump	Sex	Age	Dis
Testosterone (ng/ml)	0.79 ± 0.06	0.31 ± 0.06	0.62±0.05	0.47±0.09	0.48 ± 0.07	0.61 ± 0.07	**0.0001**	0.13	0.22
Handling tests									
MO_latency total (s)	61.1 ± 13.1	80.1 ± 8.2	75.4±7.9	65.8±13.3	67.2 ±11.6	74.0 ± 10.3	0.22	0.54	0.66
MO_flight reaction total (m)	5.62±1.8	4.4 ± 1.1	5.5±1.1	4.6±1.9	4.7±1.6	5.3±1.4	0.57	0.67	0.79
MO_HRavg (beats/min)	84.3 ± 4.6	82.3 ± 2.9	85.8±2.8	80.9±4.7	88.6 ± 4.1	78.1 ± 3.6	0.72	0.37	**0.06**
MO_HRpeak (beats/min)	104.7 ± 4.0	105.7 ± 6.7	108.2±3.8	102.2±6.5	111.3 ±5.0	99.0 ± 5.8	0.89	0.42	0.10
B_latency touch (s)	23.8 ± 7.5	31.7 ± 4.7	29.2±4.5	26.3±7.7	31.3 ± 4.2	24.4 ± 5.9	0.37	0.75	0.42
B_latency cross(s)	63.7 ± 22	90.1 ± 13	98.8±13	55.1±23	88.1 ± 19	65.8 ± 17	0.32	0.10	0.40
B_HRavg (beats/min)	76.9 ± 3.8	79.8 ± 2.4	80.7 ± 2.3	76.0±3.7	82.5 ± 2.4	74.5 ± 2.9	0.52	0.30	**0.07**
B_HRpeak (beats/min)	92.8 ± 4.8	100.8 ± 3.0	99.7± 2.9	93.8±4.9	101.6±4.2	92.0 ± 3.8	0.16	0.30	0.10

3.2. Correlations between hormone concentrations (testosterone and estradiol) and handling test responses

Testosterone concentrations were negatively correlated (r = -0.41, p = 0.006) to MO_flight reaction total in the mixed group, but not to any other handling test responses (Table 5). For stallions there were no correlation between testosterone and the responses in the handling tests. In the 3 and 5 year old mares, estradiol concentrations were negatively correlated to MO_latency total (r = -0.35, *p* = 0.02), MO_HRpeak (r = -0.43, *p* = 0.0002) and B_HRavg (r = -0.30, *p* = 0.04), and estradiol tended to correlate negatively to MO_flight reaction total (r = -0.28, *p* = 0.06) (Table 5).

Table 5. Correlations between testosterone (ng/ml) and handling test responses in stallions and the mixed group (mares and geldings), and between estradiol (ng/ml) and handling test responses in mares.

	3 and 5 year old mixed (mares and geldings)		**3 and 5 year old stallions**		**3 and 5 year old mares**	
	Testosterone (n = 76)		**Testosterone (n = 65)**		**Estradiol (n = 47)**	
	R	***p*-value**	**R**	***p*-value**	**R**	***p*-value**
MO_latency total (s)	-0.17	0.13	0.03	0.81	-0.35	**0.02**
MO_flight reaction total (m)	-0.41	**0.006**	0.06	0.63	-0.28	0.06
MO_HRavg (b/min)	-0.05	0.65	0.06	0.63	-0.27	0.74
MO_HRpeak (b/min)	-0.12	0.30	0.07	0.60	-0.43	**0.0002**
B-Latency touch (s)	-0.11	0.36	-0.05	0.69	0.01	0.94
B-Latency cross (s)	-0.04	0.98	-0.13	0.31	0.01	0.97
B_HRavg (b/min)	-0.13	0.25	0.07	0.61	-0.30	**0.04**
B_HRpeak (b/min)	-0.21	0.06	0.10	0.41	-0.12	0.43

3.3. Effect of response group on hormone concentrations (testosterone and estradiol).

The distribution of horses into the three response groups is shown in Table 6. There was no effect of response group on estradiol concentrations (mares only), i.e. mares that habituated with repeated object exposure did not differ in estradiol concentrations from mares that sensitized, in any of the three parameters (e.g. MO_HRpeak: Group 1: 0.60 ± 0.16 ng/ml, Group 2: 0.55 ± 0.09 ng/ml, Group 3: 0.43 ± 0.20 ng/ml, *p* = 0.80).

Table 6. Distribution of horses into the three groups: Group 1: habituated, Group 2: inconsistent, Group 3: sensitized, for the three response parameters measured: MO_Latency, MO_flight reaction, MO_HRpeak

Percentage of horses	Group 1 Habituated	Group 2 Inconsistent result	Group 3 Sensitized
MO_latency	25% (Stallions: n=16 Mares: n=11 Geldings: n=8)	59% (Stallions: n=39 Mares: n=32 Geldings: n=10)	16% (Stallions: n=10 Mares: n=8 Geldings: n=3)
MO_flight reaction	40% (Stallions: n=29 Mares: n=20 Geldings: n=8)	54% (Stallions: n=32 Mares: n=30 Geldings: n=14)	6% (Stallions: n=4 Mares: n=4 Geldings: n=1)
MO_HRpeak	45% (Stallions: n=29 Mares: n=24 Geldings: n=12)	45% (Stallions: n=29 Mares: n=24 Geldings: n=12)	10% (Stallions: n=7 Mares: n=3 Geldings: n=1)

There was no difference in testosterone concentrations between response groups 1, 2 and 3 for MO_flight reaction and MO_HRpeak. However, an interaction between sex and response group was present for MO_latency (Table 7). Stallions in group 1 had higher (p = 0.032) testosterone concentrations than stallions in group 2 and 3, but there was no difference between groups for the mixed sexes (Figure 3).

Table 7. The effect of sex and response group (MO-latency, MO-flight reaction, MOHRpeak) on testosterone concentrations (ng/ml). Values are presented as mean ± SEM.

	Sex		Response Group			P		
Test	Stallions	Mix	1	2	3	Sex	RC group	S x RC
MO_latency (s)	0.78±0.05	0.31±0.05	0.73±0.06	0.48±0.04	0.50±0.08	<0.001	0.004	**0.032**
MO_flight reaction (m)	0.80±0.07	0.27±0.07	0.56±0.05	0.56±0.04	0.50±0.12	**<0.001**	0.89	0.35
MO_HRpeak (beats/min)	0.79±0.06	0.34±0.07	0.57±0.05	0.51±0.05	0.68±0.12	**<0.039**	0.96	0.12

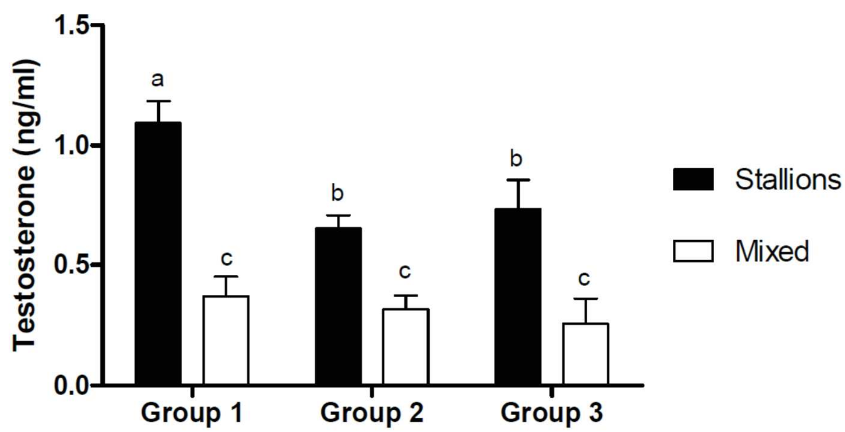

Figure 3. Stallions in group 1 (which showed a decrease in latency to resume feeding after repeated exposure to the moving object, i.e. habituation) had higher testosterone concentrations (ng/ml) than stallions in group 2 (inconsistent) and 3 (increased latency, i.e sensitization). There was no group effect for mares and geldings (mixed). Columns with different letters differ significantly at $p < 0.05$.

3.4. The relationship between testosterone, responses in handling tests and competition scores

There were no significant correlations between testosterone concentrations and competition scores, nor between responses in handling tests and competition scores for the 5 year old horses.

Only one correlation was significant; in the group of 3 year old dressage mares there was a negative correlation between latency time to touch the Bridge (B_latency to touch) and competition score (r = -0.47, $p = 0.007$).

Similarly, for the 3 year old stallions there was no significant effect of performance group on responses in handling tests, nor on testosterone levels (Performance group 1: 0.77±0.11, 2: 0.81±0.12, 3: 0.78±0.12, $p = 0.91$).-

3.5. The relationship between responses in handling tests in a familiar environment and cortisol level at competition in an unfamiliar environment

Dressage horses had significantly higher baseline cortisol concentrations than showjumpers (Munk et al., 2017), making it necessary to conduct the analysis within discipline. In showjumpers only, we found significant positive correlations between mean baseline concentration of cortisol at competition and MO_flight reaction (r = 0.49, $p = 0.04$), MO_HRavg (r = 0.50, $p = 0.04$) and MO_HRpeak (r = 0.50, $p = 0.04$), and a tendency for B-Latency to cross (r = 0.47, $p = 0.06$) (Table 7).

Table 7. Corrrelations between baseline cortisol concentrations in showjumpers and dressage horses during competition in an unfamiliar environment and responses in handling tests in a familiar environment.

	Dressage horses (n = 31)		**Showjumpers (N = 19)**	
	R	***p*-value**	**R**	***p*-value**
MO_Latency total (s)	0.16	0.34	0.17	0.50
MO_flight reaction total	0.30	0.89	0.49	**0.04**
MO_HRavg (beats/min)	-0.13	0.49	0.50	**0.04**
MO_HRpeak (beats/min)	0.06	0.77	0.50	**0.04**
B-Latency Touch (s)	0.006	0.97	0.12	0.63
B-Latency cross (s)	0.04	0.82	0.47	0.06
B_HRavg (beats/min)	0.09	0.61	0.26	0.31
B_HRpeak (beats/min)	0.02	0.90	0.12	0.65

4. Discussion

The results presented in this paper suggest that there is no clear relationship between performance, hormones concentration and behavioral responses measured in two handling tests. However, there could be an association between fear reactions in a MO test and the level of testosterone and estradiol concentrations in horses.

4.1. Correlations between hormone concentrations (testosterone and estradiol) and handling test responses

In this study, we confirmed a higher serum testosterone concentration in stallions compared to mares and geldings, but in accordance with previous studies there was no effect of sex on the responses measured in the handling tests (Wolff et al., 1997; Visser et al., 2002, 2003). However, our results suggest that steroid hormone concentrations may be related to some reactions in handling tests. We found a negative correlation between testosterone and flight reaction in the MO test in mares and geldings. This suggests that higher plasma testosterone concentration in the mare and gelding may cause the horse to be less reactive and fearful in agreement with our hypothesis. For stallions there was no correlation between testosterone levels and reactivity in any of the handling tests.

In mares the latency to resume feeding and HR were lower with increased estradiol concentration and flight reaction also tended to be negatively correlated to estradiol concentration. This indicate that higher plasma estradiol concentration in the mare may cause the horse to be less reactive and fearful, which is in agreement with our hypothesis. Plasma estradiol in the mare changes with the stage of the reproductive cycle with the highest plasma concentrations during estrous (Medan et al., 2004). To confirm our findings, future experiments could be repeated at different stages of the cycle in the same mare. In our study the actual stage of the estrous cycle was not confirmed. One previous study only looked at mares with behavioral problems in a reactivity experiment during different stages of the reproductive cycle (Hedberg et al., 2005). Hedberg et al. (2005) did not find a difference between different stages of the cycle, but the results may not be representative for behaviorally normal mares. Studies in women and female rodents have shown that attenuated anxiety is related to increases in estradiol concentration following the reproductive cycle (Marcondes et al., 2001; Mora et al., 1996; Glover et al., 2013). It is possible that correlations between testosterone, estradiol and cortisol responses in the handling tests are influenced by each other and by other hormones that are interacting throughout the estrous cycle. Behavior related to the estrous cycle in the mare is also influenced strongly by the levels of progesterone (Asa et al., 1984) wich was not measured in our study.

Testosterone secretion in stallions is seasonal and influenced by age. Our study was performed in February before start of the breeding season equally for all stallions in the experiment. We did find some variation between testosterone levels suggesting a varying degree of maturation in the group.

However, contrary to geldings and mares there was no influence of testosterone on reactivity in the handling tests in the stallions.

4.2. Effect of response group on hormone concentrations (testosterone and estradiol)

To test the hypothesis that horses with high concentrations of testosterone and estradiol habituate with repeated testing compared to horses with low concentrations, the horses were categorized into response groups depending on whether they habituated or sensitized with repeated exposures. It has to be noted that horses who did not return to the feed bucket within 60 seconds or horses that moved more than 7 m away from the bucket after three exposures were registered as inconsistent, but these horses might have either habituated or sensitized if allowed more time or a longer line. The peak HR was found to increase from exposure 1 to 3 in only 9% of the horses, whereas it decreased in 46% of the horses, suggesting that HR is very sensitive to habituation as also shown by Christensen et al. (2008a,b). Only 6% of the horses sensitized in terms of flight reaction (MO_flight reaction; Table 6). There was no difference in estradiol concentration in mares between these response groups. However, stallions that showed a decrease in the latency to resume feeding between successive exposures (habituated) had a higher testosterone level than stallions with either inconsistent or increased latency (sensitized).

Only 8 out of 65 stallions showed habituation in regards to both latency to resume feeding, flight reaction and peak HR. It is not unusual that habituation occurs at some different rates for various behavioural and HR parameters (Christensen et al., 2006). Nevertheless, the inconsistency leaves it open for debate whether the decreased latency reflected true habituation, or if other factors influenced the results. If it was caused by true habituation, we could expect the same horses to be in Group 1 for both MO_flight reaction and MO_HRpeak. However, there could be an interaction between MO_flight reaction and MO_HRpeak since it includes physical activity. If the decreased latency to resume feeding was not caused by habituation, it could be that the stallions with increased testosterone levels had more explorative and novelty-seeking behavior than stallions with lower testosterone concentration and therefore returned to the bucket faster.

4.3. The relationship between testosterone, responses in handling tests and competition scores

We aimed to investigate if either testosterone and estradiol levels or behavior in handling tests were related to competition score. Due to circumstances, the competition was performed 2-3 weeks following the handling tests and time of hormone sampling, results must therefore be interpreted with caution. Estradiol concentration was not included in the analysis due to the fact that stage of reproductive cycle in mares was not controlled and would have influenced the results. It was assumed that testosterone concentrations were more stable over time. However, we did not find any relationship between competition scores and testosterone concentrations. Ideally, blood samples should have been taken at the time of competition. However, this was not possible in this study because of restrictions regarding blood sampling at competitions.

We hypothesized that horses with lower fear reactions in handling tests in the home environment receive higher competition scores than horses with higher fear reactions. In this study, we found that an increased latency to touch the bridge was related to a low competition score in the group of 3 year old dressage mares (showjumpers were not tested due to low numbers). Visser et al. (2003) reported that an increased time to pass a bridge correlated to increased "responsiveness to environment" and decreased "attention to the rider" in 5-15 year old warmblood showjumper mares and geldings. The results from our study and Visser et al. (2003) suggest that the bridge test could be valuable in evaluating the temperament of a horse in relation to the expected behavioral responses in a competition situation.

We were unable to detect any other relationships between competition score and responses in the handling tests. This could be caused by the composition of the competitions scores for the young horses with parameters such as gaits and conformation included in the score. It is possible that rideability is a better reflection of the actual temperament of the horse.

4.4. The relationship between responses in handling tests in a familiar environment and cortisol level at competition in an unfamiliar environment

We further investigated the hypothesis that horses with more pronounced fear reactions in the handling tests have an increased salivary cortisol response in a competition environment. We found that showjumpers having a strong flight reaction as well as a higher HR in the MO test also had higher baseline cortisol concentrations at competition. There was also a tendency that showjumpers with a high salivary cortisol concentration spend longer time passing the bridge.

Increased cortisol levels are widely used as a physiological and psychological stress indicator in animals. The same stressor may cause different responses in different horses, and the perception of a potentially stressful situation depends on a combination of genetic predisposition and experience of the individual as well as a range of other factors (Möstl and Palme 2002). Increased cortisol concentration has been related to an increased reactivity score in horses exposed to disturbing procedures as clipping and the sound of fireworks (Young et al., 2012) and reactivity score in a novel object test (Anderson et al., 1999). Anderson et al. (1999) did also find that horses scored as "easy-to-spook" in a questionnaire had a higher baseline plasma cortisol than horses scored "hard-to spook". This suggest the importance of identifying horses with temperamental traits such as "easy to spook" in their home environment to make special individual preparation for future competitions.

The effect of repeated exposure or "habituation" to competition has been shown by Cayado et al. (2006) who found that the cortisol response to competition was influenced by competition experience in both dressage and showjumpers. The difference between dressage horses and showjumpers in reaction in handling tests as well as cortisol response has been further investigated and both Cayado (2006) and Munk et al. (2017) found that dressage horses had higher cortisol response to a competition environment than showjumpers. In line with Cayado et al. (2006) and Munk et al. (2017), Von Borstel et al. (2010) found that 3-17 year old showjumpers had a reduction in time to resume feeding as well as an attenuated reaction to a moving object compared to dressage horses, and in our study dressage horses tended to have a higher heart rate than showjumpers in both handling tests.

There is a tradition for more frequent competition exposure in showjumpers compared to dressage horses, which might explain why more studies have found decreased cortisol response and attenuated fear responses in showjumpers compared to dressage horses. There is no obvious explanation why we were only able to show a relationship between reactivity in handling tests and baseline cortisol concentration at competition in showjumpers. Von Borstel et al. (2010) suggested that showjumping breeding lines were selected for traits as fearlessness and courage, however, it is possible that the definition of discipline based on pedigree is not as important as the previous experience of the horse. Compared to the horses in von Borstel et al. (2010) the horses in our study were young and only separated into discipline according to pedigree and owner's expectations, most of them had only limited competition experience.

Munk et al. (2017) found an indication of a positive relationship between baseline cortisol at competition and competition score and in a group of 3 year old Danish warmblood mares, similar to our study population, Rothmann et al. (2014) found that highly reactive showjumpers received lower grades in rideability and free jumping than less reactive horses, but reactivity was not associated with performance traits in dressage. In contrast, Lansade et al. (2016) found that more fearful show jumpers cleared more fences but had more refusals than less fearful horses during a competition. This highlight that research is needed to develop behavioral tests that can be used to test horses prior to competition, so that training can be optimized and stress-induced decreased performance can be prevented. Furthermore, we need more studies to investigate the link between hormone concentrations and performance in both handling tests and competition.

Conclusion

The results from the present study indicate that estradiol and to a limited extent testosterone, may influence heart rate and behavioural responses to short term handling tests. Mares with high concentrations of estradiol had some reduced fear responses to the handling tests and stallions with higher testosterone concentrations showed reduced latencies to resume feeding with repeated object exposure. Competition score was not correlated to testosterone concentrations and responses measured in the handling tests. Cortisol concentrations at competition correlated to measures in the moving object test for showjumpers, but not dressage horses.

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An exploratory study of competition scores and salivary cortisol concentrations in Warmblood horses

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ABSTRACT

The main objective of this explorative study was to describe the relationship between competition scores and salivary cortisol concentrations in young horses during dressage and showjumping competitions. The study also investigated whether the diurnal rhythm of salivary cortisol concentrations was affected by competition over consecutive days compared with the home environment. Saliva samples were collected from 126 dressage horses and showjumpers in their home environment and at 3 different events. The relationship between scores given by judges at the competition and cortisol concentrations at the event was assessed. The results demonstrated that competition scores correlated positively to baseline cortisol concentrations at one of 3 events ($r = 0.53$, $P < 0.001$). Salivary cortisol concentrations followed a diurnal rhythm with the highest concentrations measured in the morning and the lowest in the evening, both at home and in the competition environment ($P < 0.05$). Salivary cortisol concentrations were greater during the competitions than at home ($P < 0.05$) except at one event where showjumpers did not increase between home and competition. Dressage horses had the highest baseline cortisol concentrations at competition, and exercise caused cortisol concentrations to increase in both showjumpers and dressage horses ($P < 0.001$). In conclusion, the diurnal rhythm in salivary cortisol concentrations was maintained in the novel environment. Dressage horses demonstrated greater baseline cortisol concentrations at competition than showjumpers, suggesting that they may perceive the novel environment as more stressful. Furthermore, there was no consistent relationship between baseline salivary cortisol concentrations and competition scores across the events.

1. Introduction

Increased cortisol levels are widely used as a physiological stress indicator in animals [1,2]. Cortisol increases in horses in response to both physical and psychological stressors such as transport [3–5], exercise [6–9], and competition [10,11]. During exercise, an increase in hypothalamic–pituitary–adrenal (HPA)-axis activity (resulting in eg, cortisol secretion) stimulates substrate mobilization by enhancing gluconeogenesis, protein catabolism, and the mobilization of free fatty acids [12,13]. The cortisol response to exercise increases with intensity and duration and decreases after repetitive training and with the competition experience of the horse [6,7,14–16]. Little is known however, about the relation between HPA-axis activity and competition performance in horses. Quantifications of cortisol in saliva can be used as a noninvasive estimate of plasma cortisol, as these have been shown to correlate well across the diurnal rhythm in humans [17] and horses [18,19].

In humans, there is some evidence for a relationship between performance in sport competitions and HPA-axis

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activity. A positive correlation was found between postexercise salivary cortisol concentrations and performance during weightlifting competitions [20], and an anticipatory rise in salivary cortisol before competition was associated with better performance in Judo athletes [21] and rowers [22]. In elite female athletes, baseline cortisol concentrations were greater than in nonelite athletes [23].

In horses, a previous report has shown a positive association between postexercise salivary cortisol concentrations and placing in a showjumping competition in an unfamiliar environment [24]. However, the relationship between baseline cortisol, exercise-induced cortisol release, and competition scores in an unfamiliar environment has not yet been studied in horses. Discipline-related differences in cortisol response to exercise have been identified in one previous study: a greater plasma cortisol response was measured in dressage horses compared with showjumpers when introduced to a new environment and after the competition [10]. In addition, the level of competition experience has been found to decrease cortisol concentrations in response to exercise at competitions in both dressage horses [10] and showjumpers [10,11].

Horses kept in a familiar environment accustomed to management, and exercise routines have shown a constant diurnal rhythm in plasma and salivary cortisol concentrations, with the highest concentrations measured in the morning and a decrease throughout the day (eg, [25–28]). There is some evidence that the diurnal rhythm can be disturbed by removing the horse from its familiar environment, or other acute or chronic physical and/or psychological stressors [1,29]. To our knowledge, there is no published literature concerning the effect of consecutive days at competition in an unfamiliar environment on the diurnal rhythm of salivary cortisol concentrations in horses.

The main objective of this explorative study was to investigate the relationship between salivary cortisol concentrations and competition scores in dressage horses and showjumpers. We further aimed to investigate whether the diurnal rhythm of salivary cortisol concentrations was affected by consecutive days of competition compared with the home environment.

2. Materials and methods

The study was in agreement with the ‘guidelines for ethical treatment of animals in applied animal behavior and welfare research’ from the ethics board of the International Society of Applied Ethology (www.applied-ethology.org).

The data presented in this article were collected on-site at 3 large competition events in Denmark as well as in the various home environments of the horses and should be considered an exploratory study. The 3 events were: (1) the Danish National Stallion Show for 3-yr-old stallions (n = 23) held jointly with the National Championship for 5-yr-old Danish Warmblood horses (n = 21) in 2013 (SH13), (2) the Danish National Stallion Show for 3-yr-old stallions (n = 30) in 2014 (SH14), and (3) the Young Horse Championship (n = 56) in 2013 (YHC). All participants in the 3 events were offered the chance to participate in the study, and horses were included if both the trainer and the owner signed an agreement form. At each event, the horses were assigned to a specific competition according to age.

Table 1
Distribution of sex, discipline, and age in horses at 3 separate events: the Danish National Stallion Show and National Championship for 5-yr-old Danish Warmblood horses in 2013 (SH13), the Danish National Stallion Show in 2014 (SH14), and the Young Horse Championship in 2014 (YHC) and in the home environment.

Event	Item	SH13	SH14	YHC
Sex	Stallion	23	30	19
	Mare	7	0	12
	Gelding	10	0	25
	Total	40	30	56
Discipline	Dressage	22	24	34
	Jumping	18	6	22
Age	3 yr	19	30	0
	4 yr	0	0	22
	5 yr	21	0	15
	6 yr	0	0	17
	Range	3–5	3	4–6
	Mean age of dressage horses	4.1	3	4.8
	Mean age of showjumpers	4.0	3	5.1
Day of collection	Home	39	22	56
	Event day 1	40	30	56
	Event day 2	40	30	0
	Event day 3	40	30	0
	Event day 4	0	24	0
Time of collection at home	Morning 06:00–08:00	Yes	None	Yes
	Midday 11:00–13:00	Yes	Yes	Yes
	Evening 17:00–19:00	Yes	None	Yes
	Pre-exercise	Yes	None	None
	Postexercise 10 min	Yes	None	None
Time of collection at event	Morning 06:00–08:00	Yes	None	Yes
	Midday 11:00–13:00	Yes	Yes	Yes
	Evening 17:00–19:00	Yes	None	Yes
	Pre-exercise	Yes	Yes	Yes
	Postexercise 10 min	Yes	Yes	Yes

2.1. Description of events, horses, and procedures

All horses were Warmblood riding horses (Hanoverian, Oldenburger, Holstein, Danish, or Dutch Warmblood), bred for either dressage or showjumping. Details on age distribution, sex, and discipline at the different events are presented in Table 1. All horses were trained and ridden by professional riders. In the 7 different home environments, the horses were stabled in individual pens and trained daily.

2.1.1. Danish National Stallion Show 2013 (SH13)

Stallions that qualified for the Danish National Stallion Show and horses that qualified for the National Championship for 5-yr-old Danish Warmblood horses in March 2013 were included in the study (n = 40; Table 1). All included horses were sampled 3 times per day (as described in Section 2.2), both at home and on Days 1, 2, and 3 of the event. Furthermore, pre-exercise and postexercise samples were collected at home and on Days 1 and 2 of the event. For the 3-yr-old stallions, home exercise on the day of sampling involved approx. Fifteen min of lunging divided equally by trot and canter. Event exercise consisted of approximately 10 min of free exercise in the show arena on Day 1, and 15 min of lunging divided equally by trot and canter with side reins on Day 2. The 3-yr-old stallions were regarded as either dressage horses or showjumpers based on their pedigree, but the event exercise did not differ between the disciplines. The 5-yr-old horses were ridden and their exercise at competition consisted of approximately 25 min of warm up and 10 min of either showjumping or dressage, and home exercise was set up to equal the effort at competition.

2.1.2. Danish National Stallion Show 2014 (SH14)

Stallions that qualified for the Danish National Stallion Show in March 2014 were included (n = 30) (Table 1). Saliva samples were collected at midday both at home and at the event on Days 1, 2, 3, and 4. Saliva samples were also collected postexercise on Day 2 of the event. Exercise consisted of 15 min of lunging divided equally by trot and canter with side reins. The stallions were regarded as either dressage horses or showjumpers based on their pedigree, but the event exercise did not differ between the disciplines.

2.1.3. Young Horse Championship (YHC)

Horses that qualified for the Danish Young Horse Championship 2013 for 4-, 5- and 6-yr-old horses were included (n = 56; Table 1). Saliva samples were collected 3 times per day (as described in Section 2.2), at home and on Day 1 of the event. Saliva samples were also collected postexercise at the event, and the exercise consisted of approximately 35 min of warm up and 10 min of competition in either dressage or showjumping. In dressage, the level of difficulty—and thus the expected physical demand—of the competition increased with age; in showjumping 4-yr-old horses jumped ten 110 cm high obstacles, 5-yr-old horses jumped fourteen 120 cm high obstacles, and 6-yr-old horses jumped fifteen 130 cm high obstacles.

2.2. Saliva sampling method and analysis

Saliva was collected with synthetic swabs (Salivette, Nümbrecht-Rommelsdorf, Germany). The swabs were placed under and over the tongue with the help of an arterial clamp for approximately 1 min, until they were soaked with saliva. The swab was then placed in a polypropylene tube and stored at −18°C until analysis.

Sampling of saliva was performed in the morning (06:00–08:00 h), at midday (11:00–13:00 h), in the evening (17:00–19:00 h), and 0–1 h before (pre-exercise) and 10 min after exercise (postexercise) both at home and at the events. However, the number of samples (morning, midday, or evening) and days (at home and Day 1, 2, 3, or 4 of the events) where saliva was collected, differed between the events (Table 1) due to practical reasons. Saliva was sampled either before feeding or a minimum of 1 h after feeding and at least 2 h after exercise when the horses were resting in their stables. The morning or midday samples were used as pre-exercise samples if the horses were exercised within 1 h after either the morning or midday sample. If the morning or midday samples could not be used as pre-exercise samples, a separate pre-exercise sample was collected approximately 1 h before the exercise. Postexercise samples were collected in the stable area 10 min after the horses left the exercise arena. At all the events, the same 3 persons performed the sampling procedure.

All obtained saliva samples (after a 1:10 dilution with assay buffer) were analyzed using a cortisol enzyme immunoassay (for details see Palme and Möstl [30]) without extraction, validated for equine saliva [27,31]. Values should be interpreted as cortisol immunoreactivity, because the antiserum cross-reacts with cortisone and some cortisone metabolites. The intra-assay coefficient of variation was 5.0%, the inter-assay variation was 6.7%, and the minimum detectable concentration was 0.1 ng/mL.

2.2.1. Cortisol levels

Baseline salivary cortisol concentrations refer to the daily average salivary cortisol concentrations both at home and for the events SH13 and YHC (ie, the average of the morning, midday, and evening samples), and the midday salivary cortisol concentrations for event SH14, since only midday samples were obtained at this event. The exercise-induced increase in saliva cortisol concentrations (EXDIF) was calculated as the difference between pre-exercise and postexercise saliva cortisol concentrations. At SH13, EXDIF was measured on two different competition days (Day 1 and 2) and the EXDIF mean was used for the correlation analysis. At SH14, EXDIF was only determined on one of the competition days (Day 2). For the event YHC, the EXDIF values relate directly to the exercise that resulted in the respective competition score, the score was given on the same day as the EXDIF was measured. In the discussion, the EXDIF is further expressed as the percent increase relative to the pre-exercise values to compare the levels found in different studies.

2.3. Competition scores

In both dressage and showjumping, the performance of each horse was evaluated by competition scores given by judges. The 3-yr-old stallions in SH13 and SH14 were evaluated for the movement and conformation in 10 parameters; each parameter was scored from 1 to 10 with a total maximum score of 100. If the total score was below 70, the stallions were not approved for breeding, and the exact scores were not released and were also unavailable for this study. Seven stallions at SH13 and 9 stallions at SH14 were not approved for breeding and they were given a score of 69 in the analysis. The performance score for the remaining stallions was the summation of scores given across 3 days at the event (maximum: 100). For the 5-yr-old horses in SH13 and the 4-, 5- and 6-yr-old horses in YHC, scores were given from 1 to 10 with a total maximum of 100. In dressage, the scores were based on walk, trot, canter, capacity, and rideability, whereas in showjumpers, the scores were based on canter, technique, capacity, and rideability. Since the competition scores at SH13 and SH14 were a summation of scores across days and the scores for individual days were not released, the EXDIF values do not reflect the total amount of exercise that resulted in these competition scores.

2.4. Data analysis and statistics

Except when mentioned specifically, the statistical analyses were performed using the SigmaPlot statistics package, version 13.0 (Systat Software Inc, Chicago, USA). Normality of data was assessed using the Shapiro-Wilk test, and variance homogeneity was assessed via the Brown-Forsythe test. Post hoc testing was performed using a pairwise comparison procedure (Holm-Sidak test).

Due to the skewed distribution on age, sex, and discipline across events, we were unable to perform a multivariable analysis including all these factors. Therefore, the obtained data were analyzed in a number of separate analyses as described below. Effects were considered to be significant at $P < 0.05$ and showing a tendency at $0.05 < P < 0.10$. Values are presented as the mean ± the standard error of the mean.

Spearman's rank correlation coefficient was used to test for correlations between competition scores and cortisol concentrations (both baseline at event and EXDIF) at each separate event. P-values <0.05 were considered to be significant.

2.4.1. SH13 (N = 40)

Due to the distribution of age and sex (eg, all 3-yr-old horses were stallions), the data were initially analyzed separately per age class. Thus, data from the nineteen 3-yr-old stallions (discipline; 13 dressage horses, 6 showjumpers) were analyzed in a 1-way ANOVA test for the effect of discipline on baseline cortisol concentration and EXDIF. Data were logarithm transformed to fit normality.

Data from the 21 5-yr-old horses (4 stallions, 10 geldings, 7 mares; discipline; 11 dressage horses, 10 showjumpers) were analyzed in a 2-way ANOVA test for the effect of sex and discipline on baseline cortisol concentration and EXDIF. Data were logarithm transformed to fit normality.

Furthermore, the effects of age and 'time of the day' (morning, midday, evening) on baseline cortisol levels, and the effects of age and environment (home and event Day 1, 2, and 3) on baseline cortisol levels were analyzed in a 2-way ANOVA for repeated measures to account for repeated sampling. Data were logarithm transformed to fit normality.

2.4.2. SH14 (N = 30)

Data from the thirty 3-yr-old stallions (discipline; 24 dressage horses, 6 showjumpers) were analyzed in a 1-way ANOVA test for the effect of discipline on baseline cortisol concentration and EXDIF. Data were logarithm transformed to fit normality.

The effect of discipline and environment (home and event Day 1, 2, 3, 4) on baseline cortisol levels were analyzed in a 2-way ANOVA for repeated measures to account for repeated sampling. Data were logarithm transformed to fit normality.

2.4.3. YHC (N = 56)

Due to the limited number of horses, the data from the 56 horses (age; 22 4-yr-old-, 15 5-yr-old, 17 6-yr-old; sex; 12 mares, 25 geldings, 19 stallions; discipline; 33 dressage horses, 23 showjumpers) could not be analyzed in a 3-way ANOVA test with all the interactions. Therefore the main effects of age, sex, discipline, and time of day on baseline cortisol as well as interactions between these factors were analyzed in SAS (Statistical Analysis System, version 9.3) with a proc mixed procedure including age, sex, discipline, and time of day as well as all 2-way interactions as fixed factors and horse as a random factor. Differences between home and event and interactions between sex, age, and discipline were analyzed using the mean values from home and event with the same model except that time of day was omitted.

3. Results

3.1. The effect of sex and age on salivary cortisol concentrations at the events

For 5-yr-old horses at SH13 and horses at YHC, we did not find any effect of sex on baseline cortisol concentrations (SH13: stallions 1.21 ± 0.21, mares 2.08 ± 0.32, geldings 1.84 ± 0.22 ng/mL, $P = 0.13$, and YHC: stallions 0.67 ± 0.18, mares 0.70 ± 0.17, geldings 0.86 ± 0.10 ng/mL, $P = 0.56$) nor on EXDIF (SH13: stallions 2.20 ± 0.41, mares 2.11 ± 0.50, geldings 1.73 ± 0.33 ng/mL, $P = 0.83$, and YHC: stallions 1.78 ± 0.36, mares 2.25 ± 0.52, geldings 1.81 ± 0.31 ng/mL, $P = 0.72$).

Similarly, in SH13 and YHC, there was no significant effect of age on baseline cortisol concentrations (SH13: 3-yr-old 2.16 ± 0.41, 5-yr-old 2.05 ± 0.27 ng/mL, $P = 0.96$, and YHC: 4-yr-old 0.81 ± 0.12, 5-yr-old 0.61 ± 0.18, 6-yr-old 0.80 ± 0.13 ng/mL, $P = 0.59$). The effect of age on EXDIF could not be investigated as the level of exercise differed between the age groups.

3.2. The relationship between competition scores and salivary cortisol concentrations

There was no correlation between baseline cortisol concentrations and competition scores at the events SH13 (Fig. 1A) and SH14 (Fig. 1B). However, at YHC, there was a positive correlation between baseline cortisol concentrations and competition scores (Fig. 1C). The correlation between EXDIF and competition scores was low and nonsignificant for all the events (r <0.14; $P > 0.58$).

3.3. Diurnal rhythm in salivary cortisol concentrations in the home environment and at events

Saliva cortisol concentrations followed a diurnal rhythm, with the highest concentrations measured in the morning and the lowest concentrations in the evening both at home (SH13) and at the events (SH13 and YHC; Fig. 2). There was no effect of age, sex, or discipline (data not shown).

3.4. Baseline cortisol concentrations at home and at the event

The effect of day (home and Day 1, 2, 3, 4 at the events) on the baseline saliva cortisol concentrations is shown in Table 2. Baseline saliva cortisol concentrations were lower

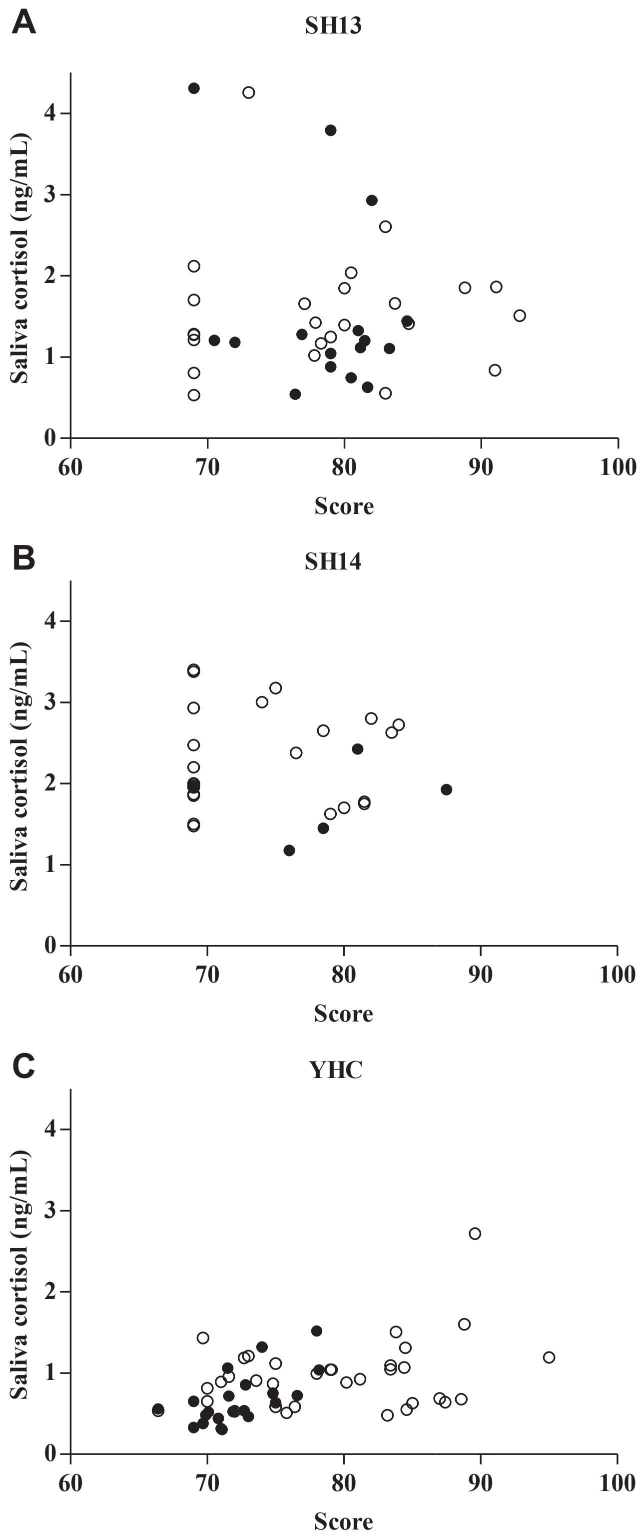

Fig. 1. The correlation between baseline cortisol concentrations (ng/mL) and competition scores; (A) SH13 (overall r = 0.08, P = 0,63, in dressage r = 0.08, P = 0.7 and showjumping r = 0.02, P = 0.95), (B) SH14 (overall r = −0.05, P = 0,81, in dressage r = 0.04, P = 0.84 and showjumping r = 0.60, P = 0.35), and (C) YHC (overall r = 0.53, P = 0.0003, in dressage r = 0.33, P = 0.05 and showjumping r = 0.63, P = 0.002). The symbols ○ and ● indicate dressage horses and showjumpers, respectively. YHC, Young Horse Championship.

at home than at event in SH13 and SH14 for both dressage horses and showjumpers (Table 2), whereas at event YHC, only dressage horses had a greater baseline cortisol concentrations at the event than at home (dressage horses; home: 0.61 ± 0.07, event: 0.93 ± 0.07, P < 0.0001, and showjumpers; home: 0.55 ± 0.09, event: 0.59 ± 0.09, P = 0.61). The average saliva cortisol concentrations increased during the Days at SH13 and were significantly greater on Day 3 than on Day 1. For the event SH14, there was no difference in saliva cortisol concentrations between Day 1, 2, 3, and 4 at the event (Table 3). Neither age nor sex (SH13 and YHC) had an effect on baseline cortisol concentrations.

3.5. The effect of discipline on baseline cortisol concentrations and EXDIF

The effect of discipline on the baseline cortisol concentrations and EXDIF is shown in Table 3. In SH13, we found that exercise at home increased salivary cortisol concentrations by 89% compared with pre-exercise concentrations, and during the 3 d of the event, exercise increased salivary cortisol by 135%–139% compared with baseline cortisol concentrations. In SH13, there was no difference between the disciplines for the 3-yr-old stallions, but the 5-yr-old dressage horses had greater baseline and EXDIF cortisol concentrations than showjumpers (Table 3). In SH14, dressage horses had greater baseline and EXDIF cortisol concentrations than showjumpers (Table 3). There was also an effect of discipline on baseline cortisol concentrations at YHC with dressage horses having greater baseline cortisol concentration than showjumpers, and dressage horses tended to have a greater EXDIF than showjumpers (Table 3).

4. Discussion

The results from this study indicate that young sports horses in particular dressage horses have an increased cortisol response to an unfamiliar environment compared with home. However, the diurnal rhythm was maintained in the novel environment. In general, there was no effect of age and sex on salivary cortisol concentrations but the results suggest that especially 4–6-yr-old showjumpers had lower saliva cortisol concentrations than dressage horses.

4.1. The relationship between salivary cortisol concentrations and competition scores

In the present study, we found no significant correlation between EXDIF and competition scores in any of the 3 events, nor between competition scores and baseline cortisol concentrations for the horses participating at SH13 and SH14. However, at event YHC, there was a significant but rather weak biological relationship between competition score and baseline saliva cortisol concentrations. A study by Peeters et al [24], found that the number

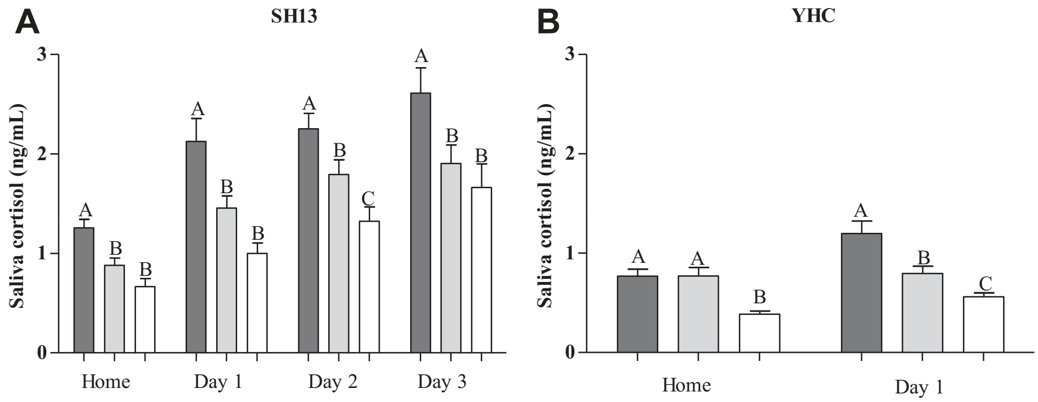

Fig. 2. The effect of time of the day (morning, midday, or evening) on saliva cortisol concentrations (ng/mL) at home and at the events (A) SH13 and (B) YHC (within days, columns with different letters differ significantly [$P < 0.05$]). YHC, Young Horse Championship.

of penalties in a showjumping competition was negatively correlated (r = −0.44) to salivary cortisol concentrations measured 20 min postexercise, however, they did not correlate scores to baseline cortisol or EXDIF in their study. The negative correlation was suggested to reflect that the stress induced during the competition (in a familiar environment, without any transport or change in stabling) was lower than the limit where stress should become distress and potentially have a negative influence on performance.

It has been suggested in human studies that cortisol is related to behavioral patterns important for competitions including aggression, arousal, and mobilization of physiological resources [20–22]. This could also be the situation for the horses in YHC where the competition score was based on physical exercise, sensitivity to rider cues and movements. In regard to the 3-yr-old stallions in SH13 and SH14, part of the competition score was based on conformation to which cortisol is likely unrelated.

It could be assumed that a high-cortisol response to exercise would be associated with a high-competition score in competitions depending on physical parameters but in this study, we did not find a positive relationship between EXDIF and cortisol. Since EXDIF was measured only on one (SH13) or two days (SH13) of the event whereas competition scores related to the entire event, it is not surprising to find that these parameters were unrelated. Further studies that control for the level of physical exercise and include behavioral parameters are required to investigate the potential relationship between competition scores and cortisol responses in horses.

4.2. Diurnal rhythm in saliva cortisol concentrations in the home environment and at events

In the present study, we found a diurnal rhythm in baseline cortisol concentrations in the home environment. There was a difference between morning and evening samples, with a decrease in salivary cortisol concentrations throughout the day. This is in accordance with other studies [25,27,31]. It has been reported that stressful events can disrupt the diurnal rhythm of cortisol concentrations in animals [29,32]. However, this disruption was not apparent in the present study, where horses were housed in an unfamiliar environment at an event for one day (YHC) to three days (SH13). At the events, horses were kept in temporary stables where they could see and hear other horses and with physical and vocal contact with unfamiliar neighbor horses. Despite these conditions, the diurnal rhythm was preserved at the events. The diurnal rhythm of plasma cortisol was also demonstrated in trained racehorses both before and after the exposure to jetlag (change of daylight cycle) [25] and in horses housed in individual stalls or in groups [29]. Other studies have failed to detect a diurnal rhythm in an indoor-stabled group and an outdoor control group of horses [33]. The lack of diurnal rhythm in these studies was interpreted by the authors as being the result of an underlying stressful situation, however, cortisol concentrations can be influenced by feeding time and metabolic requirements that have not been controlled in the studies. In our study, the diurnal rhythm was not disrupted, despite a significant increase in cortisol concentrations at the events, compared with at home (SH13 and YHC).

Table 2
Baseline saliva cortisol concentrations (ng/mL) at home and during 3 different events: the Danish National Stallion Show and National Championship for 5-yr-old Danish Warmblood horses in 2013 (SH13), the Danish National Stallion Show in 2014 (SH14).

Event	Baseline cortisol					
	Home	Day 1	Day 2	Day 3	Day 4	*P*-value
SH13	0.94 ± 0.06^{C}	1.53 ± 0.13^{B}	1.77 ± 0.12^{A,B}	2.03 ± 0.18^{A}	-	<0.001
SH14	1.34 ± 0.32^{B}	2.29 ± 0.22^{A}	2.21 ± 0.14^{A}	2.35 ± 0.22^{A}	2.23 ± 0.18^{A}	<0.001

Values are presented as mean ± SEM.
Home, home environment; Day 1, 2, 3, 4, days of the event. Baseline saliva cortisol concentrations represent midday cortisol concentration in event SH14 and mean cortisol concentration (morning, midday, and evening) for event SH13.
^{A,B,C}Values with no common letters differed significantly within the event ($P < 0.001$).

Table 3
The effect of discipline on baseline saliva cortisol concentrations (ng/mL) and on exercise-induced increase (EXDIF) in saliva cortisol concentrations (ng/mL) in the different age groups at the 3 events: the Danish National Stallion Show and National Championship for 5-yr-old Danish Warmblood horses in 2013 (SH13), the Danish National Stallion Show in 2014 (SH14), and the Young Horse Championship in 2014 (YHC).

Event	Age	Baseline cortisol		*P*-value	EXDIF cortisol		*P*-value
		Dressage	Showjumping		Dressage	Showjumping	
SH13	3	1.76 ± 0.14	1.73 ± 0.21	0.904	1.91 ± 0.23	3.02 ± 0.99	0.281
	5	2.28 ± 0.23	1.40 ± 0.19	0.012	2.56 ± 0.41	1.30 ± 0.33	0.007
SH14	3	2.30 ± 0.12	1.64 ± 0.14	0.015	4.04 ± 0.77	0.73 ± 0.43	0.045
YHC	4	0.93 ± 0.10	0.71 ± 0.18	0.009	1.80 ± 0.37	2.09 ± 0.68	0.074
	5	0.87 ± 0.13	0.61 ± 0.16		2.43 ± 0.51	0.83 ± 0.62	
	6	1.15 ± 0.14	0.69 ± 0.12		2.45 ± 0.54	1.39 ± 0.46	

Values are presented as mean ± SEM.

4.3. Baseline salivary cortisol concentrations at home and during consecutive competition days

We further aimed to investigate the extent to which competition horses adapt to an unfamiliar environment over consecutive days at an event. At SH13, the baseline cortisol concentrations were greater at the event than at home, and it increased over the 3 consecutive days. At SH14, the baseline cortisol concentrations were also greater at the event than at home and remained at the same elevated level across the 4 consecutive competition days. It should be noted, however, that we only obtained midday samples at SH14, whereas the results from SH13 are based on three daily samples and may therefore be more precise. Thus, there were no indications that the horses habituated to the competition environment during the events. At YHC only dressage horses had an increased baseline cortisol concentration, and this could relate to the age distribution of the horses at this event. The 5- and 6-yr-old showjumpers would potentially have more competition experience than the dressage horses from the same event and than the younger horses from SH13 and SH14. Other studies have shown that repeated exposure to a stressor (such as training and transportation) in some cases will lead to a reduced cortisol response, possibly due to habituation [3,4,34,35].

4.4. Cortisol response to exercise

In SH13, the increase in cortisol related to exercise between home (89%) and the event (135%–139%) was most likely to be caused by the additional psychological stress perceived by the horses. It was not possible to determine the extent of the physical response, since we could not measure parameters related to physical activity such as blood lactate or heart rate. However, an exercise-induced cortisol increase of 135%–139% compared with pre-exercise concentrations is not unusually high when compared with the results of other studies, where an increase of 340% was measured in horses after finishing a cross-country course [9], an increase of 150%–360% when showjumping in an unfamiliar environment [7,24,31], or an increase of 200% during a dressage competition [31]. Transportation is regarded as mainly a psychological stressor, and transport has been found to increase cortisol concentrations by 600% compared with pre-transport concentrations [3,4].

The event with the highest exercise-induced increases in salivary cortisol was YHC, where the increase was 220% compared with pre-exercise concentrations, respectively (all age groups and both disciplines included). We did not measure any physical parameters, however, the physical demands at YHC were expected to be greater than for the horses at SH13 and SH14 since competition level increased with age, and there were no 3-yr-old horses at YHC.

4.5. Cortisol response in different disciplines

Showjumpers had lower baseline cortisol concentrations than dressage horses during events, except that there was no difference between dressage and showjumpers for the 3-yr-old stallions at event SH13. This corresponds well with the results from Cayado et al [8], who found that dressage horses had a greater plasma cortisol concentration than showjumpers after the transportation to a new environment, as well as an increased plasma cortisol concentration before exercise at the competition. This may suggest that dressage horses are more sensitive to these types of stressors.

Von Borstel et al [36] investigated the difference in reactivity and time to resume feeding after exposure to a moving object in showjumpers and dressage horses and found that showjumpers reacted less than dressage horses. This difference in fear reactions was independent of training level and therefore genetic heredity was suggested as a reason. Similarly in this study, the difference in baseline cortisol between dressage horses and showjumpers found at SH13, SH14, and YHC may also reflect genetic heredity, but this requires further studies. Management could also explain the difference in cortisol concentrations between dressage horses and showjumpers, because even though the majority of horses at SH13 and SH14 were 3-yr-old horses, they could already have been trained differently.

Furthermore, we found differences in cortisol responses to exercise between disciplines as dressage horses had a greater EXDIF than showjumpers in two of three events (SH13, 5-yr-old only, and SH14) and a tendency in one event (YHC). However, since the values are based on a very low number of horses (only 6 showjumpers participated in SH14), and there is a lack of standardization of exercise between the events the results should be interpreted with caution.

Studies [37,38] have reported that dressage horses tested with and without spectators (only 6 horses), or showjumpers tested in a competitive and a non-competitive setting did not have significantly different postexercise cortisol responses, suggesting that spectators may not be perceived as a stressor in horses. However, it is difficult to distinguish between the physiological cortisol release related to exercise and the psychological-induced cortisol release caused by environmental factors and since it was not possible to standardize exercise and warm up in our study and we did not have any measures on physical performance, the influence of discipline on EXDIF in this study should be interpreted with caution and require further studies.

5. Conclusion

A diurnal rhythm in salivary cortisol concentrations was confirmed in the home environment and also when stabled in an unfamiliar environment during consecutive days at competition. As expected, baseline salivary cortisol concentrations were greater at the events than at home. Dressage horses generally had greater baseline cortisol concentrations at competition than showjumpers and an acute bout of exercise lasting approximately 30 min, significantly increased cortisol concentrations, with a generally greater response in dressage horses. However, due to the nature of this exploratory study, a large number of factors could not be controlled for, and further studies are required to investigate the potential relationship between competition results, discipline, and cortisol responses.

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The effect of sex and time of day on testosterone concentrations in equine saliva and serum

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RESEARCH ARTICLE

Abstract

In terms of exercise, testosterone is important for the growth and maintenance of skeletal muscle mass. Sampling saliva could be a non-invasive alternative to blood sampling for the quantification of testosterone levels in horses. The objective of this study was to compare testosterone concentrations in saliva and serum (sampled simultaneously) from horses of different sexes and at different times throughout the day. A total of 67 warmblood riding horses (21 geldings, 22 mares and 24 stallions) were included in the study. Saliva and blood samples were collected in the morning (06:00-08:00), at midday (11:00-13:00) and in the evening (17:00-19:00). The results demonstrated a weak correlation between saliva and serum testosterone concentrations (r_s=0.25, P=0.04). Stallions had higher serum testosterone concentrations than mares and geldings (P<0.001), but there was no significant effect of sex on salivary testosterone concentrations. The time of day did not affect the concentration of testosterone in either saliva or serum. In conclusion, our results indicate that saliva samples cannot be recommended for measuring testosterone levels in horses. However, further research is needed to identify the disturbing factors.

Keywords: horse, hormone, gelding, mare, stallion, testosterone

1. Introduction

Testosterone is a key anabolic hormone with numerous physiological functions in the body. It is especially important in the growth and maintenance of skeletal muscle, bone and red blood cells during exercise (Crewther *et al.*, 2006; Zitzmann and Nieschlag, 2001). Immunoassays designed to measure salivary testosterone in humans are well documented (Granger *et al.*, 1999), and saliva sampling can be used as a non-invasive alternative to blood sampling for the quantification of testosterone concentrations (Cook *et al.*, 2012; Nunes *et al.*, 2011). Furthermore, it allows for straightforward repeated sampling.

A correlation between testosterone concentrations in saliva and blood has also been documented in captive monkeys under anaesthesia (Arslan, 1984) and in monkeys trained for the sampling of blood and saliva (Kutsukake *et al.*, 2009), whereas no such correlation was found in guinea pigs (Fenske, 1996). Saliva from monkeys was sampled by passive drool (Arslan, 1984; Kutsukake *et al.*, 2009) and cotton swabs were used for the guinea pigs (Fenske, 1996).

Currently, testosterone concentrations in horses are typically measured in the blood (Cox *et al.*, 1973; Soma *et al.*, 2008), but Khalil *et al.* (2009) have also shown that faeces are suitable for measuring differences in equine testosterone concentrations. Saliva samples have been used in one study with a limited number of horses (n=3), but it was concluded that salivary mucopolysaccharides interfered, giving unreliable results (Boudene *et al.*, 1976). Whether saliva can reliably be used to assess testosterone concentrations in the blood requires further study. Blood testosterone concentrations from stallions follow a diurnal rhythm, peaking in the morning and with a nadir in the evening (Sharma, 1976), but no existing literature describes a diurnal rhythm in the saliva or blood testosterone concentrations of geldings or mares.

The objectives of the present study were to: (1) investigate a potential correlation between testosterone concentrations in serum and saliva; (2) assess the effect of sex; (3) determine whether diurnal variation could be measured in serum and salivary testosterone concentrations in geldings and mares. It was hypothesised that: (1) there is a positive correlation between serum and salivary testosterone concentrations; (2) saliva samples will demonstrate the expected difference in testosterone levels between the sexes; (3) there is a diurnal rhythm in the secretion of salivary testosterone as well as in serum testosterone concentrations in stallions, though not in mares or geldings.

2. Materials and methods

The experimental procedures followed Danish national legislation, in addition to guidelines on the protection of vertebrate animals, approved by the member states of the Council of Europe (Anonymous, 1986). Furthermore, this study conformed to the 'Guidelines for Ethical Treatment of Animals in Applied Animal Behaviour and Welfare Research' upheld by the ethics board of the International Society of Applied Ethology (www.applied-ethology.org).

Horses

A total of 67 privately owned horses (21 geldings, 22 mares and 24 stallions) from 10 different yards were included in the study. The age of the horses ranged from 3 to 24 years (mean age ± standard deviation SD: geldings: 8.3±3.1, mares: 8.4±2.7, stallions: 9.4±5.7 years). All horses were warmblood riding horses (Hanoverian, Oldenburger, Holstein, Danish or Dutch Warmblood), bred and trained for either dressage or showjumping. Samples were obtained during the non-breeding season (January), and the mares were non-pregnant and anoestrous, as confirmed by ultrasonography.

Sampling of blood and saliva

Blood and saliva samples were collected from each of the 67 horses while stabled in individual boxes in their home environment. Sampling was performed in the morning (06:00-08:00), at midday (11:00-13:00) and in the evening (17:00-19:00). Within each yard, samples from all horses were collected on the same day, but different yards were sampled on different days. Blood samples were obtained by venipuncture of the vena jugularis with the use of S-Monovette 7.5-ml Z tubes (Sarstedt, Nümbrecht, Germany), followed by centrifugation (2,000×*g* for 10 min). Serum was stored at -18 °C until analysis. A saliva sample was obtained using synthetic swabs (Salivette®, Nümbrecht-Rommelsdorf, Germany) immediately after blood sampling. The swabs were placed under and over the tongue with the help of an arterial clamp for approximately 1 min, until they were soaked with saliva. The swabs were then placed in a polypropylene tube and stored at -18 °C within 20 min of sampling. Saliva samples were further processed by thawing and centrifugation (2,500×*g*). Saliva were then transferred to new tubes and again frozen until analysis. The recovery of the synthetic swabs (Salivette) in the freezing/thawing cycle was separately determined (n=10) by adding tritium labelled testosterone. It was found to be 52.3±2.5%. Saliva and blood samples were obtained either prior to feeding or at least 1 h after feeding, and at least 2 h after exercise, when the horses were resting in their boxes.

Analysis of testosterone in saliva and serum

Serum samples (0.5 ml) were extracted with diethyl ether (5 ml) by shaking for 30 min, followed by centrifugation (2,500×*g* for 15 min), freezing (-20 °C) and decanting the ether phase into a new vial. After evaporating the ether to dryness (at 40 °C under a stream of nitrogen), extracts were redissolved in assay buffer and an aliquot measured in a testosterone enzyme immunoassay (EIA; see below). An aliquot of the saliva samples was directly measured using the same assay (assay readings were corrected for testosterone loss of the swabs). The antibody was raised in a rabbit against testosterone-3-CMO-bovine-serum-albumin, and a biotin-streptavidin system was utilised as label. Details of the assay procedure and the testosterone EIA, including cross-reaction of the antibody, can be found elsewhere (Möhle *et al.*, 2002; Palme and Möstl, 1994). The sensitivity of the EIA was 0.2 pg/well. The intra- and inter-assay coefficients of variation were 7.9 and 12.9%, respectively. All samples were run in duplicate.

Statistics

The statistical analysis was performed using the SigmaPlot statistics package, version 13.0 (Systat Software Inc., Chicago, IL, USA). The normality of the data was assessed using the Shapiro-Wilk test, and variance homogeneity was assessed via the Brown-Forsythe test. Data did not fit normality and/or had unequal variance therefore the non-parametric Friedman Repeated Measures Analysis of Variance on Ranks was used to analyse changes in testosterone concentrations throughout the day.

The average of the morning, midday and evening samples of testosterone in saliva and serum were calculated for each horse and a Kruskal-Wallis One Way Analysis of Variance on Ranks was used to analyse the effect of sex on testosterone concentration. Post-testing was performed with Dunn's Method (All Pairwise Multiple Comparison Procedures). Spearman's rank correlation coefficient was used to test for correlations between average testosterone concentrations in saliva and serum. *P*-values <0.05 were considered to be significant. Data was not normally distributed and therefore values are presented as medians (25th and 75th percentiles).

3. Results

All horses tolerated the procedures well, and sampling of saliva and blood was straightforward. There was a weak correlation between salivary and serum testosterone concentrations (r_s=0.25, P=0.04), and the average salivary and serum concentrations of testosterone from each horse (n=67) are shown in Figure 1.

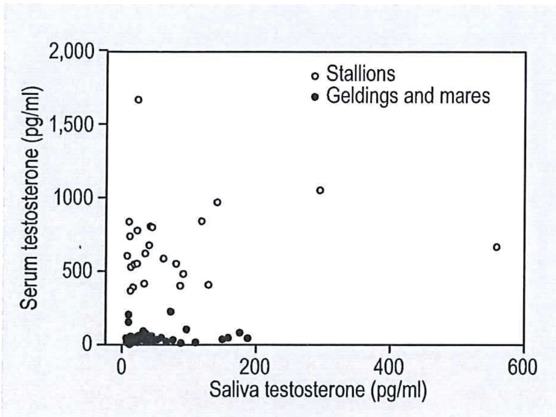

Figure 1. Average of morning, midday and evening samples of saliva (x-axis) and serum (y-axis) testosterone concentrations in stallions (n=24) and geldings and mares (n=43) (r_s=0.25, P=0.04).

There was an effect of sex on the concentrations of testosterone in serum, with stallions having higher concentrations than mares and geldings (P<0.001; Table 1). However, this effect of sex was not observed in the salivary testosterone concentrations. No diurnal rhythm was detected in testosterone concentrations, either in serum or in saliva (Table 2).

4. Discussion

Salivary testosterone reflects the free fraction of testosterone in blood that diffuses through the salivary glands into saliva. Saliva samples are frequently used as a non-invasive substitute for blood samples to measure testosterone levels in humans (Maso *et al.*, 2004; Nunes *et al.*, 2011), and it has been reported that salivary testosterone levels correlate well with free testosterone levels in serum when sampled simultaneously (Vittek *et al.*, 1985). To our knowledge, only one published study has measured salivary testosterone in horses (Boudene *et al.*, 1976), and the authors concluded that testosterone could not be measured accurately in saliva due to interference with mucopolysaccharides. However,

Table 1. The effect of sex (geldings, mares and stallions) on testosterone concentrations in serum and saliva. Values are presented as medians [25th; 75th percentiles].

	Sex	n	Median [0.25; 0.75][1]	*P*-value
Serum (pg/ml)	geldings	21	33.7 [18.7; 51.4] [b]	<0.001
	mares	22	41.5 [32.4; 56.4] [b]	
	stallions	24	613.0 [494; 804] [a]	
Saliva (pg/ml)	geldings	21	15.0 [10.4; 62.9]	0.23
	mares	22	26.0 [14.9; 58.0]	
	stallions	24	37.1 [16.9; 90.0]	

[1] Values in the same column with different superscript letters differ significantly (P<0.05).

Table 2. The effect of time (morning, midday and evening) on testosterone concentrations in serum and saliva. Values are presented as medians [25th; 75th percentiles].

	Sex	n	Time	Median [0.25; 0.75]	*P*-value
Serum (pg/ml)	stallions	24	morning	607 [388; 958]	0.76
		24	midday	668 [389; 991]	
		24	evening	519 [372; 710]	
	mares and geldings	43	morning	36.2 [16.0; 51.0]	0.80
		43	midday	31.0 [16.0; 54.4]	
		43	evening	37.4 [19.4; 57.0]	
Saliva (pg/ml)	all	67	morning	25.1 [10.0; 76.2]	0.23
		67	midday	14.8 [10.0; 44.1]	
		67	evening	17.3 [10.0; 44.5]	

studies in humans have found that a freeze-thaw cycle followed by centrifugation removes mucopolysaccharides from the saliva sample (Arregger *et al.*, 2007), but that centrifugation might decrease salivary testosterone levels in comparison to unprocessed saliva (Durdiaková *et al.*, 2013). Therefore we applied such a freezing/thawing cycle to the saliva samples prior to the analysis with the testosterone EIA and added a recovery experiment to correct for testosterone loss of the whole saliva collection method. Unfortunately, also in our study the correlation between testosterone concentrations in serum and saliva was weak.

The weak correlation observed in the current study could be related to the saliva sampling method. In humans, the correlation between salivary testosterone and total testosterone in serum is dependent on the saliva-collection method (Fiers *et al.*, 2014; Granger *et al.*, 2004). Passive drool has been found to increase the correlation between serum and salivary testosterone significantly in both men (r=0.68) and women (r=0.52) compared to saliva sampled with cotton swabs (both sexes: r=0.43) (Fiers *et al.*, 2014). In contrast to humans and monkeys, passive drool is not a practical option in horses, but it might be possible if saliva production could be stimulated pre-sampling, thus avoiding sampling with cotton swabs. However, there are also conflicting results regarding the use of saliva stimulation with regard to testosterone measurements. In humans, stimulating the saliva flow by touching the tongue with cotton swabs treated with 2% citric acid increased saliva production, but did not affect the concentration of salivary testosterone in comparison to cotton swabs (Durdiaková *et al.*, 2013). Other studies state that citric acid should be avoided due to interference with the method used for testosterone analysis (Granger *et al.*, 2004; Shirtcliff *et al.*, 2001), and that stimulation of saliva flow increases salivary testosterone concentrations (Dabbs, 1991; Granger *et al.*, 2004). However, a high correlation has been observed between salivary testosterone and free testosterone concentrations in human serum, regardless of immunoassay method (Granger *et al.*, 2004; Riad-Fahmy *et al.*, 1982; Wang *et al.*, 1981).

Additional factors potentially affecting testosterone analysis include the handling and storage of samples. One study found that storing samples at room temperature or between -20 and -80 °C, for up to 1 month did not affect the testosterone concentrations (Durdiaková *et al.*, 2013), whereas another study found that testosterone concentrations increased by 20% after 4 weeks of storage at 4 °C, and decreased after 24 months of storage at -20 °C (Granger *et al.*, 2004). In the current study, saliva samples were stored at -18 °C within 20 min of sampling, and were analysed within 3 months, so storage is not expected to have influenced the results.

It is possible that there is a time lag before responses measured in blood can be measured in saliva. Human studies demonstrating a positive correlation between testosterone in saliva and blood were sampled simultaneously (Fiers *et al.*, 2014; Johnson *et al.*, 1987), and following exogenous testosterone administration, testosterone concentrations in saliva and serum rose abruptly and in parallel (Wang *et al.*, 1981). Salivary pH and flow differs between horses and humans, with equine salivary pH ranging from 7.4 to 7.9 (Alexander, 1966), compared to 5.5-6.0 in humans (Horner, 1976). Saliva is continuously produced in humans, but is stimulated when horses masticate (Alexander, 1966; Burgen and Emmelin, 1961), with the rate of saliva flow influencing electrolyte concentrations (Alexander, 1966). However, information on the influence of saliva flow (and pH) on testosterone concentrations in saliva of horses is lacking. However, this could be a potential reason for the lack of correlation between saliva and plasma testosterone concentrations. Thus future research should determine whether there is a time lag between saliva and blood in terms of testosterone concentrations in horses, in order to ensure that saliva is sampled at the optimal time.

As expected, serum testosterone concentrations were higher in stallions than mares and geldings, and it was anticipated that this difference also would be observed in saliva. However, this difference between the sexes could not be detected in the saliva samples. Average salivary testosterone concentrations from stallions were two times higher than the values measured in mares and geldings, but due to large variations, the differences were not significant. Further research is needed to locate the problem.

In humans, a diurnal rhythm in testosterone concentrations has been confirmed (Granger *et al.*, 2004), and this has also been measured in blood from stallions, with peak testosterone concentrations in the morning and a nadir in the evening or at night (Kirkpatrick *et al.*, 1976; Sharma, 1976). However, the results are conflicting, as others did not find any diurnal rhythm in blood testosterone concentrations (Bono *et al.*, 1982). This is similar to the results from the present study, where diurnal rhythms in serum and salivary testosterone levels could not be detected.

5. Conclusions

In conclusion, salivary testosterone concentrations did not correlate well with serum testosterone concentrations. Stallions had higher serum testosterone concentrations than mares and geldings, but there was no significant difference between the sexes in salivary testosterone concentrations. It was not possible to detect a diurnal rhythm in serum or salivary testosterone concentrations, regardless of sex. The results from this study indicate that blood samples are more reliable for measuring testosterone concentrations in horses. Presently, saliva samples cannot be recommended for measuring testosterone levels in horses. However, further research is needed to identify the disturbing factors.

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8. General Discussion

The results in this thesis does not provide strong evidence to confirm an existing relationship between competition score in young horse competitions and either steroid hormone concentration or fearfulness. However, there are indications and tendencies to be discussed. Salivary cortisol concentrations did increase as a response to a competition environment and to exercise, however, a diurnal variation was maintained in the competition environment. Salivary testosterone had only a weak correlation with serum testosterone, and it cannot be recommended to use saliva as a proxy for serum when measuring testosterone levels in horses. A diurnal variation in serum or salivary testosterone concentration could not be detected. Below I discuss some aspects of the relationship between steroid hormones and performance i.e; the use of competition score as the performance goal, as well as the association of steroid hormones with measured fear responses in handling tests and the relationship between responses in handling tests and performance. Furthermore, factors that can influence steroid hormone concentrations (e.g. diurnal rhythm, age, sex, experience, discipline and exercise) are discussed. Finally, methodological considerations (e.g. the choice of fearfulness as a temperament trait, handling tests and the use of saliva for steroid hormone measurements) are also discussed.

8.1 Steroid hormones and performance

The first important result of this thesis to be discussed is the unclear relationship between steroid hormone concentration and competition score. There are indications that an increased baseline cortisol concentration at competition can be related to a higher competition score in competitions not including conformation score. Throughout this thesis, competition score is used as a measure of performance. Competition score is a relevant performance goal for dressage horses and show jumpers, and the competition results are often used to set the value of the horse. The value of using a competition score as a measure of performance in scientific investigations in young dressage horses and show jumpers could be challenged due to the complexity of the score's composition. The variation between the components included in the overall competition score given to each horse at the respective competitions caused difficulties of comparison and challenged the interpretation of results in this thesis. The competition score at the various events included different traits, incl. the physical ability of the horse (measured by gait and technique), and indirectly the temperament traits of the horse (measured as rideability).

The results of my own studies show that at one event (YHC) out of three there was a significant but rather weak biological relationship between competition score and baseline saliva cortisol concentration for both show jumpers and dressage horses (Paper II). These inconsistent results could be caused by the different components of the competition score between competitions. At YHC, the competition score was based on physical exercise, sensitivity to rider cues, and movements, whereas part of the competition score for the 3-year-old stallions in SH13 and SH14 was based on conformation, to which cortisol is not likely to be related.

It could be speculated whether the relationship between baseline cortisol concentration and performance follows an inverted U-shape as has also been suggested for arousal levels and performance in human athletes (the Yerkes-Dodson law; Harmison et al., 2006; Salehi et al., 2010; Chaby et al., 2015). This theory could also be applied in the study by Peeters et al. (2013) who found a negative correlation between cortisol concentration and faults in a show jumping competition (which was held in a familiar environment, without any transportation or change in stabling). Leading to the assumption that the cortisol measurement in Peeters study was at the top or close to the top of the inverted U-shape curve. The results in Paper II indicates that the horses participating in YHC also had cortisol concentrations around the top of the curve whereas the horses in DCY and SH13 would have cortisol concentration en either end of the curve.

The design of Study 1 and Study 2 did not allow for a good comparison of competition performance between stallions, geldings and mares, therefore only the relationship between competition performance and level of steroid hormone within sex was investigated. However, there were no relationship between testosterone concentration and competition score at any time (Paper I). To test the influence of testosterone level on physical performance the competition might need to have a high demand for explosiveness and strength as in the study by Knobbe et al. (2015) where two race winning mares had higher testosterone concentration than the non-winning mares. In human athletes, an increased baseline testosterone concentration is related to superior performance in high level explosive sports (Wood and Stanton, 2012), and likely the physical demands of the competitions for 3-6-year-old horses is not demanding enough to favorize horses with higher testosterone concentration. In high level competitions, superior performance of eventing stallions was found compared to mares and geldings (Whitaker et al., 2003, 2008), however, this was measured as a summation of points over the year and not at a specific competition. In the competitions investigated in this thesis, testosterone would be more likely to have an influence on competition score in competitions for the 3-year-old stallions, since one of the parameters in the assessment of conformation is stallion stamp and a positive relationship between testosterone and stallion stamp would be expected. However, the total competition score also includes multiple scores for conformation and therefore the influence of the stallion stamp score is diluted in the total score.

In Paper II there was no correlation between cortisol EXDIF and competition scores at any of the events. This is surprising since an increase in cortisol concentration often reflects an increased exercise intensity or an increased physical effort which is often related to superior performance in human sports (Passerlergue et al., 1995, Passerlergue and Lac., 1999). A similar situation would be expected in show jumping where superior performance is related to a fast time to complete the course as well as an explosive jump or in dressage where energetic expressive gaits are rewarded with more points. However, again this could be the results of the components of the scoring system in young sports horse competitions where other parameters than physical exercise are involved. Another factor that could influence the EXDIF measured in this study and thereby also the non-existing relationship between EXDIF and competition score is the fact that the actual physical training state of the individual horses were unknown. Studies in horses have reported that the response to exercise (EXDIF) is dependent on the level of long-term or chronic training the horse has been exposed to and some studies agree that the EXDIF will decrease in more trained (Marc et al. 2000) or over-trained horses (Golland et al. 1999). The 3- to 4-year-old horses measured in Paper II might not have been exposed to a high level of physical exercise prior to the competitions whereas this could be the case for the 5- and 6-year-old horses. Difference in long-term training and preparation also exist within age group, making a clear interpretation of results difficult. It would be of interest to investigate the relationship of both testosterone and cortisol to competition performance in show jumping competitions where a high level of physical effort and exercise intensity are involved. Further research is also required to identify whether an ideal performance state exists for the individual horse, where circumstances causing a raise in cortisol such as aggression, arousal, and mobilization of physiological resources are controlled or measured.

Various other factors can have influenced the results, such as the fact that the testosterone concentration used for correlation was measured at the time of the handling test, 2-4 weeks prior to the competition. Salivary samples collected at events for cortisol analysis were also analyzed for testosterone concentration, but based on results from Paper III, the data were regarded as invalid and this was not investigated further. Due to competition regulations, it was not possible to obtain blood samples for testosterone analysis on the day of competition and it would be an interesting addition to the current knowledge to measure testosterone and estradiol concentrations at competition.

8.2 Steroid hormones and fearfulness

The second important result of this study is the indication of a relationship within sex between estradiol and testosterone concentration and fear responses in handling tests. The relationship between sex and both physical and behavioral performance in dressage and show jumpers is up for debate amongst riders. A general perception is that stallions are more aggressive, less sensitive to the rider and more distracted by the environment than mares and geldings and it is also a common perception that mares can have rideability issues related to the estrous cycle (Hedberg et al., 2006). In addition, riders want "brave" horses for both dressage and show jumping (DW, 2017). Therefore, the aim of this thesis was partly to investigate if fearfulness was related to steroid hormone concentrations. Based on the influence of testosterone on behavioural parameters such as aggression and dominance in humans (Passerlerque and Lac, 1999; Castro and Edwards, 2016) and on rats, chimpanzee and sheep, with males showing less fear reactions than females or castrated males (Archer, 1975; Buirski et al., 1978; Vandenheede and Bouissou, 1993; Vandenheede and Bouissou, 1996) it was expected that stallions would have lower fear responses in handling tests than mares and geldings. However, there was no influence of sex on the fear responses measured in the handling tests (Paper I). Only a few other studies in horses have tested both mares, geldings and stallions in handling tests. Wolf et al. (1997) and Visser et al. (2002) found no difference between sexes in 2-36 month old horses, neither did Von Borstel et al. (2011) in horses between 3-19 years of age.

Not only is testosterone the hormone responsible for most of the differences between the sexes (Zitzmann and Nieschlag, 2001), an increased testosterone concentration within the same sex has also been shown to have an impact on increased aggression, novelty seeking and reduction of fear in humans and rodents (Frye and Seliga, 2001; Maattanen et al., 2013) and in ewes and heifers androgen administration decreased fear responses (Boissy and Bouissou 1994; Vandenheede and Bouissou, 1993). In Paper I, mares and geldings with an increased testosterone concentration had reduced fear reactions in the moving object test compared to mares and geldings with a lower testosterone concentration. Results also showed that mares with an increased estradiol concentration resumed feeding faster than mares with lower concentrations and their heart rate was lower in both tests, which may be similar to the effect on fear that estradiol has in women and rodents (Walff et al., 2009; Glover et al., 2013). In mares, individual differences in the response of steroid hormone concentration to an ACTH challenge test has been recognized (Hedberg et al., 2007a) and it is possible that the sensitivity to the level of testosterone and estradiol is much higher in mares and in geldings. In terms of future temperament testing in mares, it would be of interest to investigate the relationship between reproductive stage and responses in handling tests. Only one previous study studied mares with behavioral problems in a reactivity experiment during different stages of the reproductive cycle (Hedberg, 2005). Hedberg (2005) did not find a difference in responses in a fear test measured at two different stages of the cycle, but the results were based on 7 mares with behavioural problems and only 5 control mares and may not be representative for behaviorally normal mares. Studies in women and female rodents have shown that attenuated anxiety is related to increases in estradiol concentration following the reproductive cycle (Mora et al., 1991; Marcondes et al., 2001; Glover et al., 2013). It is possible that correlations between testosterone, estradiol and cortisol responses in the handling tests are influenced by each other and by other hormones that are interacting throughout the estrous cycle. Behavior related to the estrous cycle in the mare is also influenced strongly by the levels of progesterone (Asa et al., 1984) wich was not measured in our study.

Further it can be questioned whether fearfulness is the temperament trait most important to measure in relation to competition performance. The answer to that question is that fearfulness is the best described temperamental trait in equine studies and therefore this parameter was chosen. It can also be questioned if the two handling tests were the best for measuring fearfulness and whether the reactions measured as fear response were the correct reactions to measure fearfulness, however, the selection of handling test were justified in the state of art section and it was manageable in the test situation in study 1. It is difficult to interpret whether the reactions measured in the Bridge test was related to fear or novelty seeking, or a combination of these, however this was also questioned by Visser et al. (2001). Whether latency to feed reflects fearfulness in Paper I is debatable. Christensen and colleagues (2008a,b, 2011) describe latency to feed as a valid parameter of fearfulness in young Warmblood horses. However, these horses were habituated to the test arena and to feeding from the bucket for 1 month prior to testing. The horses in Paper I were familiar with the test arena but no systematic habituation to the arena or feeding from the bucket was performed due to time restrictions. Horses with a long latency showed a variety of different behaviours while away from the bucket, some horses had increased activity, while others spent time investigating the ground, and some concentrated on the entrance to the arena. Latency to resume feeding could be associated with hunger or the motivation to eat, which was also discussed by Von Borstel et al. (2010), who found that horses fed higher amounts of concentrate returned more quickly to feeding, and that horses ridden more often (up to seven times/week) had shorter latencies than horses ridden less often. Neither of these parameters were controlled in Paper I.

8.2.1 Habituation

In Paper I, it was also an aim to investigate if hormone concentrations were related to habituation or senzitization to repeated exposures to the moving object. The results in Paper I showed no difference in estradiol concentration in mares between groups. However, stallions that showed a decrease in the latency to resume feeding between successive exposures (habituated) had a higher testosterone level than stallions with either inconsistent or increased latency (sensitized). It is likely that the decreased latency to resume feeding between successive exposures in stallions with higher testosterone levels was caused by other factors than habituation, since the stallions that habituated in regard to latency to resume feeding were not the same as the horses who habituated in regard to fear reaction or heart rate. If the decreased latency to resume feeding was not caused by habituation, it could be that the stallions with increased testosterone levels were hungrier or had more explorative and novelty-seeking behavior than stallions with lower testosterone concentration and therefore returned to the bucket faster. In the study by Von Borstel et al. (2010) the horses experienced 5 exposures to the moving object. Due to time restrictions and owners constraints, the horses studied for Paper 1 where only exposed to the moving object 3 times and the results should therefore be interpreted with caution.

8.2.2 Cortisol and fearfulness

Another interesting result presented in Paper I is that show jumpers with an increased fear response and a higher HR in the moving object test and a tendency to spend longer time passing the bridge, also had higher baseline cortisol concentrations at competition. This is an indication that horses who perceives a competition environment as stressful can be identified in a handling test in the home environment and precautions can be made. There is no obvious explanation why this relationship was confirmed in show jumpers and not in dressage horses therefore this requires further investigation.

8.3 Fearfulness and performance

Only a single relationship between competition score and responses in the handling tests was confirmed in Paper I, in which the latency time to touch the bridge was shorter in the 3-year-old dressage mares that received the highest competition scores. As previously discussed the composition of the competition score could affect the results, and it is possible that responses in the handling tests should be correlated to scores related only to the temperament traits or behaviour. However, the fact that more fearful and highly reactive horses perform inferior to less fearful horses in ridden competitions and free jumping have also been confirmed by Visser et al. (2008) and Rothman et al. (2014). In a study by Lansade et al. (2016) it was again confirmed that the most fearful horses were the most difficult horses to ride during training and also the horses with more refusals during show jumping competition. However, very important was the fact that the most fearful horses jumped higher over fences and knocked down fewer bars in a show jumping competition than less fearful horses. In two studies by von Borstel et al. (2011b, 2012), young warmblood stallions were tested in novel object tests during a stallion performance test. Surprisingly, they did not find a relationship between shying or other reactivity measures in the test and current scores for temperament. Based on the literature it appears that the relationship between the temperament of the horse and performance at competition is highly dependent on performance scoring system. Whether more or less fearful individuals are related to better competition performance are dependent on the scoring system as well as the rider and horse combination.

8.4 Steroid hormones, fearfulness and performance

To summarize and combine information on fearfulness, cortisol concentrations, performance in handling tests and performance at competition; it appears that there is a relationship between increased cortisol concentration measured after a riding session (Anderson et al., 1999) or during a period of fireworks (Young et al., 2012) and horses characterised as agitated, anxious, active and aggressive by riders (Anderson et al., 1999; Young et al., 2012) and as fearful in handling tests (Paper I). In addition, more fearful horses have a higher baseline cortisol concentration in a competition environment (Paper I). However, in Paper II there was an indication of a positive relationship between competition performance and baseline cortisol concentration. This is in line with the results from Lansade et al. (2016) where more fearful horses jumped higher and knock down fewer bars than less fearful horses. It is possible that the selection for more extravagant gaits and increased sensitivity to the rider in dressage horses (Von Borstel et al., 2010) and for "carefulness" in show jumpers might also have indirectly selected for temperament traits like sensitivity to the environment shown as an increased cortisol concentration. However, this has an impact on the horse-rider match and also in regards to the health of the animal. A study by Malmkvist et al. (2012) found a relationship between grade of gastric ulceration and increased cortisol response to a novel object in the box. In this study, horses were divided into groups based on the occurrence of ulcers and subsequently exposed to the novel object. It is possible that the horses in the group with ulcers would have been identified as more fearful individuals if tested. Therefore, the ability to identify horses that are fearful and reactive to stressful situations could provide valuable information on which horses could benefit from specific training and habituation to situations like competition and new environments thus increasing welfare of the horse as well as performance at competitions. It should also be emphasized that the preference for more or less fearful horses and the successful outcome at competition is dependent on the experience of the rider.

8.5 Factors influencing steroid hormone concentration

The third important result in this thesis is that the young horses can maintain the diurnal rhythm of cortisol in a stressful competition environment. In Paper II it was hypothesised that the stressful environment during competition, with approximately 350 horses stabled under the same roof, with 24 hours of activity and light in the stable, and frequent vocalization from other horses would disturb the diurnal rhythm of cortisol. The hypothesis was not confirmed since a diurnal rhythm of cortisol was measured both in the home environment and in two different competition environments (Paper II). However, the baseline cortisol concentration at the events increased compared to the home environment for the entire duration of the events in agreement with Erber et al. (2013) who found an elevation in baseline cortisol concentration after a change from group to individual housing in 3-year-old mares in training. The maintained diurnal rhythm was unexpected, as studies have shown that stressful situations such as a change of housing conditions can interfere with the diurnal rhythm (Harewood and McGowan, 2005; Irvine and Alexander, 1994). It is possible that the horses have been habituated to competition situations by previous experience like horses habituated to repetitive transportation and therefore maintained the diurnal rhythm. This could be the case for the 5-6 year old horses but it is not likely in the 3-4 year old horses. The increased baseline from home to event can i.e. reflect a stress response to the new environment. Increased cortisol concentration was also found when stabling young stallions with mixed breeding experience within a short distance in other studies (Aurich et al., 2015; Villani et al., 2006). It is also possible that the increase in baseline cortisol concentration reflects anticipation of “competition” as has been found in a study by Cayado et al. (2006) where baseline cortisol concentration also was higher at competition in an unfamiliar environment and in a study by Becker-Birck et al. (2013) where cortisol response to competition in a familiar environment was investigated. An anticipatory cortisol increase has also been reported in horses before transportation (Schmidt et al., 2010b,c). Most likely, multiple factors are contributing to the difference in cortisol concentration between the home environment and a competition environment.

Another factor that might affect cortisol concentrations and need mentioning, is feeding. It has been found that horses fed above maintenance requirements for energy and protein had higher plasma cortisol concentrations than horses fed below the requirements (Glade et al., 1984; Sticker et al., 1995). Jensen et al. (2017) found in a study with a standardized feeding and exercise program that horses fed fiber-based diets had lower cortisol concentrations that horses fed starch-based diets, however, exercise had a larger impact on the cortisol response than diet composition. It was not possible to control diet composition, feed intake or feeding times of the horses used in this thesis, and it should just be kept in mind that other factors like feeding might also have influence on the results.

The diurnal rhythm of testosterone described by Sharma et al. (1976) and Kirkpatrick et al. (1976) in stallions could not be detected in Paper III. Neither could a diurnal rhythm in mares or geldings, however, the fact that there are no previous reports on the diurnal variation of testosterone in mares and geldings makes discussion rather difficult. The lack of a diurnal variation in stallions was also found in a study by Bono et al. (1982). The blood samples used for testosterone analysis in Paper III were collected in the home environment during the non-breeding season, and stressful conditions that could have influenced the testosterone concentrations were avoided. In future studies it could be relevant to include more samples during 24 hours to test the diurnal variation. It is possible that the time of year could influence the diurnal variation in testosterone concentration since steroid hormones can undergo seasonal variation and can be influenced by the reproductive cycle. Knowledge about the rhythmicity, seasonality and association between the reproductive cycle and hormone concentration is important when comparing concentrations and changes from interventions over days or longer periods. In Paper I and II, the competitions were selected by the riders or horse owners, and we were therefore not able to control the seasonal variability. In Paper II, cortisol was sampled in October and March during the first year and in March the following year. According to Aurich et al. (2015), who studied diurnal and seasonal rhythm of cortisol and the seasonal rhythm of testosterone from December to May, cortisol concentration was highest in December and lowest in March for 3-year-old mares, and in May for young stallions and geldings. Due to the seasonal variation between the events described in Paper II, cortisol data were not pooled across events.

In Paper I and III, testosterone samples were taken in January and February to represent the non-breeding season in Denmark. Testosterone concentration in stallions is lowest from January to March (Aurich et al., 2015) and this increases over the reproductive season (Johnson et al., 1983) to a greater extent in breeding stallions than other stallions (Aurich et al., 2015). In Paper I and II, the effect of using breeding stallions was ignored since the competitions were held during the non-breeding season, where breeding stallions had been excluded from breeding for at least 2 months.

In mares, both testosterone (Ginther et al., 2007) and estradiol (Wu et al., 1976) are influenced by pregnancy and the ovarian cycle, but Aurich et al. (2015) did not find any variation in cortisol concentration associated with the reproductive cycle. The mares in Paper I and II were classified as non-pregnant by the owners, but the stage of the individual mare's reproductive cycle was not confirmed. In Paper III, the mares were in anoestrus or transitional, as confirmed by ultrasonography. Neither the seasonality nor the reproductive stage is likely to have had an influence on the cortisol or testosterone results in this study. However, there are disagreements in the literature describing the relationship between the ovarian activity and testosterone concentration (Silberzahn et al., 1978; Ginther et al., 2007; Meinecke et al., 1987) and therefore the effect of season on diurnal variation cannot be neglected. Estradiol is associated with the ovarian cycle in mares, and it would have added valuable information to the study if the reproductive stage of the individual mares were known in Paper I. However, since increased estradiol concentrations to some extend was associated with decreased fear responses, we can hypothesize that the reproductive stage of a mare could influence the outcome of a fear test.

8.5.1 Sex

In accordance with a recent study on Warmblood horses by Aurich et al. (2015), cortisol concentration was not influenced by sex (Paper II). However, a variation in cortisol concentration between male and females would not have been surprising, since reproductive status can affect cortisol levels. In stallions, exposure to vocalization from other stallions can increase cortisol concentrations (Villani et al., 2006) and Aurich et al. (2015) found an increased basal cortisol concentration in active breeding stallions to be caused by the housing of sexually active stallions in adjacent boxes in the stable, which represents a stress-inducing challenge. During the events in Paper I and II, the stallions were stabled so they could hear, smell, see and occasionally have contact with unfamiliar stallions, but we still did not detect a difference between the sexes. As expected, testosterone concentrations were higher in stallions than in mares and geldings, with no difference between mares and geldings (Study 1 and Study 3).

8.5.2 Age

Neither testosterone or cortisol concentration were influenced by age in this thesis. With regards to cortisol this is in agreement with other studies (Aurich et al., 2015; Janczarek et al., 2013; Kang and Lee, 2016) but testosterone have been found to increase with age (Johnson et al., 1983; McDonnell and Murray, 1995; Khalil et al., 2009; Aurich et al., 2015). However, the age range from 3- to 6-year old horses in this study might be too narrow to show age variation in particular when samples were taken in the non-reproductive season. Studies have found that previous competition experience influences cortisol concentration in both dressage horses and showjumpers (Cayado et al., 2006) and in marathon driving horses and eventers (Mircean et al., 2007). Previous experience of training and transportation also leads to a decreased cortisol response when training and transport are repeated (Schmidt et al., 2010a,b,c). Previous training experience rather than age was also found to cause attenuated reactions in fear tests (Von Borstel et al., 2010), as well as a lower HR when exposed to a novel object (Visser et al., 2002). Based on these studies it is likely that previous experience will influence cortisol concentration more than the age of the horse. Unfortunately, the level of competition experience and the handling prior to testing (Paper I and II) were not controlled. Due to the population of 3- to 6-year old horses in this study, competition experience would not be very high. However, there will be differences between the number of competition experiences for the 3-year-old horses compared to the 4- to 6-year old horses. The 4- to 6-year-old horses had all qualified for a top-level competition within their age group. The qualifying competitions were between 2 and 6 months prior to events DYC and YHC. It can therefore be assumed that the horses had experienced a high level of training for at least 6 months before the final events. The 3-year-old horses were mostly inexperienced, with the inclusion criteria that they had been stabled and trained for at least 1 month at the place where the handling tests were performed. The history of the horses prior to this 1 month of training was unknown. Some horses might have been handled on a daily basis for 3 years, whereas others might not have experienced any handling until arrival 1 month prior to the tests. Throughout the studies in this thesis, there was no association between age group and steroid hormone level or responses in handling tests. However, knowledge about previous training and competition experience would have added useful information to Paper I and II.

8.5.3 Discipline

Differences in cortisol concentration and fearfulness between the two disciplines were found (Paper I and Paper II). Dressage horses tended to have a higher average HR during the handling tests than show jumpers (Paper I) and dressage horses had a higher baseline cortisol concentration at competition than show jumpers (Paper II), therefore the assumption of dressage horses being more fearful than show jumpers, was made. This was also confirmed in studies on other Warmblood sport horses (von Borstel et al., 2010; Hausberger et al., 2011; Cayado et al. 2006). Von Borstel et al. (2010) found that both trained and untrained show jumpers reacted less fearful than other horses in a moving object test, and the authors suggested that it could have been caused by genetics. This could also explain that baseline cortisol concentration at event were lower in show jumpers than in dressage horses (Paper II) and that dressage horses experienced a higher heart rate than show jumpers during handling tests (Paper I). Though, the difference between discipline could also be due to the large variation in management between disciplines, where show jumpers traditionally participate in more competitions than dressage horses and are more exposed to environmental changes, such as different jumps during training. However, in a study by Cayado et al. (2006) the cortisol response observed during both transportation to the competition arena and preparation for competition was higher in dressage horses than in show jumpers, but this decreased with experience in both disciplines, indicating that there could be a true difference in fearfulness between disciplines. As expected there was no difference in testosterone concentration between discipline. The increase in testosterone concentration observed in elite athletes is associated with strength training and high intensity exercise (Kraemer et al., 1992; Cadore et al., 2010; Cook et al., 2012). Even though show jumping requires strength and explosiveness the physical demands during training and competition of the young show jumpers in this study would not be strenuous enough to induce a testosterone response.

8.5.4 Exercise

Desmecht et al. (1995) measured post-exercise plasma cortisol and lactate in show jumpers, eventers, Thoroughbreds, Standardbreds and endurance horses, and found that the degree of exercise-induced cortisol concentration was related to both the intensity and duration of exercise for all five sporting events. As described in Paper II, salivary samples were collected prior to exercise and 10 minutes post-exercise, and the difference between cortisol concentration before exercise and after exercise (EXDIF) was calculated. Dressage horses had a higher EXDIF than show jumpers for the 5-year-old horses in SH13 and the 3-year-old stallions in SH14 (Paper II), and there was a tendency for EXDIF to be higher in dressage horses than in show jumpers in YHC. Cayado et al. (2006) also found dressage horses to respond more to exercise than show jumpers, but this is in contrast to the general perception that cortisol concentration increases with increased intensity (Church et al., 1987; Jimenez et al., 1998) and power demand, such as the increasing height of the fences in show jumping (Ferlazzo et al., 2012). When interpreting these results caution should be taken due to the lack of proper measures for exercise intensity. To be able to control and adjust for exercise intensity measures of lactate production and HR parameters can be used and this provides valuable information as to whether the cortisol response to exercise is caused by exercise intensity or environmental factors impacting the cortisol response to exercise (Visser et al., 2002; Schmidt et al., 2010a,b,c,d; Becker-Birk et al., 2013). The horses in Study 2 had an increase in cortisol concentrations in response to exercise both at home and at competition, but the EXDIF was higher at competition. In the home environment, the horses completed an acute bout of exercise that was supposed to be of same duration and intensity as during competition, though there were no physical measures (such as lactate concentration or HR monitoring) to control the exact intensity. Becker-Birck et al. (2013) found that dressage horses and show jumpers competing in their home environment had a higher cortisol concentration during competition days than during training days when completing the same course, and Covalasky et al. (1992) found that the response to exercise was significantly higher after exercise at competition compared to at home. It is possible that the increased response found at competition in the horses in study 2 was induced by the competition situation, as described in the previous studies (Covalesky et al., 1992; Cayado et al., 2006), but could also be due to an increased physiological demand. However, there was no distinction made between the physiological and psychological effects in either these studies or in Paper II.

Another parameter that can influence cortisol response to exercise is the actual physical training level of the horse. In highly trained human athletes, the response to acute exercise reflects the actual training level of the athlete and the stress of competition (Healy et al., 2014). There are very few studies in sport horses investigating the baseline cortisol response to chronic training or the response to an acute bout of exercise after a period of chronic training. In one study, a high level of fitness caused a decrease in cortisol concentration during periods of acute exercise, and post-exercise levels of cortisol were reported to be higher in less fit young Warmblood horses when exercising on a treadmill than in fit horses (Marc et al., 2000). The physical training status of the horses in paper II was not confirmed, but it is likely that the horses aged 5 and 6 years experienced more frequent training sessions of longer duration than those aged 3 and 4 years and they had also been doing controlled physical exercise for a much longer period of time that 3 -year old horses. However, the largest variation in training intensity and duration is thought to be between disciplines, despite a large variation being found between riders (Egenvall et al., 2013; Lönnell et al., 2014). Dressage horses are trained at a lower intensity and a longer duration than show jumpers (Murray et al., 2010), and professional riders have been shown to train show jumpers at a higher intensity and shorter duration than amateur riders (Munk et al., 2013; Sommer et al., 2015).

Horses with a high level of physical training will often have experienced more training sessions and possibly also have participated in more competitions. Therefore, it can be difficult to identify whether an attenuated cortisol response to exercise in more highly trained horses is due to an increased physiological training state, or if it is caused by a decreased cortisol response in a horse more habituated to environmental changes. Mircean et al. (2007) divided a group of eventers and a group of marathon driving horses into groups of ‘fit’ and ‘less fit’ horses, based on standardized exercise tests. They found that the group of fit horses had lower cortisol responses to an acute bout of exercise, but they also had more competition experience, so it was not possible to determine whether the cortisol response was caused by fitness level or competition experience.

In conclusion, the cortisol response observed in the horses in Study 2 is comparable to the response seen in other studies on dressage horses and show jumpers, but the reason why there are differences between disciplines is difficult to explain. It can be caused by a combination of an unexpected increased physical demand in the dressage competitions compared to show jumping or an increase in sympatho-adrenal medullary activity caused by environmental factors.

8.5.5 Sampling time

It is also possible that the timing of cortisol sampling post-exercise could cause a variation in results between studies. With blood sampling, maximum cortisol concentrations are usually recorded 20 to 30 min after the end of exercise (Foreman and Ferlazzo, 1996; Marc et al., 2000; Hamlin et al., 2002). It is important to determine whether a reported cortisol concentration is a true maximum cortisol concentration (Cmax) identified after repeatable measures. After a cross-country phase and after marathon driving, Cmax was reported 15 minutes post-exercise in eventers (Mircean et al., 2007), and dressage horses had Cmax 0-5 minutes post-exercise (Christensen et al., 2014). In general, there is considerable variability between studies with regard to the time of post-exercise sampling, and this can affect results like in the study by Peeters et al. (2013), where the peak cortisol concentration was actually the first post-exercise sample obtained 20 minutes post-exercise and therefore not a true peak concentration.

In Paper II, the samples were obtained 10 minutes post-exercise. Post-exercise sampling time was based on practical considerations, as the horses were back in the stable area and away from the public after 10 minutes, and they were not allowed into the box to feed or drink, which could potentially interfere with the salivary cortisol concentration. The pre-exercise samples were obtained approximately 1 hour prior to competition, when the horses were resting in the stable. At that point, most horses were standing quietly without interference from the groom. However, it is possible that the horses were anticipating something happening, since the grooms might have prepared the horse beforehand. It has been shown that the anticipation of exercise induces an increase in cortisol in some horses (Alexander and Irvine, 1991).

In conclusion, the cortisol response to exercise observed at the events (Paper II) could be caused by either physical exercise or the influence of environmental stress, and could be affected by both the training state and competition experience of the individual horses. Heart rate and lactate measurements could have provided additional information on the interaction of physiological and psychological aspects on cortisol response, and this could potentially have influenced the relationship between EXDIF and competition score. The true cortisol response might not be reflected in the EXDIF since Cmax was not determined through frequent measurements.

8.6 Salivary cortisol and testosterone

The fourth important result in this thesis was that salivary testosterone concentration has a rather low correlation with serum testosterone concentration in horses and should therefore be further investigated before used in performance assessment as well as in scientific studies. The decision to measure cortisol and testosterone concentrations using saliva samples in this thesis was based on different factors. Firstly, side effects of sample collection should be avoided in studies that investigate the effect of stressful events. In addition, non-invasive samples that are easy to obtain with no or only minor resistance from the horse and owner are necessary in field studies that focus on competitions and involve horse owners. A literature search on the relationship between performance, training, behavior and concentrations of cortisol and testosterone in humans revealed a relatively large number of studies on salivary cortisol and testosterone. Despite the conflicting evidence on the relationship between testosterone in serum and saliva in human studies, salivary sampling is still extensively used in human research (Arregger et al., 2007; Budde et al., 2010; Sedghroohi et al., 2011; Nunes et al., 2011; Crewther et al., 2013). Despite indications from human literature and from Unit of Physiology, Patophysiology and Experimental Endocrinology, University of Veterinary Medicine, Vienna that the sampling method used would give valuable results, an analysis of over 750 samples (Study 1 and Study 2, data not shown) did not reveal significantly higher levels of testosterone in stallions than in mares. A subsequent validation study confirmed a very poor correlation between testosterone in serum and saliva, as discussed in Paper III. The use in human research, however, is also based on studies with a large variation in the correlation coefficients from to r= 0.43 (Fiers et al., 2014) to r= 0.92 (Lane and Hackney, 2015). The difference between sampling methods, sex and methods of analysis has been discussed in human literature and should also be considered in further studies in horses. Especially the components in and properties of equine saliva should be investigated. Saliva sampling for cortisol measurements has been extensively used in horses and will not be discussed further.

9. Conclusions

This thesis provided new knowledge regarding the steroid hormones cortisol, testosterone and estradiol in young warmblood horses and their relation to competition performance and fear reactions in handling tests. The main findings in this thesis are:

- Testosterone concentration was unrelated to competition score.
- There was no relationship between cortisol response to exercise and competition score, and no consistent relationship between baseline salivary cortisol concentrations and competition scores across events.
- Stallions did not have a lower fear response than mares and geldings
- An increased testosterone concentration in mares and geldings was associated with an attenuated fear reaction and a lower peak heart rate in a handling test, and mares with a higher estradiol level showed attenuated fear responses compared to mares with lower levels.
- In stallions, an increased testosterone concentration was associated with a decrease in latency to resume feeding after each exposure.
- A high competition score was related to a shorter latency to touch the Bridge for 3 years old dressage mares.
- An increased baseline cortisol concentration at competition was associated with increased heart rate and more pronounced fear reactions in handling tests in show jumpers but not in dressage horses.
- Young dressage horses and showjumpers maintained the diurnal rhythm in cortisol in a competition environment. However, baseline cortisol concentration was increased during competition days compared to the home environment.
- Dressage horses had higher baseline cortisol concentrations at events than show jumpers, suggesting that they may perceive the novel environment as more stressful. This was supported by a tendency to an increased heart rate in dressage horses compared to show jumpers when exposed to a sudden movement of a novel object.
- Stallions had higher serum testosterone than mares and geldings, but no diurnal rhythm could be measured in horses.
- Salivary testosterone did not correlate with serum testosterone, and it cannot be recommended to use saliva as a proxy for serum when measuring testosterone levels.

Furthermore, an important outcome of this PhD study is that the industrial PhD candidate has gained valuable knowledge that support Højgård Equine Hospitals business activities in respect to providing the newest evidence-based guidance on how to manage sports horses. Stutteri Ask and Blue Hors need this guidance to be able to successfully produce, educate, compete and sell horses for international show jumping and grand prix dressage.

10. Perspectives

There are about 200.000 horses in Denmark and 75.000 people organized in the Danish Riding Association (Dansk Ride Forbund). The main goals for most of these horses and riders today are to reach the optimum sports performance, and this must occur through understanding of behavior and training physiology of the horse. Steroid hormones play an important role in both training physiology and behavior of the individual, and as we have experienced in this thesis there are links between serum levels of testosterone, cortisol and estrogen in horses, fear reactions in handling tests and the performance at competitions.

Further studies are needed to analyze the details of these links. From the human literature, we know that individual temperament traits have an impact on competition performance. This is also indicated in horses and therefore further studies are needed to identify temperamental traits that influences sport horse performance. We also know that combined measures of sports performance, behaviour and steroid hormone concentrations are valuable in monitoring training and also in determining overtraining. An interesting parameter to measure in the future is the relationship between serum testosterone and cortisol (the T/C ratio) as it has been done in human athletes. Cumulative fatigue can cause variation in the concentration of testosterone and cortisol, and a decrease in the T/C ratio is regarded an indication of fatigue. A decrease in the T/C ratio can originate from an increase in cortisol and/or decrease in testosterone. Information on the T/C ratio may allow athletes to alter their sleeping, dietary and training habits in order to be best prepared for training and competitions. It was originally one of the aims for this thesis to analyze the T/C ratio; but due to methodological problems with the measurements of salivary testosterone this was not possible.

The difference in steroid hormones between and within the sexes and their relation to performance in competition and daily training also needs further consideration. It is well known that male human athletes have superior strength, motivation and competitive ability compared to female human athletes, and that these differences are ascribed to the difference in expression of steroid hormones and hence the difference in the development of sexes. In equestrian sports, mares, geldings and stallions compete on equal terms disregarding any differences there may be between performance and behavior of the three sexes. The results of this study suggest that testosterone does influence the behavior and temperament of mares and geldings, as increased serum testosterone level leads to a lower fear response in a challenge test. Castrating stallions obviously has a major impact on testosterone levels, but how the steroid hormone levels influence trainability and performance is not known in sports horses of either sex. Future research should be performed in order to address the different physiologic training needs for mares, geldings and stallions, and the effect of castration on physical performance and trainability would be another interesting topic to explore.

The reproductive stage of the mare and the relationship between steroid hormones and both behavior and performance has received little attention in horses. The natural fluctuations in steroid hormones in the mare may affect the results in both competition and in handling tests. In humans, it is well known that hormone fluctuations affect both behaviour and performance. Reflecting some of the differences in behavior that may exist during the reproductive cycle, we did find that high testosterone or high estrogen levels in mares were correlated with a decreased fear response in the fear challenge tests. In the recent years, various methods of suppressing natural cycling in mares during the time they are ridden and shown in competitions are used, because excessive sexual expression or altered behavior in general is disturbing for the training and performance of the mare. Various methods (hormone vaccines, hormone suppressing implants and progesterone agonist therapy) have been well studied in regards to safety, future reproductive capability for the mare and expression of altered (sexual) behavior during the treatment. However, no studies have investigated the effect of suppressing natural cycling on training physiology and consequences for the physical performance during or after the treatment. Since improved physical performance as a result of any of these treatments could be regarded as a performance-enhancing treatment (doping), future studies within this field are very important.

Finally, further research in steroid hormone concentrations in relation to behavior and fitness testing of horses over time would be of interest for both researchers, veterinarians and horse owners. This thesis took the initial steps and opened for new research questions on the differences between behavior and performance in young performance horses of different sexes. It would be of great interest to follow up our results in more standardized set-ups on adult horses or follow a group of maturing horses in training over time, to compare the developments in hormone levels in relation to development of fitness and behavior and future success in performance.

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