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## REVERSIBLE DOWNREGULATION OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN THE STALLION WITH A THIRD-GENERATION GNRH ANTAGONIST

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REVERSIBLE DOWNREGULATION OF HYPOTHALAMIC-PITUITARY-  
GONADAL AXIS IN THE STALLION WITH A THIRD-GENERATION  
GNRH ANTAGONIST

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THESIS

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A thesis submitted in partial fulfillment of the requirements for  
the degree of Master in Science in the College of Agriculture,  
Food and Environment at the University of Kentucky

By

Gabriel Monteiro Davolli

Lexington, Kentucky

Director of thesis: Dr. Edward L. Squires

Lexington, Kentucky

2015

## ABSTRACT OF THESIS

### REVERSIBLE DOWNREGULATION OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS IN THE STALLION WITH A THIRD-GENERATION GnRH ANTAGONIST

The objectives of this thesis were: (1) to evaluate the downregulation of the stallion hypothalamic-pituitary-gonadal (HPG) axis by a GnRH antagonist (acyline) based upon endocrine, seminal, testicular and behavioral effects, and (2) to assess recovery after treatment. Stallions were treated for 50 days (n=4; 330 $\mu$ g/kg acyline q 5d) and controls (n=4) received vehicle alone. Stallions were assessed pre-treatment and for 72 days after last treatment. Treatment induced declines (p<0.05) in FSH, LH, testosterone (to castrate levels) and estrone sulfate. Gonadotropins and testosterone returned to control values within nine days and estrone sulfate by 14 days after treatment discontinuation. Acyline-treated stallions failed to respond with FSH, LH and testosterone increase after exogenous GnRH stimulation (25 $\mu$ g gonadorelin, IV) compared to pre-treatment and control stimulation. Total sperm numbers and motility were reduced in acyline-treated stallions, as well as total seminal plasma protein and testicular volume (p<0.05). Time to ejaculation was increased in acyline group (p<0.5). Testicular, sexual behavior and most seminal parameters regained normal levels within 72 days after treatment ceased. Sperm output of acyline-treated stallions was regained within seven months after ending treatment. Acyline reversibly suppressed the stallion HPG axis, thus has potential for treating the androgen-dependent Equine-Arteritis-Virus carrier state and as behavior modulator.

**KEYWORDS:** Hypothalamic-pituitary-gonadal axis, GnRH antagonist, Stallion, Equine Arteritis Virus (EAV), Sexual Behavior.

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## Chapter One: Introduction

Elimination of EAV (equine arteritis virus) carrier state and alteration of sexual/aggressive behavior are often cited as reasons to suppress gonadal activity in stallions. Various outbreaks of equine viral arteritis (EVA) in the past decades have highlighted the important role of persistently infected stallions in disseminating the disease through infective semen (Timoney et al., 1986; Timoney et al., 1987; Timoney et al., 2006; Barrandeguy, 2010; Mischczak et al., 2012). As many as 30 to 60% of stallions develop a persistent carrier state after recovery from the acute phase of the infection (Neu et al., 1987; Timoney and McCollum, 1993). Establishment and maintenance of the viral shedding state has been shown to be androgen dependent (Little *et al.*, 1991; Holyoak *et al.*, 1993; McCollum et al., 1994), and removal of endogenous testosterone results in termination of persistent carrier state (Little et al., 1991). Castration has historically been the method of choice to manage stallions that present excessive display of sexual or aggressive behavior; even before such behavior was linked to estrogens and androgens (Thompson Jr. et al., 1980; McDonnell, 1986; McDonnell et al., 1989). Orchiectomy seems to resolve most of the cases where sexual behavior may compromise training or performance of stallions (Line et al., 1985). However, the obvious irreversibility of surgical castration precludes the use of stallions for breeding after retirement from a successful athlete career.

As an alternative to castration, different approaches for down-regulation of the hypothalamic-pituitary-gonadal (HPG) axis in the stallion have involved the use of the progestogen, altrenogest (Brady et al., 1997; Squires et al., 1997; Johnson et al., 1998; Goolsby et al., 2004) or immunization against GnRH (Dowsett et al., 1996; Malmgren et al., 2001; Turkstra et al., 2005; Janett et al., 2009). There is some uncertainty about the reversibility of reproductive function after discontinuation of these methods of treatment.

More recently, attempts to suppress the HPG axis of stallions with GnRH antagonists yielded a decline in FSH, LH and estradiol and seemed to decrease libido after a single injection of Antarelix (Hinojosa et al., 2001). Furthermore, Antarelix and a second GnRH antagonist, Cetrorelix, given daily for 35-38 days, achieved a reversible decrease in testosterone, which was suggested to have had a causal relationship with EAV clearance in shedder stallions (Fortier et al., 2002).

A potent, third-generation GnRH antagonist, acyline, was shown to decrease FSH and LH for at least 15 days, and to keep testosterone at castrate levels for 2 weeks after a single injection in men (Herbst et al., 2004). In male dogs, one dose of acyline caused FSH, LH and testosterone to decrease for at least nine days (Garcia Romero et al., 2009). Acyline treatment in dogs reversibly affected libido and erectile function (Valiente et al., 2007) and suppressed response of the HPG axis to exogenous GnRH stimulation (Garcia Romero et al., 2012b). This GnRH antagonist has been used in mares to suppress FSH and retard follicular growth (Checura et al., 2009; Checura et al., 2010); however, there are no reports of its use in stallions.

To address the hypothesis that acyline treatment in stallions would reversibly down-regulate the HPG axis, this study aimed to (1) determine the effects of acyline treatment on endocrine, seminal, and behavioral parameters and testicular volume of stallions; (2) determine responsiveness of acyline-treated stallions to exogenous GnRH stimulation; and (3) to examine the degree of recovery of these parameters after withdrawal of acyline treatment.

## Chapter Two: Literature Review

### 2. Manipulation the Hypothalamic-pituitary-gonadal (HPG) axis

#### 2.1. Justifications for interference of reproductive endocrine function in stallions

There are a number of different reasons why it would be of interest to down-regulate the hypothalamic-pituitary-gonadal (HPG) axis in the stallion, including contraceptive measures. However, for this review we will focus on the control of **equine arteritis virus** and **behavior modulation** as reasons for suppression of the HPG of stallions.

##### 2.1.1 Equine Viral Arteritis

###### *A. History, epidemiology and economic impact*

The first equine arteritis virus (EAV) isolation (Doll et al., 1957b) was associated with an outbreak of respiratory disease and abortion in a Standardbred breeding farm in Ohio (Doll et al., 1957a). However, not many investigators considered equine viral arteritis (EVA) a disease of veterinary or economic significance until another outbreak occurred in 1984. It was the first EAV outbreak recorded in the Thoroughbred population in the U.S.A and generated widespread interest and major concern to the equine industry (Timoney and McCollum, 1993).

The importance of the disease was emphasized by a number of significant outbreaks of EVA that occurred in the last decade in North and South America as well as Europe. A 2006 outbreak of EVA in the USA affected horses from different breeds – though initially American Quarter Horses – and spread across nine states (Timoney et al., 2006). The outbreak that occurred in Normandy, France, that affected only draft and saddle horses (Pronost et al., 2010; Mischczak et al., 2012); and the large 2010 outbreak in Argentina which affected mainly sport-horses, involved abortion rates up to 50% and resulted in 38

stallions becoming seropositive for EAV (Barrandeguy, 2010). All of these outbreaks could be traced back to EAV carrier stallions.

Equine Arteritis Virus infection occurs in horses on every continent. Seroprevalence rates vary considerably, not only among countries, but among horse populations and breeds. In the USA, the infection is believed to be endemic in Standardbreds, with an average prevalence of 80%, whereas in Thoroughbreds, the seroprevalence is 2-3% (McCollum and Bryans, 1973; Timoney and McCollum, 1993). A serologic survey of horses imported to California from several countries demonstrated that the prevalence of EAV infection was also high in European Warmbloods – around 18% (Hullinger et al., 2001).

Among the consequences associated with EAV infection, the most significant are the risk of abortions and the establishment of the carrier state in stallions. There are direct financial losses attributable to outbreaks of EAV-related abortion; rates can reach between 50 and 60% of pregnant mares at risk. In addition, carrier stallions and ,(Timoney and McCollum, 1993) infective semen (Barrandeguy, 2010; Timoney, 2011) cannot be exported unless to the USA. Other financial consequences include veterinary and laboratory expenses; disruption of races, shows and other competitions; and inconveniences that result from movement restrictions (Barrandeguy, 2010).

### ***B. Virus transmission***

The development of clinical signs following natural or experimental infection of EVA can vary considerably in severity among individual horses and outbreaks, though the majority of cases are asymptomatic. Regardless of the severity of clinical signs, all naturally infected adult horses recover spontaneously from infection (Timoney and

McCollum, 1993; Timoney, 2011). Abortion rates between 10 and 60% have been reported in the case of natural outbreaks of the disease and they are not necessarily preceded by clinical signs of EVA (Timoney and McCollum, 1993).

Transmission of EAV occurs mainly by the respiratory route from an acutely infected animal (Doll, Bryans, and Knappenberger, 1957; McCollum et al., 1971) or by the venereal route through the acutely or chronically infected stallion (Timoney, McCollum, Roberts, et al., 1986; Timoney et al., 1987). Transmission via the respiratory route requires direct contact between susceptible horses and acutely infected animals or their infective body secretions (Timoney and McCollum, 1993; Timoney, 2011).

During the acute phase of the infection, stallions may experience diminished libido, in addition to impairment of sperm production, decreased sperm concentration and motility, and an increased percentage of morphologically abnormal sperm that may persist for up to 6-7 weeks or longer. Such a period of temporary subfertility have been shown to be due to increased testicular temperature associated with scrotal and preputial edema rather than a direct effect of the virus on the testes (Neu et al., 1992; Campos et al., 2014).

Stallions acutely or persistently infected with EAV play a major role in the venereal transmission of EAV to mares during natural breeding and artificial insemination with fresh, cooled or frozen semen (Timoney et al., 1986; Timoney et al., 1987; Balasuriya et al., 1998). Approximately 85 to 100% of mares bred to long-term carrier stallions seroconvert to EAV within 28 days after breeding (Timoney and McCollum, 1993). Recent work has shown that embryo transfer from a donor mare inseminated with EAV infective semen to naive recipient mares could also represent a risk of EAV transmission, under experimental conditions (Broaddus et al., 2011).



### ***C. EAV carrier stallion and persistent infection mechanisms***

After resolution of acute phase of infection with EAV, intact males may become chronically infected with EAV and constantly shed the virus in the ejaculate, despite high antibody titers to the virus in the circulation. It has been shown that the carrier state may last for several weeks, months or years (Timoney et al., 1986; Timoney et al., 1987; Timoney and McCollum, 1993). Studies of horse populations that experienced outbreaks of EVA indicated that stallions shedding EAV in their semen serve as a reservoir for the virus within equine populations (Timoney et al., 1986; Timoney et al., 1987). Timoney and colleagues verified that 47% of the stallions naturally infected with EAV during the 1984 disease event became persistent carriers of the virus (Timoney et al., 1987). Population surveillance in the years following also indicated that the carrier rate was associated with breed, seemingly higher in Standardbreds than Thoroughbreds (Timoney and McCollum, 1993, 2000) and overall the frequency of the carrier state after infection with EAV was between 30 and 60%.

It has been demonstrated that the virus persists in the carrier stallion harbored in the accessory sex glands, and the ampulla of the vas deferens has the highest viral titers (Neu et al., 1987). The mechanism of EAV persistence, even though still not clear, was first shown by Little and collaborators to be testosterone dependent. In their study, long-term EAV carrier stallions ceased to shed the virus in the ejaculate between 4 and 24 days after surgical castration, while castrated carriers supplemented with exogenous testosterone maintained viral output in the semen (Little et al., 1991). Additional data underscores the direct or indirect role of testosterone in the carrier state: persistent infection was not established in colts exposed to the virus before the onset of puberty (Holyoak et al., 1993), or in castrated males experimentally infected with the KY-84 strain of the virus (McCollum

et al., 1994). The event which induces spontaneous clearance of the shedding state in the persistently infected stallion is still unclear, but there is no indication that a stallion could revert back to shedding or become an intermittent shedder once it has stopped shedding the virus in its semen (Timoney and McCollum, 2000).

One possible mechanism for long-term persistence of EAV infection in the reproductive tract of mature stallions could be a presumed suppressive activity of testosterone. Based on *in vitro* studies, testosterone is thought to reduce B-cell lymphopoiesis, diminish inflammatory cytokine production and inhibit the phagocytic capacity of macrophages (Muehlenbein and Bribiescas, 2005). It could also be that, as accessory sex glands depend on testosterone for their normal function (Thompson Jr. et al., 1979), the hormone provides the trophic conditions for the virus population to thrive. It is also assumed that there is a continuous re-infection of susceptible tissues by the virus in the reproductive tract (Balasuriya et al., 1999).

Recently Go and collaborators demonstrated that a common haplotype of CD3<sup>+</sup> T cells is associated with resistance/susceptibility *in-vitro* to EAV (Go et al., 2011) and that such *in-vitro* susceptibility marker corresponded to the pattern of immune response *in-vivo* in infected horses. Populations of horses could also be divided into susceptible and resistant (Go et al., 2012b). Finally, the same author showed that *in-vitro* susceptibility of CD3<sup>+</sup> T lymphocytes indicates a predisposition of naturally infected stallions to become persistently infected with EAV (Go et al., 2012a).

A method to cope with EAV carrier state in order to utilize sperm of stallions still shedding the virus was studied by Morrell and coworkers. Virus particles were mechanically separated from sperm cells in extended semen through the use of

centrifugation - once or twice - over a single colloid (Androcoll-E) layer. After centrifugation, the infective virus numbers were reduced drastically, with no adverse effect on sperm motility. Nonetheless, there was still viral load present in the supernatant, which precludes the potential use of the procedure to process infected semen (Morrell et al., 2013).

### **2.1.2. Aggressive/sexual behavior modification**

#### ***A. Source of aggressiveness: From natural to artificially induced***

Environmental and dominance status as well as sex and age affect the type of aggressive behavior displayed by male horses. Stallions can be threatening to subordinate horses in conflict situations during sexual interactions. Interactions between stallions often involve ritualized displays and are of normal occurrence between disputing mature, free-ranging stallions. This is thought to be a way to keep spacing and maintain social structure. It seems that interactions between two harem stallions tend to be less intense than between a harem and a bachelor stallion (Waring, 1983).

Violent, non-ritualized aggression occasionally occurs between stallions. That happens with sudden charge of stallions toward one another, followed by biting, foreleg striking and hind leg kicking. Bloody wounds and broken bones can occur. Soon one of the individuals withdraws or is chased away. As expected, most aggressive interactions observed are from disputes over the harem male position in a herd (Waring, 1983; Beaver, 1986; Keiper, 1986).

Problems associated with dominance can occur in a number of situations in which horses interact with other horses or humans. When horses are exposed to new social groups, dominance hierarchies form as they would among band members in the wild. New horses introduced to a group must find their place within the social order, and during that short

time, threats and fights will occur between individuals of a herd (Keiper, 1986). With the limited opportunities for interactions environmental setting, aggressive acts may happen, and often unexpectedly (Keiper, 1986)

In the specific case of colts and stallions, the natural occurrence of harem status influences reproductive endocrinology and function, including an increase in androgen levels and sexual/aggressive behavior. On the other hand, bachelor status imparts suppression the reproductive axis. Given the constant re-accommodation of sociosexual dynamics, as the dominance establishment mentioned above, it is no surprise that male horses, especially young ones, will often switch from one status (bachelor or harem) to the other (McDonnell, 1986; McDonnell and Murray, 1995). However, the transition encompasses displaying the behavior for a certain status, which can be in the form of rather unexpected behavior (McDonnell, 1986).

In-hand breeding procedures are also a source of contrast between the feral and domesticated environment. Also in this scenario, human interference can exacerbate the contrast experienced by the horse, characterized mainly by limitations of display of normal ethology that the horses would not find in the wild, or in a domestic context with little human intervention (McDonnell, 1986, 2000). Considering the marked difference in the opportunities for interaction before copulation compared to a free-range situation, it is quite remarkable that most domestic stallions can have what is considered normal domestic breeding career with such minimal precopulatory contact with mares. Some breeding farm managers recognize and accommodate these apparent needs, but some expect all stallions to do well with limited contact which may lead to various responses (McDonnell, 2000). Restraints applied to the mare often appear to provoke a change in response in mares that,

instead of being otherwise receptive, may show fear and resistance. While safety is an important concern, over restraint of the mare, modifying the interaction during copulation, can frustrate some stallions (McDonnell, 2000). As a result, abnormalities in the sexual behavior of stallions range from excessive biting and aggressiveness to the various forms of impotence and even fear of mares (Waring, 1983). Excessive aggression is an aberrant behavioral problem of some stallions. Frustration of high libido stallions caused by incomplete sexual interactions and repeated non-ejaculatory copulations appears involved in most cases (Waring, 1983). It has been reported (McDonnell, 1995) that continuous teasing exposure to mares consistently increased testosterone concentrations and higher levels of sexual/aggressive behavior.

***B. Concern with aggressive stallion behavior in the industry***

Humans may encounter stallion aggression in two main ways: people may be injured when aggressive encounters occur between horses or when a horse considers itself dominant to the person (Beaver, 1986). In more extreme situations, although biting, striking and kicking are normal elements of precopulatory sequence, savage aggressiveness is one of the most serious behavioral problems. Many stallions are consistently aggressive, but some breeding stallions are uncontrollably or unpredictably aggressive toward mares or handlers (McDonnell, 1986). This sexual and aggressive behavior of stallions often inhibits the performance potential of these individuals and may also be dangerous to other horses, handlers, riders or others (Johnson et al., 1998; Stout and Colenbrander, 2004). Colts or stallions in training or competition that exhibit these undesired traits are considered by trainers to have adversely affected performance (Stout and Colenbrander, 2004)

### ***C. Role of steroid hormones on sexual behavior***

Androgens are generally necessary for expression of stallion-like behavior. Even though administration of exogenous testosterone to intact normal stallions does not seem to improve sexual behavior it may lead to increased aggression (McDonnell, 1986). In addition, the very high concentration of estrogens in stallions, compared to males of other species (Raeside, 1969), mainly in the form of estrone (Seamans et al., 1991), led to experiments designed to determine the roles of testosterone and estradiol in the regulation of sexual behavior. Thompson and colleagues found that treatment of castrates with testosterone alone or combined with estradiol benzoate restored libido, and a similar effect was found with a high dose of estradiol benzoate. In the geldings supplemented with estradiol benzoate alone, libido appeared to be restored to a much greater extent than to the ability to ejaculate (Thompson Jr. et al., 1980). The authors suggested that peripheral or central aromatization of testosterone occurred. This observation agrees with the hypothesis that effects of testosterone in sexual behavior of males are mediated by aromatization to estradiol within the target neurons (Naftolin et al., 1971) and with the recent finding of aromatase expression in the brain of the stallion (Lemazurier et al., 2001). However, the possibility of a direct effect of testosterone on libido cannot be excluded.

McDonnell and co-workers found that GnRH induced precopulatory behavior (*viz.* attention, Flehmen response, erection duration) is testosterone dependent. The study illustrated that exogenous GnRH treatment influenced sexual behavior, though it required testosterone priming to occur (McDonnell et al., 1989).

Puberty and an abrupt rise of circulating testosterone in colts around 80 weeks of age (about 1.5 years), ranging from 56 to 97 weeks, (Naden et al., 1990) in light-breed, cross-bred colts. In many breeds, like Thoroughbreds and Quarter horses, the occurrence

of those changes then coincides with the age when young horses are at some point of training, or even about to start racing or competing in shows.

#### ***D. Castration as means of reducing aggressive behavior***

Male horses not intended for breeding are usually castrated during their second year of life or before, although some owners prefer delaying surgery until a later age, presumably to allow more substantial physical development and development of male secondary sexual characteristics (Trotter, 1989). Surgical castration is performed as an attempt to prevent development of objectionable sexual libido and aggression characteristic in stallions. A survey of horse owners evaluating castration as therapeutic measure to eliminate objectionable sexual/aggressive behavior toward people, found castration to be 60 to 70% effective, which means that about 30% of castrated stallions continued to display some kind of aggressive behavior. When castration was done prior to puberty (before two years of age) to preclude development of such behavior, no difference was found compared to castration performed after three years of age (Line et al., 1985). Despite the originality of the publication, it does not seem like “prepuberty” would not be the correct term, since most colts will have reached puberty months before 2 years of age (Naden et al., 1990).

Besides the likelihood of sexual display in castrated males, castration has some associated risks, including: hemorrhage, septic funiculitis, and eventration, among others (Getman, 2009; Schumacher, 2013). Complications associated with equine orchiectomy have been cited as the most common cause of malpractice claims against veterinarians in North America (Wilson and Quist, 1992).

According to The Jockey Club, approximately 25% of Thoroughbred racing horses are geldings, and many of these geldings were castrated because of behavioral problems

which interfered with training or racing. Although many of these geldings would not have become successful sires had they not been castrated, numerous other geldings that have had successful race careers (e.g. Kentucky Derby winners), could have been genetically important sires. Also in the Quarter horse industry (Goolsby et al., 2004) owners and trainers are frequently faced with a dilemma of whether to castrate when dealing with a colt that is not performing or training up to expectations. To have the ability to temporarily suppress sexual and aggressive behaviors during training might permit the time necessary to efficiently evaluate the performance and breeding potential of the animal before a decision is made on whether to castrate the animal (Johnson et al., 1998). Some pharmacological approaches to castration in male horses are used to some extent as will be discussed below.

## **2.2. Pharmacological approaches to down-regulate the Hypothalamic-pituitary-gonadal (HPG) axis**

### **2.2.1. Steroids: progestogens and androgens**

The rationale for the use of progestagens for blocking the hypothalamic-pituitary axis in the mare is based on these compounds having a negative feedback on GnRH release, resulting in a decrease in circulating LH and FSH, as endogenous progesterone would (Garcia and Ginther, 1978; Ginther et al., 2006). While different compounds can be used for this purpose, the oral progestin altrenogest is the most widely used progestagen in mares (Roberts and Beaver, 1987). Even though the administration of altrenogest to stallions is off-label, it is also used in the equine industry (Heninger et al., 2001) to modify sexual and aggressive behavior.

As discussed above, the aggressive behavior exhibited by some stallions during performance can harm their success in shows or at the race track. Potentially, progestins



could be used to decrease libido and aggressive behavior. However, there is question on how effective they are in modifying behavior and concern about long-term detrimental effects that might result from such treatment in stallions.

A study using the label dose of altrenogest for estrous suppression in mares (0.44 mg/kg daily) for a 30 day treatment in stallions showed no differences in semen quality or behavior for stallions greater than five years of age (Miller et al., 1997). Another study (Johnson et al., 1998) performed in young stallions (2-4 years old), using a high dose of altrenogest (0.88 mg/kg) for 57 days found that the progestin treatment decreased testicular size and function and compromised libido (Flehmen response, penis exposure and erection) without affecting body development. Testosterone and estradiol were reduced in this study during treatment but recovered after cessation of treatment. However, their data did not show reversibility of the detrimental effects on daily sperm output (DSO), scrotal size nor libido within an 8-week recovery period after the end of treatment (Brady et al., 1997)

One long-term study by Squires and colleagues investigated the effects of a 150 or 240 day altrenogest (0.88mg/kg SID) treatment on semen characteristics, testicular size, circulating LH and testosterone and libido of 3 to 18 year old stallions. Treatment decreased all parameters, including libido as measured: time for erection, time for ejaculation and frequency of failure to ejaculate. During the 90 day recovery period, adverse effects on scrotal width, DSO and motility were no longer seen. However, resumption to normality after the prolonged treatment did not occur for LH and testosterone concentrations, sperm morphology or libido parameters. The authors suggested that since the experiment ended in a time of seasonal recrudescence (March) a longer recovery period could have allowed for restoration of all of the parameters (Squires et al., 1997).

Heninger and co-workers (Heninger et al., 2001) undertook a 67-day treatment of 2-year-old colts using the mare dosage of altrenogest (0.44mg/kg SID). No differences were seen in libido, subjective parameters like sniffing/licking, biting/chewing, vocalization, aggressive behavior towards mare or handler were analyzed and found to be unaffected by administration of the progestagen. Neither were there differences in testosterone due to treatment, but estrogen concentrations, thought to govern libido in the stallion (Thompson Jr. et al., 1980) were decreased by treatment and recovered after cessation. Heninger and co-authors stated that dose of altrenogest is associated with the efficacy of behavioral modification in the stallion.

A survey (Goolsby et al., 2004) collected data on the use of altrenogest in in Quarter horse in training across the USA during 2002; out of 63 respondents, 29% reported the use of altrenogest in their stallions. Only 11% owners/trainers administered the progestagen to stallions being used for breeding, but 89% were treating stallions with the intention of using the stallion for breeding in the future. The main reason for altrenogest administration was to create a more focused animal in a performance setting, and also to reduce aggressiveness or create an easier stallion to handle. Even though altrenogest is widely used off-label, the available data does not warrant reversibility of the adverse effects of treatment.

Trainers and owners may also administer androgens to stallions for improvement of performance, growth acceleration (particularly muscle mass) and an increase in libido. Such treatments cause a negative feedback on the hypothalamus (Muyan et al., 1993), and as a consequence, prolonged treatment (88 days) with exogenous testosterone causes a severe suppression of the hypothalamic/pituitary axis and reproductive capacity (Berndtson et al., 1979). In this study, testicular weight was reduced to 59% of controls

and spermatogenesis was impaired by a 57% reduction of sperm production; sperm motility was also negatively affected. Interestingly, the authors did not observe any detrimental nor beneficial influence of testosterone supplementation on libido.

Data on half of stallions from the previous study, which were not castrated, were collected for 90 days after end of supplementation (Squires et al., 1981). Within that period, all of the adversely affected testicular parameters returned to values comparable to controls. Squires et al. concluded that the practice by the horse industry of administering androgens to stallions to promote libido is ineffective, at least for normal stallions. Concurring findings, though lacking the recovery period component, were published on anabolic-steroids by Squires et al. (Squires et al., 1982). Administration of either boldenone or nandrolone for 15 weeks treatment led to reduction in LH concentrations during treatment.

### **2.2.2. Immunization against GnRH**

After the first report on immunization against GnRH in a cryptorchid stallion (Schanbacher and Pratt, 1985), Rabb and colleagues conducted a study (Rabb et al., 1990) aimed at determining the effects of GnRH vaccination on relative changes in LH and FSH. A serial vaccination with carbodiimide-GnRH in bovine serum albumin (BSA) versus plain BSA, over 32 weeks, showed an anti-GnRH titer capable of reducing plasma concentrations of LH and FSH by 86 and 59%, respectively. Moreover, following a bolus injection of a GnRH analogue, immune-competent animals had a 90% reduction in LH response, while the FSH response to GnRH stimulation was unaffected. Even though the study was performed in geldings, their data provides validation on the mechanism that leads to decrease testosterone in the male horse through vaccination against GnRH.

Some pioneer studies on active immunization against GnRH specifically in the intact male horse were performed by Dowsett and co-workers in the early 1990's. A preliminary study (Dowsett et al., 1991) was performed in pre-pubertal colts to evaluate the effects of an ovalbumin GnRH conjugate in mineral oil given in two injections, 11 weeks apart. Effective antibody titer (1:1000, according to the authors) was obtained seven weeks after first injections, and the peak antibody response to immunization was reached 4-5 weeks after the booster injection. Anti-GnRH titers remained above 1:1000 in vaccinated colts for 28-32 weeks. During this high antibody period, immunization prevented a testosterone rise in vaccinated colts in a comparable manner to the pre-pubertal colts used as controls. A testosterone rise was observed in the immunized colts 28 weeks from the first injection, in synchrony with the decrease in antibody titer in these colts. The growth rates in the colts was not affected by immunization against GnRH; however, at 17 and 19 months, when the colts were castrated, testicular sizes in treated colts were smaller than controls, and morphometric evaluation showed lower Leydig cell volume. This indicated that reproductive function was not recovered after vaccination, at least with the time allowed for recovery. Lastly, there were severe reactions in the tissue injected with the GnRH vaccine.

Dowsett and colleagues (Dowsett et al., 1993) pursued different modes of vaccine delivery and the effect of GnRH immunization in pubertal rather than pre-pubertal colts. Their studies involved the application of an implant formulation of the vaccine, as well as a water-soluble formulation, which they tested in two-year-old colts, pre-pubertal colts and two-year-old fillies; for this trial, only the latter group had controls. The results are confusing, but a high anti-GnRH antibody titer was achieved with the different forms of

administration and, as expected, serum testosterone concentrations were inversely proportional to the rise and decline in antibodies. In pubertal colts, a decline in testicular volume during the period of high antibody titer was reversed after the titers decreased. Overall, there was confusion in the publication as to what constituted the recovery period. In addition, there were variations in length of response to vaccine, particularly in the water-soluble version of the vaccine, which unfortunately was the formulation resulting in least tissue reaction. Moreover, boosters were given as needed based on antibody titers, so treatment periods were not consistent, and neither were the endpoints. Therefore, this study did not answer the question of the time needed for recovery.

In order to determine the optimal frequency of vaccination, a broader comparison of different doses of the previous studied water-soluble, ovalbumin-linked GnRH vaccine was pursued (Dowsett et al., 1996) once more in two-year-old colts. Similar results were found regarding antibody titers, which peaked 2-4 weeks after booster injection (four weeks after first immunization). Serum testosterone concentrations were decreased to 90 pg/mL at six weeks after primary injection and remained suppressed (gelding levels, < 200pg/mL) until week 31 to 43. Serum androstenedione was also decreased and reached a nadir concentration of 80 pg/mL at four weeks after primary vaccination and rose above 248 pg/mL at 32 weeks after primary vaccination. During the testosterone suppression period, the authors failed to collect the subject stallions within a 15 min time-frame; only one attempt to collect semen was made while the immunity to GnRH lasted (more than 30 weeks). Whatever change in libido reported was no longer noticed when colts regained normal stallion testosterone concentrations. Besides the poor effort to collect colts during vaccine effective period, inappropriate controls (pre-pubertal) were used and without those

to compare one cannot rule out factors like seasonality. Since no ejaculation was achieved during the high-titer / low-testosterone period, changes in sperm count could not be characterized throughout all of the experiment. However, total sperm number before and after vaccination were comparable, so if any change happened in sperm number due to vaccination, it seemed to be reversed at the time of the experiment. The validity of these finding is debatable, however, when single semen collections were compared and no stabilization of extra-gonadal sperm reserve was attempted.

Later, another group of investigators (Malmgren et al., 2001) investigated the effect of a water-soluble BSA-conjugated GnRH vaccine with a commercially available adjuvant, Equimune (Vetrepharm), in mature stallions. Although methodology was explained, in detail, because of the small number of animals, lack of controls – data compared before and after vaccination – and experimental design, little can be inferred other than provide the descriptive findings of the study. Among three immunized 4 or 5 year old stallions, one failed to develop satisfactory GnRH antibody titer: peak of 10% binding capacity at 1:6400 dilution compared to 70 % and 80% in the other two, even when all of the stallions received four booster injections after the first, at two or four week intervals. Testosterone and estrone sulphate concentrations were reduced to castrate levels (<129 pg/mL and <1.46 ng/mL, respectively) during the high titer period of the two responding stallions (starting four weeks after first injection). Sexual behavior seemed to be affected, though some of the evaluations (e.g. handleability) were subjective, while the objective parameters (elapsed time for ejaculation and number of mounts per ejaculation), could have been affected by seasonality and were not properly compared to control values. Sperm production, sperm motility and sperm morphology were affected in stallions carrying high

titers against GnRH; likewise testicular size. Parenchymal alterations due to immunization seemed to involve lipid droplet accumulation in the Leydig cells; degeneration of Sertoli and germ cells and scarce number of spermatids and spermatocytes. There was local tissue reaction to the vaccine in all treated stallions, especially those with high titers. The stallions were only followed for 3 weeks after the last injection, so no inferences can be made on reversibility of the effects of immunization since the study was not designed for that.

Because of tissue reactions to the GnRH vaccine, and because of inconsistency in response to vaccination, another study was conducted in mature (four-year-old) pony stallions (Turkstra et al., 2005). A tandem GnRH dimer conjugated to ovalbumin in PBS was tested using two different adjuvants: CoVaccine<sup>TM</sup> HT or Carbopol). The former adjuvant proved efficacious in all subject stallions in inducing antibody titers with 56-76% binding at 1:2000 dilution. This high titer was achieved by two weeks after booster vaccination, six weeks after the initial immunization, and decreased testosterone levels for six weeks (until the end of experiment). It also decreased motility of sperm in 3 of 4 stallions treated with the formulation. The authors reported a reduction in testis size, as well as reduced seminiferous tubule diameter, absence of tubular lumen and reduced number of tubules with elongating spermatids and spermatozoa, which indicated severe effects on spermatogenesis. Though the study was not designed to evaluate effects on libido, failure to ejaculate was recorded in one stallions for each vaccine form. Finally, injection site reactions were not reported regardless of the titer achieved by immunization, although antibody titers depended on the type of adjuvant used.

Another report (Clement et al., 2005), based upon the CoVaccine<sup>TM</sup> HT adjuvanted immunization, described a decrease in testosterone in half of subject stallions. Testosterone

concentrations were recovered within six months after the last booster. No effect on libido, time for erection, time for ejaculation or number of mounts was seen in any of the stallions. The effects on total testes width and semen parameters including total sperm number, morphologically abnormal sperm and sperm motility were noticed in some but not all stallions. Recovery in sperm motility did not occur in all stallions. Interestingly, although the study did not target EAV shedding stallions, one of the stallions that responded well to vaccination was an EAV carrier stallion; 6 weeks after first vaccination the stallion's semen was virus negative; however, semen were EAV positive at two months after the last vaccination.

Burger (Burger et al., 2006) reported on the effects of two commercial vaccines, Equity® or Improvac®, on sexual behavior, semen quality, testis size and EAV shedding state in adult stallions. In the first experiment, using Equity®, vaccinated stallions showed a reduction in testosterone concentrations under 200pg/mL, but by 30 weeks from the last vaccine booster, some stallions still had not regained pre-treatment testosterone values. Antibody titers remained high (value not disclosed) until the end of the experiment. Libido was affected (parameters no disclosed) with individual variation; scrotal size, total sperm number, morphologically normal sperm and motility were decreased after vaccination and tended towards pre-vaccination values 10-12 months after the beginning of the study. A second experiment consisted of a questionnaire based on a field study in which vaccines were given to stallions of various breeds, with a booster 28-35 days after first vaccination. Seventeen out of nineteen stallions with behavioral issues responded with the desired decrease in libido. Endpoints involved subjective parameters such as distraction level and aggressiveness towards humans. A third experiment consisted of vaccination of four EAV



long-term carrier stallions. The virus could no longer be isolated from ejaculates of the stallions 6 months (as long as subjects were monitored) after the first vaccination (booster was given 4 weeks later). One stallion died 6 ½ months after first vaccination, but, 12-17 months after the 1<sup>st</sup> vaccination, the remaining stallions were each bred to two mares, which failed to seroconvert the virus within 28 days after breeding. The published abstract lacked statistical analysis, important details, non-vaccinated EAV-carriers as controls and had subjective evaluation of endpoints. Accordingly, discretion should be exercised if extrapolate from these results. It was, nonetheless, a study designed to evaluate anti-GnRH vaccination in order for eliminating the EAV carrier state in stallions.

More recently (Janett et al., 2009), the commercial vaccine, Equity®, was tested in sexually mature stallions (6-15 years old) for its effect on testicular function and sexual behavior. After receiving three immunizations at four and eight week intervals, the stallions were monitored weekly for a year. One of five stallions failed to develop antibody titers above 15 % at a 1:40 dilution, compared to the 30-44% peak binding found in the remaining four animals. In the vaccinated stallions, peripheral testosterone, sperm motility and morphologically normal sperm were decreased, except for the one stallion with a low antibody titer. Four of the immunized stallions presented changes in libido (elapsed time to achieve erection and ejaculation), and during the suppression period, four of five stallions failed to ejaculate in a 10 minute time-frame, while that trend was not seen in the controls. Resumption of reproductive activity was not seen in two of the subjects, whose testosterone concentrations did not return to normal; one stallion had a prolonged decline in sperm count, and another could not be collected. In this study, only minor reactions at injection sites were reported.

Based on available reports, thus far no GnRH vaccine was able to both generate adequate anti-GnRH titers and permit full recovery of testicular function. It appears that sexual behavior is affected, but not in all of the subjects, neither is libido recovered in all stallions that were affected. Were found in mares similar results regarding the effects of immunization against GnRH on behavior and gonadal activity. Ovulatory function and display of estrous behavior failed to resume to normality (Imboden et al., 2006; Elhay et al., 2007), even after recovery periods longer than 90 weeks (Imboden et al., 2006). This may serve as an illustration of the prolonged effects of immunization against GnRH in the equine species.

### **2.2.3. GnRH agonists**

Since 1972, soon after the molecular structure of mammalian GnRH was characterized, work was undertaken to synthesize analogs of GnRH. The half-life of GnRH is very short – about 2-4 min – and more potent and longer-acting analogs were considered more suitable for many of the clinical applications in humans. Various amino acid replacements to the original decapeptide GnRH sequence were shown to increase resistance to enzymatic degradation of the molecule and to enhance receptor affinity. For instance, substitutions at positions 6 and 10 can lead to superactive peptides, such as Buserelin, (Schally, 1999), which may be 20-100 times more potent than native GnRH (Clayton and Catt, 1980; Conn and Crowley, 1991; Schally, 1999).

Although a single injection of superactive agonists of GnRH induced a sustained release of FSH and LH, repeated administration produces inhibitory effects (Schally, 1999). In various species, the continuous exposure of the anterior pituitary to high doses of GnRH or to potent analogues of GnRH leads to a non-responsive state (Fraser, 1982). Continuous stimulation of the pituitary by prolonged administration of GnRH or its

superagonists inhibits the hypophyseal-gonadal axis through down-regulation (a reduction in number) of pituitary receptors for GnRH, decreased gene expression, desensitization of pituitary gonadotrophs and suppression of circulating levels of LH and sex steroids (Schally, 1999).

In humans, important clinical applications from sustained administration with GnRH agonists include treatment for central precocious puberty, polycystic ovarian disease, benign prostatic hyperplasia, endometriosis and others. Also, therapy for sex hormone dependent malignant neoplasms in women (e.g. breast cancer, endometrial carcinoma) and men (e.g. prostate cancer) is based on the reversible medical castration and the creation of a state of sex steroid deprivation (Schally, 1999). In domestic species, it has been demonstrated that prolonged GnRH agonist treatment can induce an antagonistic effect over time in rams (Bremner et al., 1976), male dogs (Vickery et al., 1984), boars (Kauffold et al., 2010), bulls (Godfrey et al., 1989) and others (Fraser, 1982).

Much of the reluctance by clinicians and researchers to pursue GnRH therapy for stallions for agonistic purposes, in particular sub-fertile ones, came from data in other species and inconsistent results in horses (Brinsko, 1996; Brinsko et al., 1998). A case report provided data in which an infertile stallion, subject to treatment with daily GnRH for 5 months had its sperm numbers and motility increase then stabilize throughout the breeding season (Evans and Finley, 1990). On the other hand, an uncontrolled study provided data that a 30-day treatment with GnRH tended to reduce testosterone concentrations in two of three stallions, though it did not induce any beneficial or detrimental changes in sperm numbers nor libido. In the same study, a 35-day long GnRH treatment in mares appeared to decrease ovarian activity (Montovan et al., 1990). Data

from Blue et al. showed that GnRH administration from February to July, in continuous or pulsatile manner did not change LH and testosterone concentrations, scrotal width, total sperm per ejaculate or sperm motility. Pulsatile administration of GnRH was not able to improve seminal and testicular parameters in abnormal stallions, in which they were inherently decreased (Blue et al., 1991).

It seems that the only study to show that prolonged GnRH treatment could clearly down-regulate testicular function was that of Boyle and colleagues, in which implants of the potent GnRH analog, Buserelin, were administered for a period of 12 months. This treatment led to a 50% decrease in DSO compared to controls. FSH, LH, testosterone and estrone sulphate concentrations were suppressed but these fluctuated and not all of the treated stallions demonstrated the same level of suppression. No changes were seen in libido throughout the 12 months compared to controls (Boyle et al., 1991). The authors reported that 7 months after the last implant was injected, all parameters had returned to normal values in the treated stallions.

It seemed that only prolonged treatment with high doses of GnRH would achieve down-regulation of the HPG axis in the stallion when given in a continuous manner. In fact, data from mares showed that suppression of LH occurred after continuous administration of GnRH, but not after pulsatile administration. Neither treatment affected follicular development or time for ovulation (Becker and Johnson, 1992). Thus, Brinsko designed a study to evaluate the effect of pulsatile administration of high doses of GnRH (250µg) for 75 days to stallions. No reduction was reported in LH, FSH or testosterone. On the contrary, concentrations of gonadotropins and testosterone increased during the treatment period in stallions receiving GnRH. Brinsko and colleagues concluded that the

HPG axis of the stallion is remarkably resistant to down-regulation following administration of exogenous GnRH in comparison to other species (Brinsko et al., 1998).

More recently, data supporting the beneficial effects of GnRH therapy in stallions was published by Sieme and collaborators (Sieme et al., 2004), who administered the agonist Buserelin 50µg twice daily for 6 weeks in the non-breeding season. Treatment increased libido parameters, as well as sperm motility, membrane integrity and functionality. It also reduced the detrimental effects of freezing on progressive motility.

Only a few publications illustrate the clinical improvement on sperm quantity and quality by GnRH treatment in stallions; the response depends upon the appropriate form of GnRH, delivery manner, dose and length of treatment to achieve improvements on reproductive function. On the other hand, the available evidence shows that down-regulation of the HPG axis in the male horse does not match that of other species in that the horse is unusually resistant to refractoriness (Brinsko, 1996); which precludes the clinical use of GnRH to diminish libido, decrease circulating testosterone or other desired effect on reproductive function by down-regulation of GnRH.

#### **2.2.4. GnRH antagonists**

Comprehensive reviews (Karten and Rivier, 1986; Rivier et al., 1996) have been published on the historical advances and molecular modifications to native GnRH that lead to antagonist molecules of clinical applicability.

The first antagonist to GnRH was reported in 1972 (Vale et al., 1972). The work in creating such peptides started concomitantly with development of agonists. Due to requirement for high affinity (without stimulatory activity) and resistance to enzymatic degradation, the design of GnRH antagonists was slow. Small, incremental increases in potency and duration of effect were reported from time to time and appeared in the

literature as equipotent groups of GnRH antagonists (Karten and Rivier, 1986). At first, GnRH antagonists were of very low potency relative to GnRH, which resulted in large doses required to be used both *in vitro* and *in vivo* (Karten and Rivier, 1986; Behre et al., 1998). Furthermore, as the potency *in vivo* increased with new amino acid replacements in positions 2, 3 and 6 of the GnRH molecule, these antagonists, triggered histamine release from mast cells in the circulation, which caused side effects ranging from local erythema to general edema and decrease in blood pressure which rendered these antagonists unacceptable for clinical purposes. A second-generation of GnRH antagonists had further structural modifications to the polypeptide chain of GnRH, at positions 5 and 6. This change led to similar or higher potency but lower histamine release, and no systemic side-effects, though the high doses needed for clinical use still provoked local side-effects (Behre et al., 1998). The so called third-generation antagonists had modifications on up to 6 or 7 amino acids of the decapeptide chain of GnRH making available antagonists of even higher potency (Behre et al., 1998; Schally, 1999). The modifications also decreased histamine effects of these antagonists to minor or negligible local side-effects, and hence were deemed safe for clinical applications in humans. The antagonists Cetrorelix and Ganirelix were both approved by the FDA for certain applications.

The mechanism of action of the GnRH antagonists is primarily by competitive blockade of the GnRH receptor in the gonadotrophs, precluding substantial occupation (and stimulation) by endogenous GnRH (Conn and Crowley, 1991; Schally, 1999; Huirne and Lambalk, 2001) resulting in immediate inhibition. This is in contrast to GnRH agonists, which require repeated administrations to achieve this effect. It has also been shown that

down-regulation of GnRH receptor numbers (Behre et al., 1997) and of GnRH receptor mRNA (Pinski et al., 1996) may occur following GnRH antagonist treatment.

GnRH antagonists have similar clinical applications as GnRH agonists with down-regulation as the aim (Schally, 1999; Huirne and Lambalk, 2001), though one advantage of the antagonist is the absence of a transient stimulatory effect. The lack of stimulation (which happens with GnRH agonists) before down-regulation has application to avoid premature LH surge in IVF programs in women (Fluker, 2000; Ron-El et al., 2000; Fasouliotis et al., 2003) and, more importantly, avoids a clinical “flare-up” that may happen in men treated with GnRH agonist for prostate cancer, in which a transient testosterone surge could reflect that metastasis has occurred (Schally, 1999; Huirne and Lambalk, 2001; Weckermann and Harzmann, 2004; van Poppel and Nilsson, 2008). Furthermore, in the case of antagonists versus agonists, the course of treatment tends to decrease, and a more rapid and predictable recovery is possible after cessation of treatment with antagonist (Rivier et al., 1996).

Even though less than a handful of antagonists are FDA approved for use in humans there are different compounds under trials and the veterinary scientific literature reports the successful use of antagonists on down-regulating the HPG axis for various purposes in domestic carnivores (Gobello, 2012), ruminants (Gonzalez-Bulnes et al., 2005; Jimenez-Severiano et al., 2007) and pigs (Patterson-Bay et al., 1997; Zanella et al., 2000) with reversibility seen in most of the trials where it was assessed.

In horses, follicular development and ovulation was investigated in mares after the treatment with GnRH antagonist. Watson and colleagues (Watson et al., 2000) found that Antarelix successfully down-regulated the pituitary and ovarian activity in cyclic mares.

The antagonist, administered on day 8 after ovulation, caused a fall in progesterone the next day, delayed estradiol and LH surges in the cycle, as well as increased the inter-ovulatory interval, though not compromising the timing of luteolysis. Later, studies of Briant (Briant et al., 2003; Briant et al., 2004) utilized the antagonist Antarelix to suppress pre-ovulatory LH surge in mares with the aim of studying the timing of ovulation.

Reports on the use of GnRH antagonists in stallions are limited. An uncontrolled study by Hinojosa et al. (Hinojosa et al., 2001) reported the effects of a single injection of the GnRH antagonist Antarelix in pony stallions of 2-5 years of age. Plasma concentrations of LH, FSH and estradiol were significantly decreased within 48h of the treatment injection and returned to pretreatment values by 4 to 6 weeks after treatment. No changes were seen in circulating testosterone, sperm motility or morphology, though an increasing number of round spermatogenic cells was noticed. Libido seemed to be affected by the single injection, but the authors suggested that it might have been confounded by paired stabling of stallions and distance from mares.

An abstract report in 2002 described the use of Antarelix or Cetrorelix in EAV carrier stallions (Fortier et al., 2002). For this trial, the dose was increased to twice daily (100ug/kg) compared to a single dose in the aforementioned studies and was given for 35-38 days. In treated stallions, testosterone decreased to basal levels within one week, except for one stallion, then returned to pre-treatment values at one week after the end of treatment. There was a transient decrease in the quantity and quality of semen produced. Those parameters appeared to be reduced the most by 22 to 55 days after the end of treatment, and were restored to normal levels by 75 to 110 days after end of treatment. The effects on the EAV carrier state was variable among stallions and treatment groups. All of



the treated stallions became semen negative for EAV on semen isolates before the end of treatment, although one stallion assigned for treatment group spontaneously cleared just before starting treatment. There was spontaneous clearing of virus in the semen of some of the control stallions, while some treated stallions that became non-shedders during treatment recommenced shedding between 14 and 42 days later. Results were confounded by the high number of stallions that spontaneously cleared. Some treated stallions which had become EAV negative reverted to an EAV positive state after cessation of treatment, which does not occur in spontaneous cessation of shedding (**see section 2.2.1**). Therefore, the occurrence momentary clearing of viral load in ejaculates after treatment suggests a causal relationship between the GnRH antagonist treatment and the viral status of the stallions.

***A. Third-generation antagonist, Acyline***

Acyline, first described by Rivier *et al.* (Rivier *et al.*, 1995), is a member of azaline family of third-generation antagonists; it is a decapeptide like GnRH, but has modifications in amino acids 1, 2, 3, 5, 6, 8 and 10 [Ac-D-Nal-D-4-Cl-Phe-D-Pal-Ser-Aph(Ac)-D-Aph(Ac)-Leu-Lys(Ipr)-Pro-D-Ala-NH<sub>2</sub>]. Acyline resulted from attempts to increase hydro-solubility and decrease gel formation while retaining potency by introducing betidamino acids (Jiang *et al.*, 1997). These are amino acids in the beta position, presenting acyl groups which probably explains the assigned name of the drug.

Acyline has been used to interfere with the effects of GnRH in rodents (Jiang *et al.*, 1997), cattle (Ginther *et al.*, 2011), cats (Garcia Romero *et al.*, 2012a), as well as men and dogs. In some trials in men, acyline was shown to be very potent in decreasing circulating gonadotropins and testosterone. In the first report of administration of acyline in men,

administration of 75 µg/kg of acyline resulted in a seven-day suppression of peripheral testosterone, reaching a nadir at 48h, though a decline in testosterone concentration occurred already within 15h. Acyline produced a longer down-regulation of the HPG axis compared to Cetrorelix, which reaches a nadir in testosterone suppression at 12h with a return to baseline values at 48h (Herbst et al., 2002). In a subsequent trial, a single injection of the acyline (300 µg/kg) reduced gonadotropin and testosterone concentrations to those of castrates (in men, below 1.45 ng/mL) for 17 days, and these hormones remained below values of controls for 19 days, returning to baseline values at day 21 (Herbst et al., 2004). A more recent study showed potential applications for the use of acyline orally, which is unusual for GnRH antagonists since these peptides cannot endure the low pH of the stomach (Amory et al., 2009). The oral version, at doses of 20 and 40 mg, promoted and sustained suppression of testosterone for 12-24h, with return to basal levels at 48h.

In male dogs, a single dose of acyline (330µg/kg) decreased gonadotropin and testosterone concentrations for at least nine days (Garcia Romero et al., 2009). Acyline also decreased libido and erectile function for four weeks, and sperm numbers for six weeks (Valiente et al., 2007). Two of the seven treated dogs became azoospermic during weeks 1 and 2, and four of the treated dogs showed azoospermia at week two after administration of acyline. In this study an increase in round spermatogenic cells was observed at week four after injection but values decreased to control values from week six to 8. Although libido and erection recovered during the follow up, full recovery of seminal parameters was not observed in this study, which the authors attribute to a short recovery period. In a later study (Garcia Romero et al., 2012b), acyline prevented the canine gonadal axis from responding to an exogenous GnRH agonist; such challenge was performed serially with

the superagonist Buserelin. Circulating testosterone in acyline treated dogs was not incremented by GnRH stimulation. The length of testosterone suppression after a single acyline injection did not differ due to the exogenous GnRH stimulations when compared with the single-injection treatment without GnRH stimulations (Garcia Romero et al., 2009).

To our knowledge, the only published studies on the use of acyline in the horse were performed in mares (Checura et al., 2009; Checura et al., 2010). Instead of a long lasting down-regulation of the HPG axis, these studies sought a short blockade of GnRH activity with a low dose of acyline (maximum of 3mg, single dose) to investigate interactions of the antagonist with FSH release and follicular development. Such treatment was enough to decrease circulating concentrations of FSH of mares after FSH-increasing stimuli such as follicular ablation and exogenous GnRH administration and to retard growth of the dominant follicle when given right before deviation.

Data from our lab (not published) suggests that two injections of acyline (50mg in 5% mannitol solution) given 10 days apart were enough to increase inter-ovulatory intervals in cycling mares.

## **Chapter Three: Reversible downregulation of hypothalamic-pituitary-gonadal axis in the stallion with a GnRH antagonist**

### **3.1. Introduction**

Methods to suppress the hypothalamic-pituitary-gonadal (HPG) axis in stallions with resultant suppression of androgen and estrogen production have been examined for different reasons including: (1) elimination of the carrier state in equine arteritis virus (EAV) persistently infected stallions and (2) alteration of sexual/aggressive behavior. Over the last decade, equine viral arteritis (EVA) outbreaks on different continents reemphasize the important epidemiologic role that persistently infected carrier stallions play in the dissemination EAV through infective semen (Timoney et al., 1986; Timoney et al., 1987; Timoney et al., 2006; Barrandeguy, 2010; Pronost et al., 2010; Mischczak et al., 2012). After resolution of the acute phase of the, 30 to 60% of stallions may become persistent carriers of EAV and shed the virus in their semen (Neu et al., 1987; Timoney and McCollum, 1993). Persistent infection with EAV is androgen dependent (Little et al., 1991; Holyoak et al., 1993; McCollum et al., 1994) and removal of endogenous testosterone results in termination of the persistent carrier state (Little et al., 1991). Castration, on its turn, has been historically the method of choice to deal with stallions that present excessive display of sexual or aggressive behavior. Orchiectomy seems to solve most of the cases in which such behavior may compromise performance of colts during training or competition (Line et al., 1985). However, surgical castration precludes the use of stallions for breeding after retirement from a successful sports career.

As an alternative to castration, several approaches have been investigated to downregulate the HPG axis in the stallion, including treatment with altrenogest and

immunization against GnRH as well as downregulation of pituitary GnRH receptors with GnRH agonists and antagonists. Off-label use of altrenogest in training or competing stallions is very common (Goolsby et al., 2004), although recovery from a decline in libido and various reproductive parameters after treatments longer than eight weeks has not been demonstrated (Squires et al., 1997; Johnson et al., 1998). There are also regulations to limit the use of altrenogest in certain countries – e.g. European Union – where the horses may be considered a food animal. Immunization against GnRH to achieve a reduced activity of this hormone has been used in pigs and ruminants to avoid surgical castration (Adams and Adams, 1992; Dunshea et al., 2001). Immunization against GnRH has also been investigated in stallions (Dowsett et al., 1996; Malmgren et al., 2001; Turkstra et al., 2005; Janett et al., 2009), including its use as a potential treatment for EAV carrier stallions (Burger et al., 2006). Nonetheless, at least four weeks are required for adequate anti-GnRH titers to be produced after the first immunization and resumption of reproductive function is not regained by all of the treated stallions, in which suppression of libido and serum testosterone may persist indefinitely (Burger et al., 2006; Janett et al., 2009).

Even though in men and most domestic animal species prolonged, repeated stimulation of GnRH receptors with high doses of GnRH or potent analogs cause down-regulation of pituitary gonadotrophs and decreased LH and FSH release after a transient stimulatory effect (Bremner et al., 1976; Fraser, 1982; Vickery et al., 1984; Godfrey et al., 1989; Schally, 1999; Huirne and Lambalk, 2001; Kauffold et al., 2010), the stallion appears to be resistant to down-regulation by exogenous GnRH agonists (Blue et al., 1991; Boyle et al., 1991; Brinsko, 1996). However, GnRH antagonists, which lead to direct inhibition rather than activation of the GnRH receptor (Heber et al., 1982; Karten and Rivier, 1986;

Rivier et al., 1996), have been shown in a few reports to be effective in stallions. A study by Hinojosa and colleagues (Hinojosa et al., 2001) reported that a single injection of GnRH antagonist, Antarelix, in pony stallions induced a decrease of plasma gonadotropins and estradiol. Even though Antarelix administration did not decrease in testosterone, libido seemed to be affected. The use of Antarelix and a second GnRH antagonist, Cetrorelix, daily for 35 to 38 days in EAV carrier stallions (Fortier et al., 2002) achieved a reversible decrease in testosterone and a transient reduction in sperm number and quality. However, treated stallions resumed virus shedding status after momentarily clearing the virus during treatment. This suggested a causal relationship between GnRH antagonist treatment and the viral status of the stallions because resumption of virus shedding is not seen in stallions that spontaneously clear the virus (Timoney and McCollum, 1993; 2000)

A potent third-generation GnRH antagonist, acyline [Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Ac)-D4Aph(Ac)-Leu-Lys(Ipr)-Pro- DAla-NH<sub>2</sub>], was described in 1995 (Rivier et al., 1995). Initial trials in men have shown that acyline decreases FSH and LH for 15 to 20 days, and acyline reduces testosterone to castrate levels for 2 weeks after a single injection of 300 µg/kg (Herbst et al., 2004). In male dogs, a single dose of acyline (330 µg/kg) decreased FSH, LH and testosterone concentrations for at least nine days (Garcia Romero et al., 2009). Dogs treated with acyline had reduced sperm numbers for six weeks and severely reduced libido and erectile function for four weeks. Although recovery from effects on semen were not seen in the eight-week follow-up, libido and erection capability recovered during this period (Valiente et al., 2007). A more recent study showed that acyline prevented the canine HPG axis responding to repeated challenges with the superagonist, Buserelin (Garcia Romero et al., 2012b).

Although there are no reports of acyline administration to stallions, acyline (approximately 10 µg/kg) was utilized in pony mares (Checura et al., 2009; Checura et al., 2010) to investigate interactions of the antagonist with FSH release and follicular development. Acyline blockade of GnRH activity in mares decreased circulating concentrations of FSH and retarded growth of the dominant follicle.

In the current study, we hypothesized that acyline administration results in down-regulation of the HPG axis in stallions and that, in addition, the effects of such blockade would be reversed after the end of treatment. The objectives of this study were to (1) determine the effects of acyline treatment on changes in endocrine, seminal, behavioral and testicular volume parameters of stallions; (2) determine responsiveness of acyline-treated stallions to GnRH stimulation; and (3) to examine recovery of these parameters after withdrawal of acyline treatment.

### **3.2. Material and methods**

#### ***A. Animals***

All animal experimentation was conducted according to a protocol approved by the Institutional Animal Care and Use Committee at the University of Kentucky (#2011-0854). Eight light-horse stallions (3-15 years, median = 9) were housed in individual box stalls at Maine Chance Farm (University of Kentucky, Lexington, KY). Stallions received hay and water ad-libitum in addition to combined whole oats and commercial concentrate (0.4% BW), and stallions had access to salt and mineral sources. Stallions were turned out into small fenced paddocks (at least 5 d a week) with visual contact with mares in nearby pastures.

### ***B. Acyline dose determination***

A preliminary study was conducted to compare the effect of two different doses of acyline on serum testosterone concentrations. Stallions (n=2, not included in the main study) received either 100µg/kg or 330 µg/kg acyline in 5% mannitol as a single intramuscular administration, and blood was collected daily for 10 d for determination of serum testosterone concentrations. Based upon this preliminary study, a dosage of 330 µg/kg at 5-d intervals was chosen for the main experiment.

### ***C. Experimental design***

To establish pre-treatment baseline seminal parameters, stallions had semen collected daily (Monday – Friday) for two weeks. Testis volume was measured weekly via ultrasonography. Daily sperm output (DSO) was estimated based upon the total number of sperm collected on the fifth day of semen collection each week as previously described (Thompson et al., 2004). Stallions were matched based on DSO (average of pre-treatment weeks), testicular size and age, then were randomly assigned to treatment (n=4; acyline 330µg/kg q 5d, IM) or control (n=4; 10mL vehicle, q 5d, IM) groups. The treatment period was 50 days, and the recovery period was 72 days after last treatment. The experiment was conducted from mid-August through mid-December in the Northern Hemisphere.

### ***D. Endocrine evaluation***

Jugular blood samples were obtained in the morning prior to feeding and to acyline treatment, in days administered (~7AM). Blood was collected in heparinized tubes (BD, Franklin Lakes, NJ), and plasma was separated by centrifugation at 2500 xG for 10 min at 4°C. Thereafter, plasma samples were frozen and stored at -20°C until analysis.



Plasma testosterone and estrone sulfate concentrations were determined daily for the last five days pre-treatment and the first nine days of treatment; then, on the days of treatment administration (q 5d), and two days following each treatment administration through the end of treatment. During the recovery period, plasma steroid concentrations were determined once weekly. Testosterone was determined using a competitive ELISA. A standard curve with a range from 0.02 ng/mL to 10 ng/mL was used with a limit of detection of 40 pg/mL (Esteller-Vico, 2015). Estrone sulfate was analyzed using a previously described competitive ELISA (Carneiro et al., 1998). Samples were run in triplicates. Intra- and inter-assays coefficients of variation (CV) for testosterone were 13% and 11% (low-binding = 10%; high-binding = 12%), respectively. Estrone sulfate intra-assay CV was 11% and inter-assay average was 11% (low-binding = 7%; high-binding = 14%).

Follicle-stimulating hormone, (FSH) and luteinizing hormone (LH) assays were run on days -3, 0 to 7, 17, 32, 52, 53, 56 to 59, 73, 87, 101 and 112 relative to treatment (day 0 = day of initial treatment). Follicle-stimulating hormone and luteinizing hormone were determined by the heterologous double-antibody RIA method (Roser and Hughes, 1991). Sensitivity was 0.25 ng/mL for both gonadotropins, and the intra-assay CVs were 2.72% for LH and 2.46 for FSH, each run in a single assay.

Gonadotropin-releasing hormone stimulation tests were performed prior to the beginning of treatment and during the seventh week of treatment in order to evaluate the suppression of the hypothalamic-pituitary-gonadal axis. The procedure consisted of injecting 25µg of GnRH (IV, gonadorelin diacetate tetrahydrate; Cystorelin, Merial LLC,

Duluth, GA). Blood was collected 30 min before, immediately prior to and 30, 60 and 120 min post GnRH administration (Roser and Hughes, 1992).

Plasma anti-Müllerian hormone (AMH) concentrations were determined using a commercially available ELISA (AMH ELISA, MOFA Global, Verona, WI) per the manufacturer's instructions. Plasma AMH concentrations were determined weekly from day -6 to 87 relative to the beginning of treatment.

#### ***E. Semen collection and evaluation***

Semen collections were performed Monday through Friday for two weeks after onset of treatment, and then on alternate weeks until the end of the experiment. Only the fifth daily ejaculate for that week (i.e., DSO ejaculate) was used for semen evaluation data. Stallions were brought into the breeding shed and exposed to an estrogen-primed ovariectomized mare (estradiol cypionate [BET Pharmacy, Lexington, KY] 10mg, q 7 d, IM). The stallion was teased until an erection was obtained, the penis was washed with warm water, and the stallion was allowed to mount a phantom for semen collection with a Missouri model artificial vagina.

The gel portion of ejaculates was removed by filtration, and the gel-free volume was determined by weight (1g = 1mL). Sperm concentration was obtained with a densimeter (591B densimeter, Animal Reproduction Systems, Chinoe, CA). Total motility, progressive motility and other sperm motion characteristics were obtained with computer-assisted sperm analysis (CASA; SpermVision, MOFA Global, Verona, WI). Preparation of samples for motility analysis consisted of diluting a fraction of ejaculate with a skim-milk extender (EquiPRO, MOFA Global, Verona, WI) to a final concentration of  $30 \times 10^6$  sperm/mL. Eight microliters of extended sperm suspension were placed on a microscope

slide (22x22-2 mm coverslip), at 37°C and allowed to stabilize for at least 30 sec before acquiring the data. The settings of the CASA were as follows. Level 1 cell classifications: immotile = AOC (average orientation change of the head) <7; locally motile = DSL (distance straight line) <6. Level 2 classifications: hyperactive= VCL (velocity curved line) >8, LIN (linearity) <0.65 and ALH (amplitude lateral head displacement) >6.5; linear= STR (straightness) >0.9 and LIN>0.5; non-linear= STR<0.9 and LIN<0.5; curvilinear= DAP (distance average path)/radius  $\geq$ 3 and LIN<0.5. Cell identification was set for an area of 14 to 80 $\mu$ m<sup>2</sup>. Motility assessment was repeated with a minimum of 7 fields or 500 cells.

Aliquots for sperm morphology assessment consisted of 50  $\mu$ L of raw semen fixed in 1mL 10% buffered formalin. Subsequent evaluation was performed in wet mounts under DIC microscopy (Axio Imager 2, Carl Zeiss Microscopy LLC, Thornwood, NY) at 900x magnification. Seminal plasma from ejaculates collected on Tuesdays and Thursdays was obtained by centrifugation at 2000 x g for 10 min, and stored at -20°C until analysis. Osmolality of seminal plasma was determined by osmometry (Micro Osmette, Model 5004 Automatic Osmometer, Precision Systems, Natick, MA). Protein concentration in seminal plasma was determined with comassie (Bradford) protein assay kit (#23200, Thermo Scientific, Kalamazoo, MI, USA) as previously described (Bradford, 1976).

#### ***F. Testicular volume***

Weekly testicular measurements were taken throughout the study. Stallions were placed in stocks and sedated (100-150 mg xylazine, [AnaSed, Lloyd, Shenandoah, IA], IV). The height, width and length of individual testes were determined with B-mode ultrasonography (Sonoscape S8, Universal Medical Systems Inc., Bedford Hills, NY), and values were recorded as the average of three measurements. Ultrasonographic

measurements and the estimation of testis volume was performed as described by Love (Love et al., 1991).

### ***G. Reproductive behavior***

Data on breeding behavior and libido recorded for each stallion included time from entrance into the breeding shed until erection and ejaculation as well as the total number of mounts per ejaculation. If a stallion failed to mount the phantom within 10 min, the tease mare was positioned beside the phantom for additional stimulation. A total time of 15 min was allowed for the stallions to show interest in the mare, mount the phantom and ejaculate. Failure to ejaculate in that time frame was recorded as a failure of collection and data regarding time to erection and ejaculation were not included in the dataset; however, a continued attempt was still made to obtain the ejaculate for other data points. At least two different tease mares were available throughout the experiment.

### **3.3. Statistical Analysis**

Continuous data were analyzed using a random-effects mixed model (JMP, ver. 10.0; SAS Institute; Cary, North Carolina, USA) with treatment and time as fixed effects and stallion as a random effect. Where necessary, data were log transformed, and model assumptions were evaluated using normal quantile plots of residuals. Data are presented as untransformed means  $\pm$  S.E.M. Comparisons between treatment groups at individual time points were made using the test-slice function of JMP to generate preplanned linear contrasts. Changes in LH, FSH and testosterone subsequent to GnRH stimulation were evaluated as area-under-the-curve (AUC) using a random effects mixed model with treatment and period (pre- vs during-treatment) as fixed effects and stallion as a random effect; that was followed by comparison of least square means of the AUCs in a Tukey's

test. Round cell numbers were compared with a Wilcoxon/Kruskal-Wallis test. Osmolality and protein content of seminal plasma (two observations per week of collection) were assessed based upon the weekly mean value. Behavior parameters were evaluated as weekly averages.

### **3.4. Results**

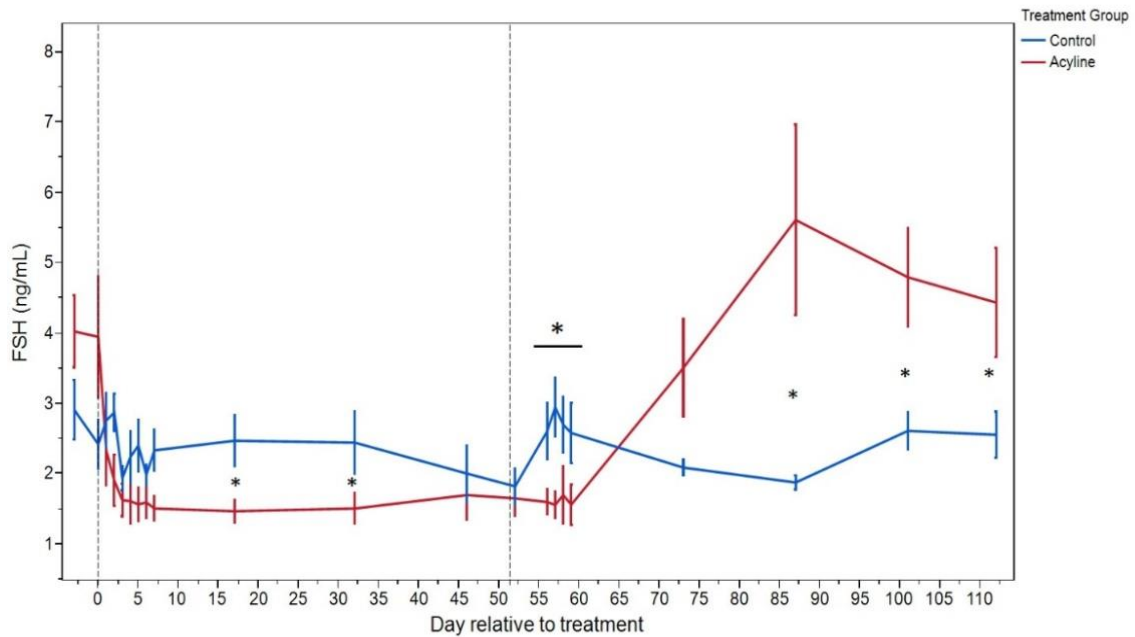
#### ***A. Endocrine Parameters***

For all data presented, days are presented relative to the initiation of treatment (Day 0). Plasma FSH concentrations in acyline-treated stallions were less than controls at day 17 and day 32 of treatment ( $p < 0.05$ ), and FSH was lower in the treatment group at days 6, 7, 8 and 9 after the cessation of treatment (Fig 1). FSH concentrations in the treated stallions were greater than control stallions at days 37, 51 and 62 after the cessation of treatment (Fig. 1). Plasma LH concentrations declined by day 2 after treatment, and concentrations were lower than controls throughout treatment measured time-points except for days 3 and 4 ( $p < 0.05$ ). Concentrations of LH displayed a rebound during the recovery period as shown by a higher ( $p < 0.05$ ) LH concentration on day 37 after the end of treatment in the acyline-treated stallions (Fig. 2).

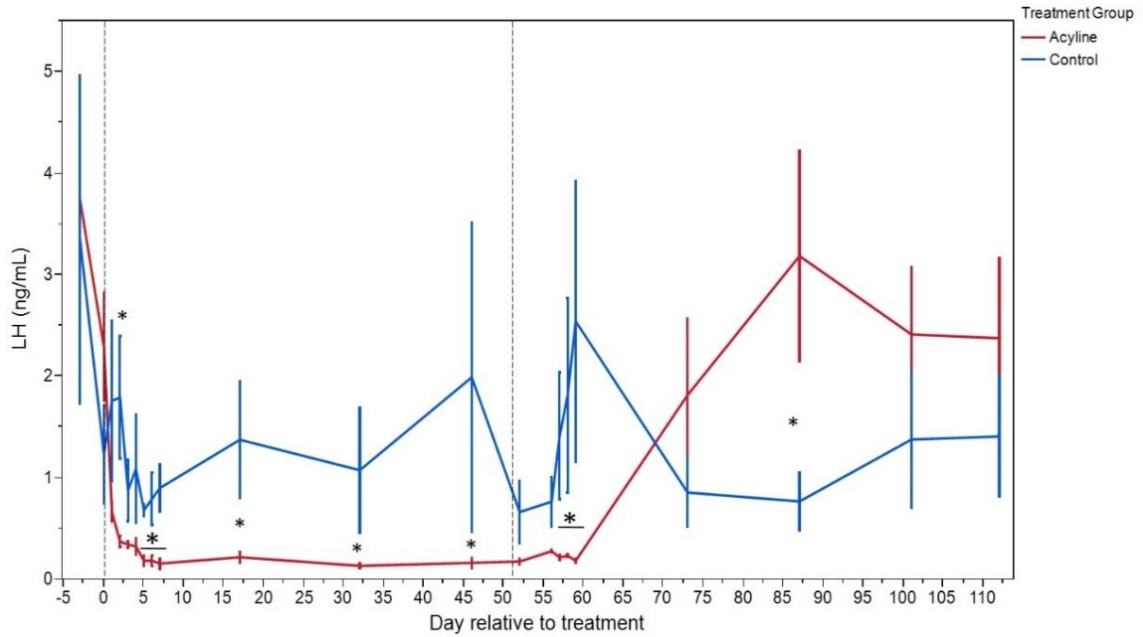
There was a significant time ( $P < 0.001$ ) and time-by-treatment interaction ( $P < 0.0001$ ) for both testosterone and estrone sulfate. Plasma testosterone concentrations declined ( $p < 0.0038$ ) by Day 1 of treatment, and testosterone concentrations remained suppressed relative to controls until the end of treatment (Fig. 3). After the last treatment, plasma testosterone concentrations in the acyline-treated stallions remained suppressed for 9 days before returning to levels comparable to controls. The acyline-treated group showed a constant, steady suppression of peripheral testosterone, with mean concentration of 129 pg/mL during treatment. There were significant time ( $P < 0.0001$ ) and time-by-treatment

interactions ( $P < 0.0001$ ) in plasma estrone sulfate concentrations, which were lower in treated than control stallions by Day 5 of treatment ( $p < 0.05$ ). There was a steady suppression ( $p < 0.001$ ) of estrone sulfate from day 6 to day 64 such that the suppression of peripheral estrone sulfate lasted 14 days after the end of treatment (day 50) (Fig. 4). Plasma AMH concentrations even though subject to a time effect ( $P < 0.0001$ ), were not affected by treatment (Fig. 5).

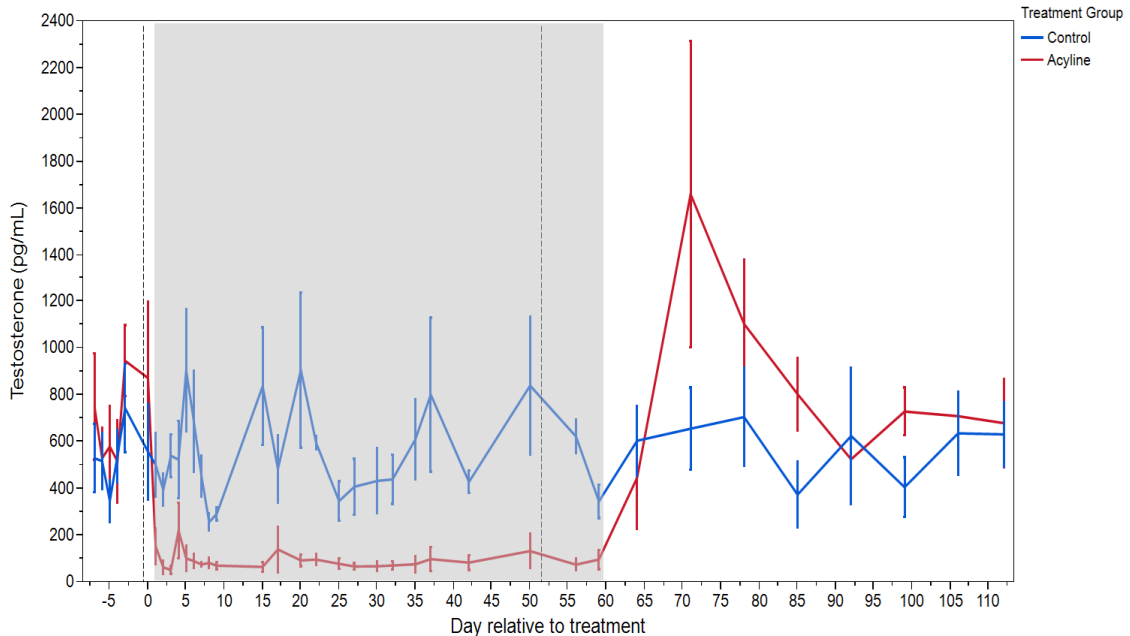
**Figure 1:** Circulating FSH concentrations in acyline-treated and control stallions. Acyline-treated stallions received 330g/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Data expressed as mean  $\pm$  S.E.M. (\*) or (\*) indicate timepoints or intervals, respectively, where  $p < 0.05$ .



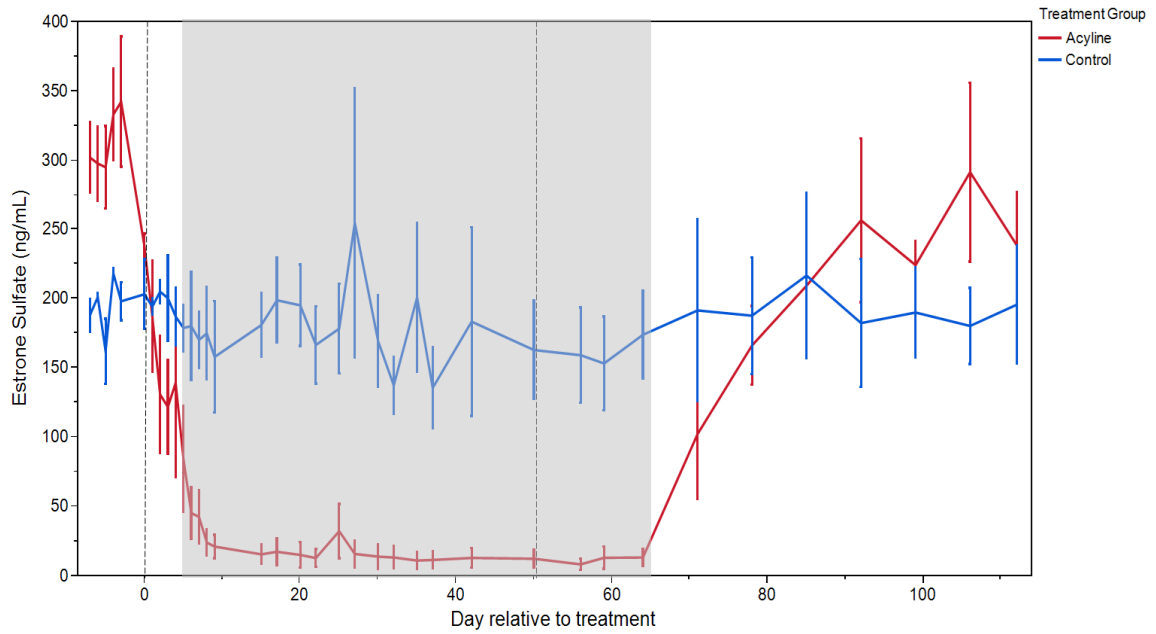
**Figure 2:** Circulating LH concentrations in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Data expressed as mean  $\pm$  S.E.M.. (\*) or (\*) indicate timepoints or intervals, respectively, where  $p < 0.05$ .



**Figure 3:** Plasma testosterone concentrations in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Data expressed as mean  $\pm$  S.E.M. The shaded area indicates the interval where  $p < 0.05$ : Day 1 through Day 59

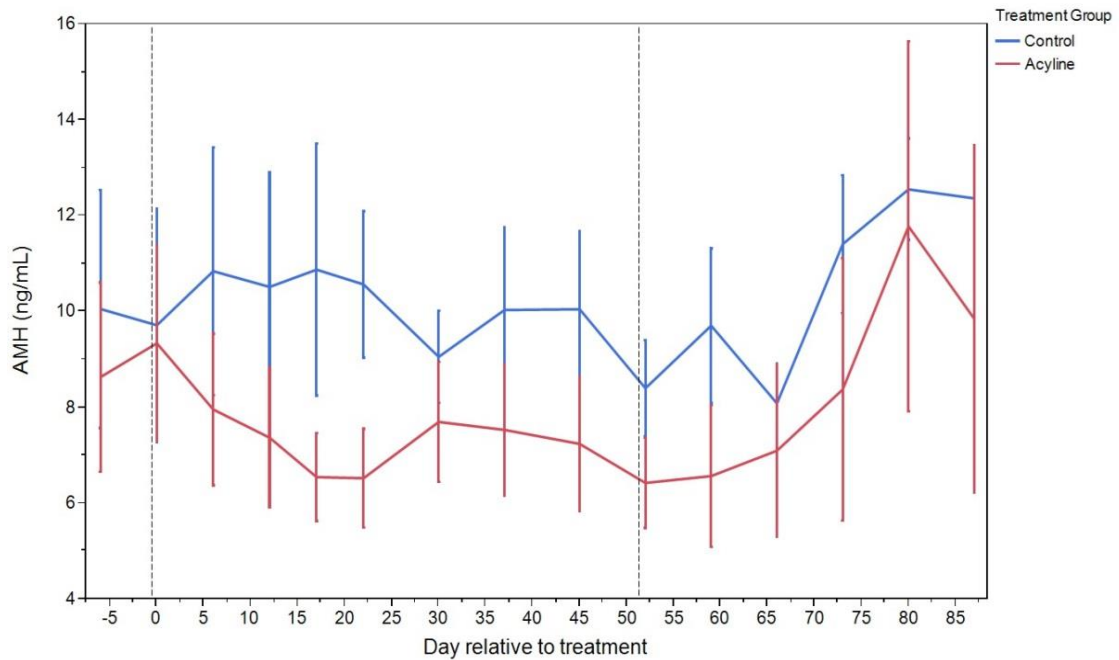


**Figure 4:** Plasma estrone sulfate concentrations in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Data are expressed as mean  $\pm$  S.E.M. The shaded area indicates the interval where  $p < 0.05$ : Day 8 through Day 64.





**Figure 5:** Plasma anti-Müllerian hormone (AMH) concentrations in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Data expressed as mean values  $\pm$  S.E.M.



Response to GnRH stimulation was evaluated as area under the curve (AUC) for FSH, LH and testosterone before and during the treatment period. There were no differences in gonadotropin or testosterone responses between groups pre-treatment and during treatment in controls. However, acyline-treated stallions did not show a rise in FSH ( $p < 0.0005$ ), LH ( $p < 0.0003$ ) or testosterone ( $p < 0.0007$ ) in response to GnRH compared to the increase in those hormones during the pre-treatment period or relative to control values (Table 1).

**Table 1:** Response of FSH, LH and testosterone (AUC) to GnRH challenge pre-treatment and during treatment.

AUC	Pre-treatment		During Treatment	
	Control	Acyline	Control	Acyline
<b>FSH</b> (ng/mL x 120min)	11.5	19.4	12.0	6.8*
<b>LH</b> (ng/mL x 120min)	10.3	21.5	11.5	0.7*
<b>Testosterone</b> (pg/mL x 120min)	3364.6	5393.3	4143.2	373.7*

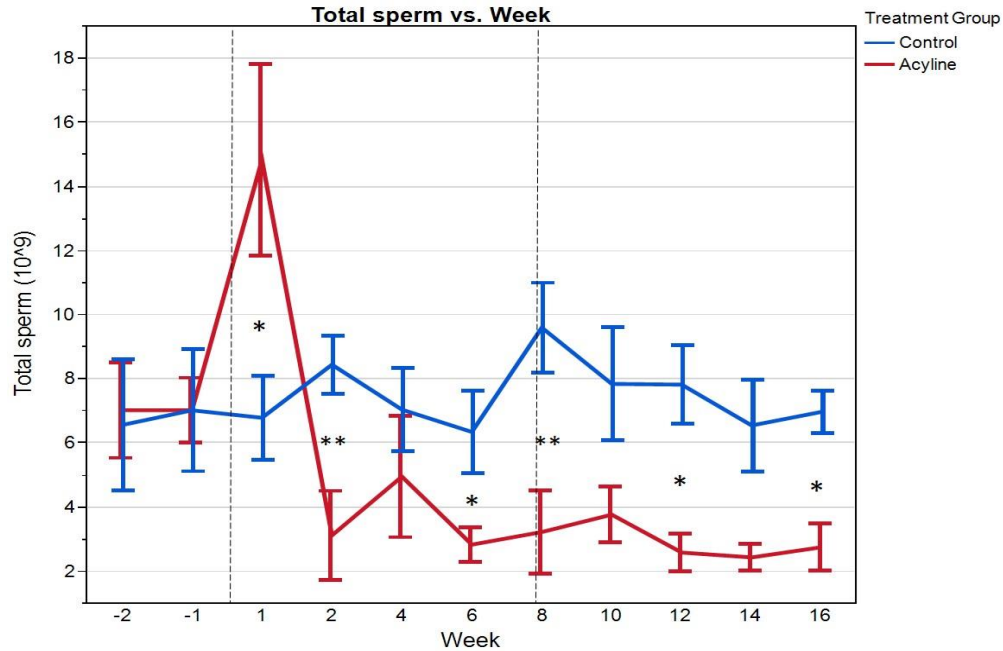
\* indicates statistical difference ( $p < 0.05$ ) in the same line

### ***B. Seminal parameters***

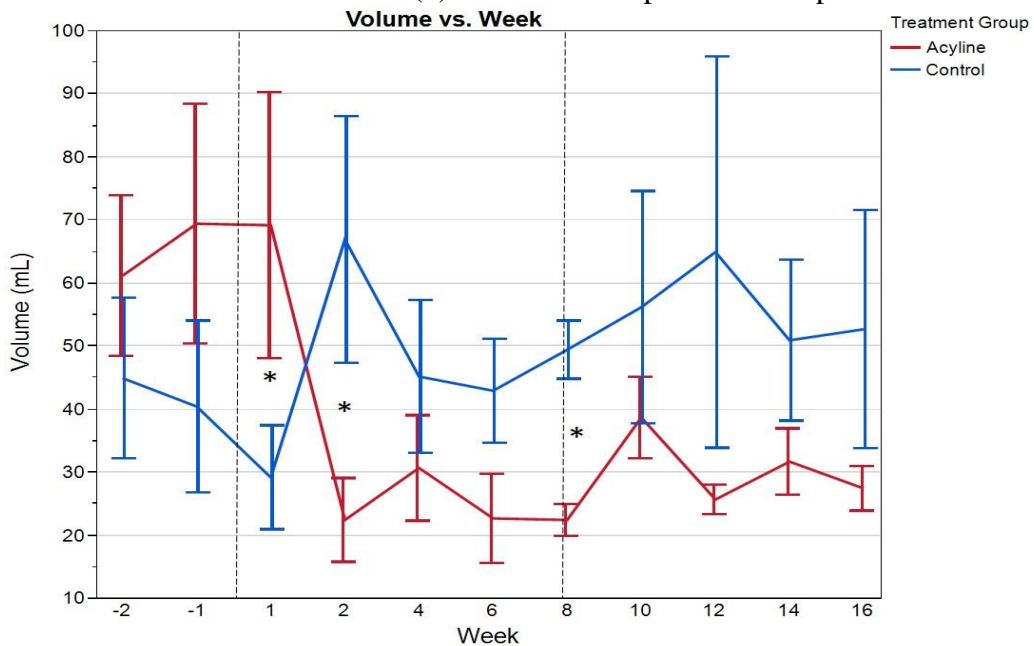
There was an effect of time ( $P < 0.0001$ ) and a time-by-treatment interaction ( $P < 0.0001$ ) on total sperm number. Total sperm number increased in treated stallions at week 1, but then declined and remained lower in treated than control stallions for the duration of the study ([Fig. 6](#)).

There was also an effect of time ( $P < 0.0001$ ) and time-by-treatment ( $P < 0.0001$ ) on semen volume. Gel free volume was higher in treated stallions in week 1 of treatment and decreased to lower than that for controls at weeks 2 and 8 but means were similar on all other weeks ([Fig. 7](#)). Concentration of sperm did not differ between treated and controls during any of the weeks of collections.

**Figure 6:** Total sperm numbers in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Sperm count for DSO ejaculate shown as mean  $\pm$  S.E.M. Dashed lines represent treatment start and finish date. (\*) indicates time points where  $p < 0.05$ ; (\*\*) indicates  $p < 0.005$ .

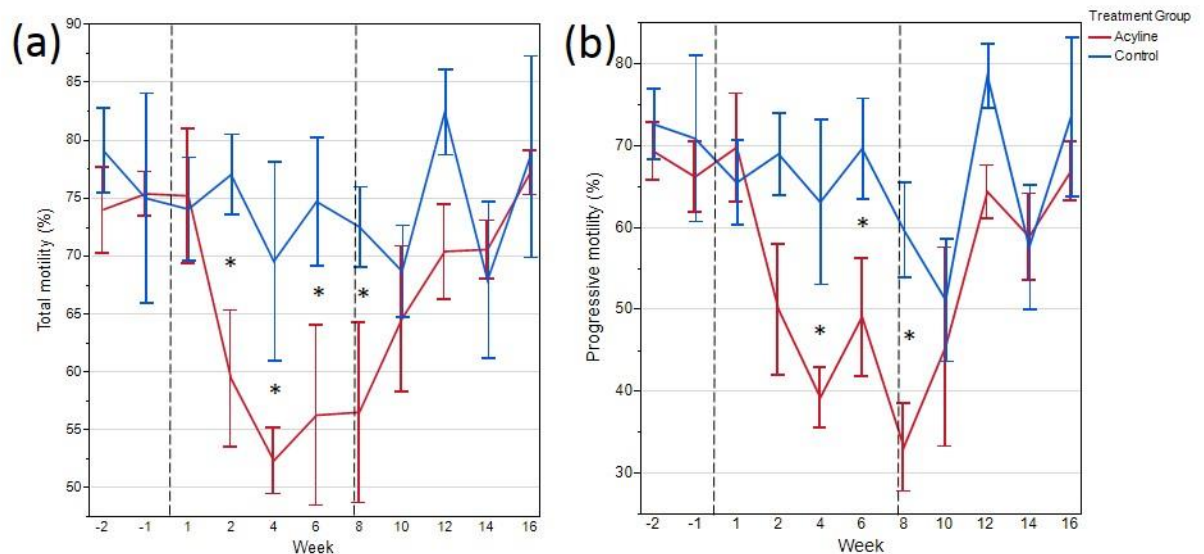


**Figure 7:** Gel-free ejaculate volume in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Dashed lines represent treatment start and finish date. Volume on DSO ejaculates shown as mean  $\pm$  S.E.M. (\*) indicates time points where  $p < 0.05$ .



Total sperm motility was lower in treated than control stallions by week 2 ( $p<0.05$ ) and remained lower than controls through the end of treatment. A similar effect was observed in progressive motility, which was lower in treated than control stallions from week 2 ( $p<0.0028$ ) to week 8 ( $p<0.003$ ), with the exception of week 4 ( $p<0.06$ ). Post-treatment, both sperm motility parameters were similar in treated and control stallions (Fig. 8). Sperm morphology evaluation did not yield any significant changes in number of morphologically abnormal spermatozoa. The number of round spermatogenic cells increased at weeks 2 and 8 in the treated group (Data not shown).

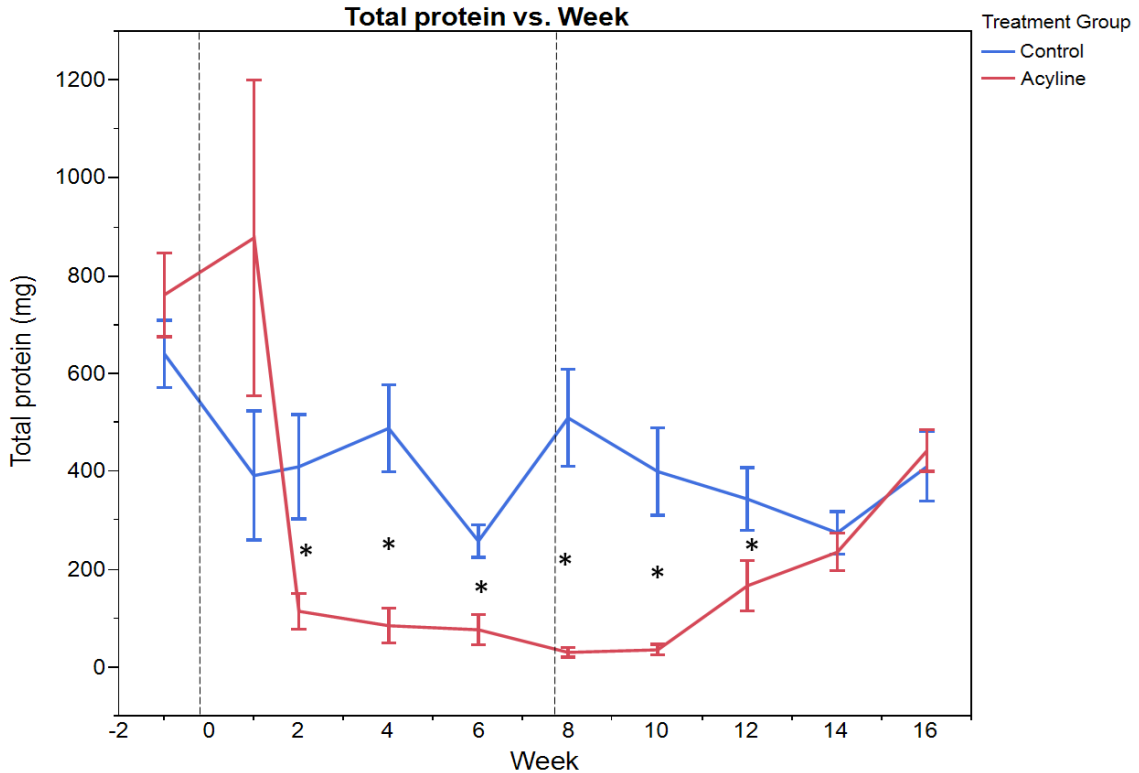
**Figure 8:** Sperm motility in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Total motility (a) and progressive motility (b), at DSO ejaculates, shown as mean  $\pm$  S.E.M. Dashed lines represent treatment start and finish date. (\*) indicates time points where  $p<0.05$ .



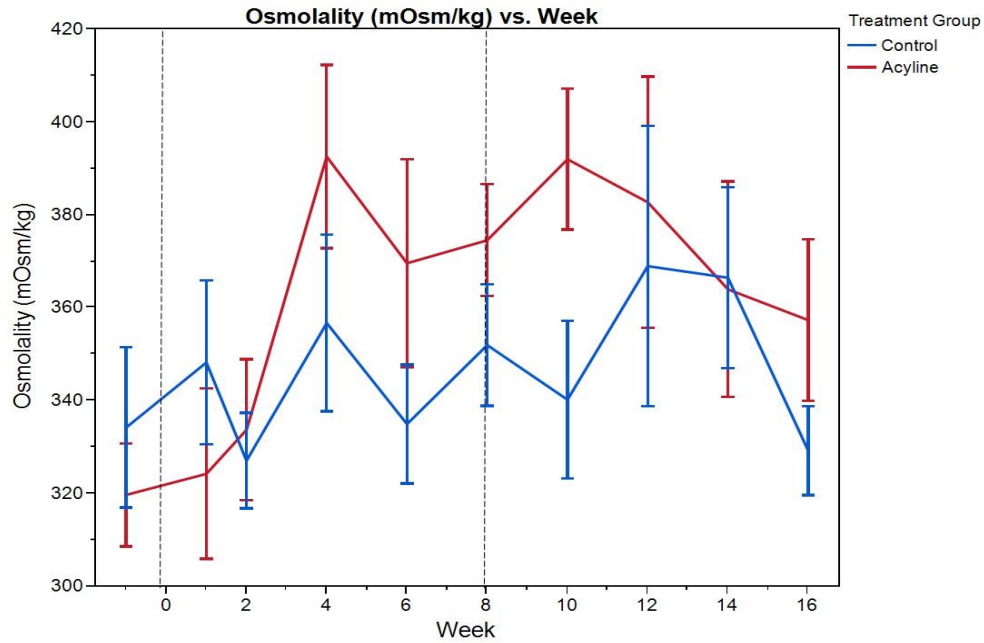
Total seminal plasma protein was lower from week 2 of treatment through the end of treatment and until the fourth week post-treatment ( $p<0.05$ ); total seminal plasma protein was similar in both groups by week 6 of the recovery period and throughout the rest of the study (Fig. 9). Osmolality of seminal plasma samples was not changed due to treatment

( $p < 0.1649$ ). (Fig. 10). There was a time effect ( $p < 0.008$ ) on osmolality with an increase during the treatment period and the beginning of the post-treatment period. Since there was no treatment by time effect, osmolality was plotted in the mixed model with number of mounts to ejaculation as a possible explanation for the unspecific fluctuation over time but no association was found between the two variables (data not shown).

**Figure 9:** Seminal plasma total protein content in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Weekly averages shown as mean values  $\pm$  S.E.M. Dashed lines represent treatment start and finish date. (\*) indicates time points where  $p < 0.05$ .



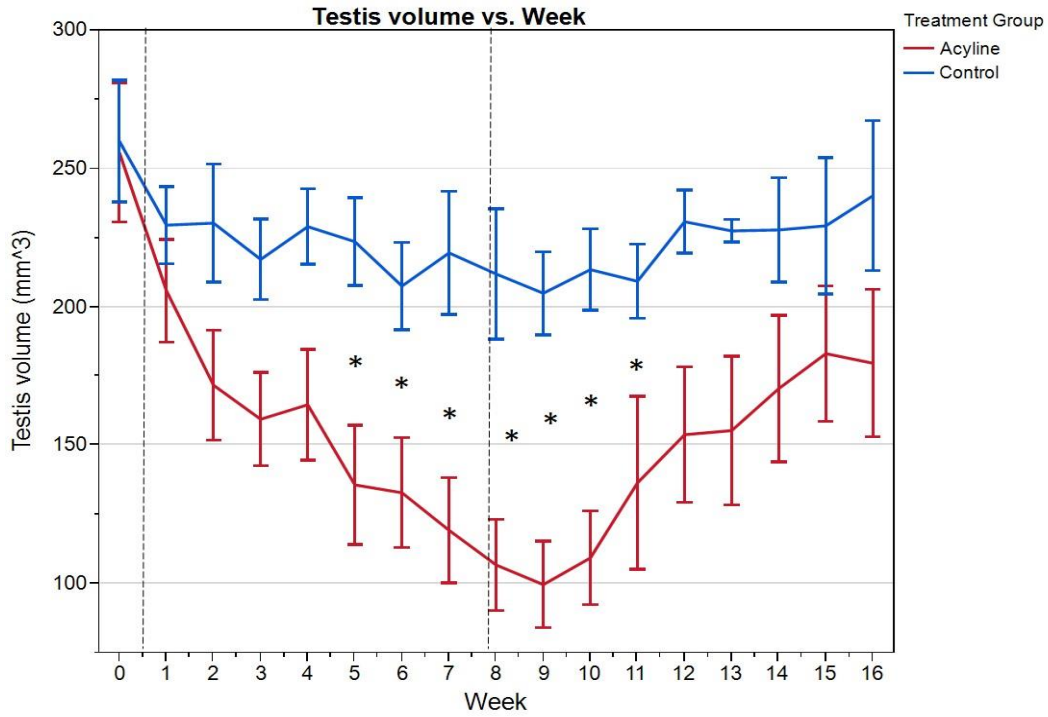
**Figure 10:** Seminal plasma osmolality in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Weekly averages shown as mean values  $\pm$  S.E.M. Dashed lines represent treatment start and finish date.



### C. Total testicular volume

There was an effect of time ( $P < 0.0001$ ) and a time-by-treatment interaction ( $P < 0.0001$ ) on testis volume. Mean testis volume was lower in acyline-treated stallions by week 5 and remained lower throughout the treatment period (Fig 11). Testis volume increased in treated stallions during the post-treatment period and was not different from controls by week 12.

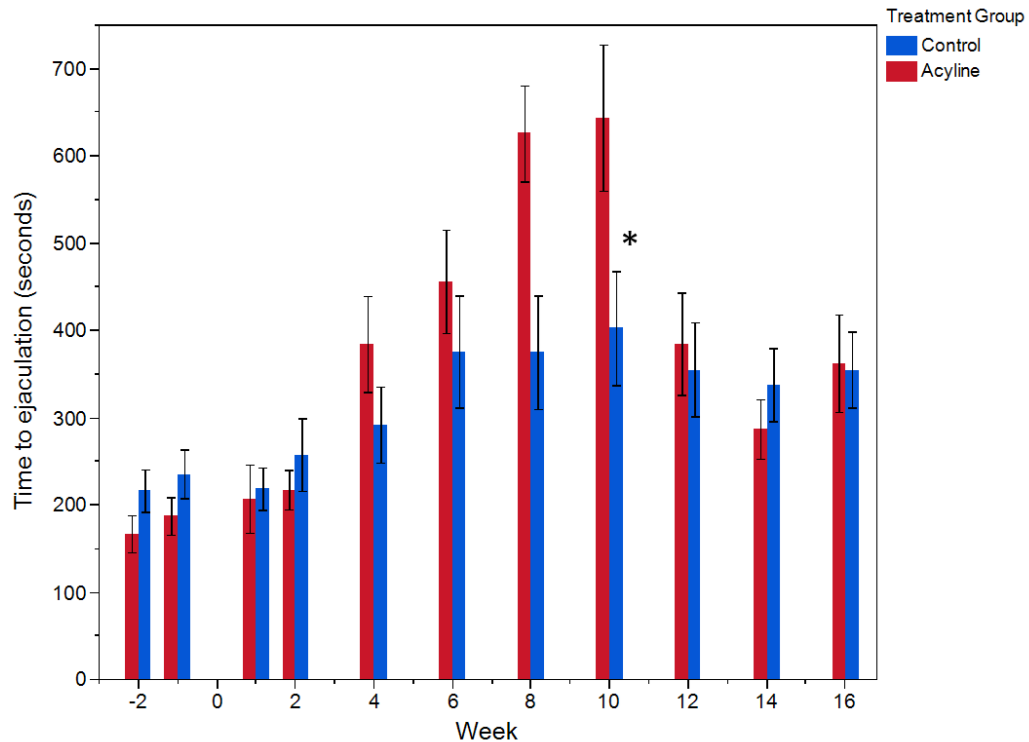
**Figure 11:** Total testicular volume in acyline-treated and control stallions. Acyline-treated stallions received 330mg/kg of acyline every five days and controls received vehicle alone. Weekly values shown as mean  $\pm$  S.E.M. Dashed lines represent treatment start and finish date. (\*) indicates time points where  $p < 0.05$ .



***D. Reproductive behavior***

There was no effect of acyline treatment on time to erection or number of mounts. Towards the end of the treatment and beginning of recovery period treated stallions tended ( $p < 0.08$ ) to require longer to achieve an erection. A similar trend was seen for number of mounts required for ejaculation. At week 10, treated stallions required longer time ( $p < 0.05$ ) to achieve ejaculation (Fig. 12). A time effect was observed on all of the three variables ( $p < 0.0001$ ).

**Figure 12:** Time to ejaculation over time. Mean times, on weekly averages, are shown  $\pm$  S.E.M. Data not transformed.



### 3.5. Discussion

The primary mechanism of action of GnRH antagonists is a competitive blockade of the GnRH receptor in pituitary gonadotrophs precluding substantial occupation and stimulation by endogenous GnRH (Heber et al., 1982; Conn and Crowley, 1991; Huirne and Lambalk, 2001) which causes an immediate inhibition. This is in contrast to GnRH agonists, which require repeated administration to achieve this effect (Clayton and Catt, 1980; Conn and Crowley, 1991) via down-regulation of GnRH receptors (Schally, 1999). Our findings clearly show a suppression of the hypothalamic-pituitary-gonadal axis in stallions treated with acyline. Down-regulation of the HPG axis was evident by a reduction in gonadotropins, testicular steroids, as well as diminished response to GnRH stimulation



during treatment with acyline. The suppression of the HPG axis was associated with changes in testis volume, DSO, total seminal plasma protein as well as sperm motility and, to some extent, behavioral modification. Discontinuation of treatment enabled the axis to resume functionality based on return of FSH, LH, estrone sulfate and testosterone to normal circulating concentrations, followed by resumption of the majority of the seminal and testicular parameters.

Administration of other GnRH antagonists has been used in the stallion to examine downregulation of the HPG axis. A single dose of Antarelix to stallions resulted in no suppression of testosterone, a slight suppression of LH and a marked reduction in FSH and estradiol (Hinojosa et al., 2001), although when Antarelix or Cetrorelix were administered twice daily for 5 weeks (Fortier et al., 2002), testosterone was decreased until a week after the end of treatment. Findings on testosterone suppression during treatment with acyline in stallions agrees with those in men (Herbst et al., 2002; Herbst et al., 2004) and dogs (Garcia Romero et al., 2009), and levels of testosterone were consistent with concentration of geldings – lower than 130-200pg/mL (Dowsett et al., 1996; Malmgren et al., 2001). The depth of suppression of the HPG axis during acyline treatment was demonstrated by a lack of response in FSH, LH or testosterone to GnRH stimulation in treated stallions compared to controls and to pre-treatment response of treated stallions. These results are similar to what has been reported in dogs (Garcia Romero et al., 2012b). Previous studies investigating downregulation of HPG in stallions have utilized different approaches, but acyline provides a more rapid and consistent downregulation when compared to altrenogest (Brady et al., 1997; Miller et al., 1997; Squires et al., 1997; Johnson et al., 1998; Heninger et al., 2001) and anti-GnRH vaccination (Dowsett et al.,

1996; Malmgren et al., 2001; Clement et al., 2005; Turkstra et al., 2005; Burger et al., 2006; Janett et al., 2009).

Anti-Müllerian hormone is produced by Sertoli cells and differentially expressed in prepubertal, postpubertal and cryptorchid stallions (Ball et al., 2008). AMH peripheral concentration decreases with sexual maturity, though secretion into circulation remains high in cryptorchids (Claes et al., 2013). Given the suppression in Leydig cell activity and spermatogenesis of acyline-treated stallions, one can assume that changes in FSH and steroid hormones are also having an impact on Sertoli cell metabolism. Not much is known about the regulation of AMH in the male, though the lack of effect of HPG suppression on AMH concentrations may suggest that regulatory mechanisms for AMH secretion are apart from the HPG axis.

Reduced sperm output was somewhat expected given the endocrinological changes and is in accordance with previous work using GnRH antagonists in the stallion where a drop of almost 50% in sperm output after a single injection was noted (Hinojosa et al., 2001). In the study by Fortier *et al.* there was a near absence of sperm in the ejaculate at the end of treatment (Fortier et al., 2002). In dogs, acyline did not suppress spermatogenesis completely in all of the subjects after a single injection, but it was sufficient to cause azoospermia in four of seven treated dogs (Valiente et al., 2007). In the present study, even though reproductive hormones, particularly steroids, were strongly suppressed, sperm numbers were still about one third of those during pre-treatment. It is intriguing that sperm output of stallions increased in the first week of acyline treatment then dropped in the following week. Since extra-gonadal reserves had been depleted, this may indicate a hastening of the outflow of sperm cells from spermiation until reaching the cauda

epididymis. There was a 10-15% loss in total motility and 10-25% drop in progressive motility, with total motility reaching a nadir of about 35%. Conversely, Hinojosa *et al.* (Hinojosa et al., 2001) did not find effects of the antagonist on sperm motility following a single injection of GnRH antagonist. In humans, contraception has been cited as a potential use for acyline, though it seems that the partial persistence of motile sperm in the ejaculate of treated stallions precludes the use of this antagonist as a contraceptive in the male equine. The decreased testicular volume during treatment with acyline is in line with the sperm numbers present in the ejaculates and was also reported in acyline-treated dogs (Valiente et al., 2007).

The appearance of round spermatogenic cells was also noticed in dogs receiving acyline (Valiente et al., 2007), and in stallions given Antarelix and Cetrorelix, in which testicular histology exhibited loss of round spermatogenic cells into the lumen 8 weeks after administration (Hinojosa et al., 2001). The increased number of round cells in acyline treated stallions at weeks two and eight, in particular, seems to agree with these previous publications. Further explanation for these findings may come from studies on GnRH antagonist in rats. As observed histologically, inhibition of meiosis in a high percentage of the seminiferous tubules resulted in only 22% of seminiferous tubules containing elongated spermatids in a 30-day long treatment (Szende et al., 1990), while in a 60-day long treatment spermatocytes were the most advanced germ cell form detected, signifying a spermatogenic arrest (Bokser et al., 1991).

Normal function of the accessory sex glands of stallions (Gebauer et al., 1976; Thompson Jr. et al., 1980) and the epididymis of various species (Amann, 2011), depends on testosterone and estrogen (Thompson Jr. et al., 1980; Parlevliet et al., 2006), and these

structures are responsible for producing the protein components of the seminal plasma (Weber and Woods, 1993; Koskinen et al., 2002; Amann, 2011). Thus, the transient decrease observed in total protein content of the seminal plasma of treated stallions seems logical.

After castration in the study by Thompson and colleagues (Thompson Jr. et al., 1980), the desire to mount was retained for longer than the ability of stallions to ejaculate. Despite the strong suppression of both testosterone and estrone sulfate seen in the present study with acyline, changes in libido were only evident in respect to the increased time to ejaculation at week 10, while there was no significant change in time for erection. That seems to agree with the idea that there are at least two aspects of sexual behavior displayed by stallions: desire to mount and thrust, and ability to ejaculate (Thompson Jr. et al., 1980; Wallach et al., 1983).

Though the study was not designed to evaluate seasonality, there was a time effect in a number of parameters, which may be explained by the months when the experiment was conducted. The general increase in time to erection and time to ejaculation in the fall months agrees with previous reports (Pickett et al., 1976; Thompson et al., 1977; Clay et al., 1987). Variations due to season have been reported in testosterone (Berndtson et al., 1974; Thompson et al., 1977; Clay et al., 1987; Claes et al., 2013), testicular size (Clay et al., 1987), sperm output (Pickett et al., 1976; Thompson et al., 1977; Clay et al., 1987) and also for AMH (Claes et al., 2013).

After discontinuation of treatment the persistence of a depression in LH, FSH, testosterone and estrone sulfate is worth noting. The suppression of testosterone for at least nine days after last treatment showed that the effect of serial administrations of the GnRH

antagonist led to a suppression of the HPG axis for about double the time it did after a single injection. A more extended suppression in FSH and LH following a series of GnRH antagonist injections compared to single injection was also seen in humans (Behre et al., 1997). It was demonstrated in rats that, in fact, the amount of GnRH receptors (Srkalovic et al., 1990; Bokser et al., 1991; Halmos et al., 1996; Murase et al., 2005) and GnRHr mRNA (Pinski et al., 1996; Murase et al., 2005) were decreased due to GnRH antagonist treatment. This contrasts with the previous idea that GnRH antagonists exerted their effects purely by competition for GnRH receptors. From a practical standpoint, doses of acyline could be progressively reduced during a treatment with repeated doses, or low doses given after a high, loading dose, as was suggested for GnRH antagonists in humans (Behre et al., 1997), thus possibly reducing the cost of treating a stallion

As observed in the present study, a rebound effect on FSH and LH after discontinuation of GnRH antagonist treatment was noticed in men (Behre et al., 1997). After a single injection of acyline in dogs, a rebound was noticed in FSH, but not LH (Garcia Romero et al., 2009); a testosterone peak was also noticed after recovery of the suppression in dogs (Garcia Romero et al., 2009; Garcia Romero et al., 2012b), but no significant rebound occurred in testosterone concentration in acyline-treated stallions.

While sperm output was study the only parameter in the present that did not return to normal levels during the recovery period, recrudescence of the testicular parenchyma might serve as an indication that sperm production would also return to baseline numbers. As postulated by Berndtson and co-authors (Berndtson et al., 1974), an interval of approximately 60 days is needed between the testicular insult affecting spermatogenesis in the stallion and appearance of a maximal response measured as sperm output. Even though

the recovery period (72 days) was long enough for production of new sperm and epididymal transit (Amann, 2011), it may not have been sufficiently long if one takes into consideration the delay in return of gonadotropins and steroids to normality. Nevertheless, a follow up in the following breeding season, as part of another experiment 7 months after the end of the recovery period, showed that all of the sperm numbers at DSO, obtained after every-other-day collections for 14 days, had returned to normal, though it is impossible to precisely determine when the recovery in sperm production occurred. Hints for the recrudescence of spermatogenesis may be gathered from reported return of sperm output to normality at 75 days after the end of treatment with Antarelix and Cetrorelix in stallions (Fortier et al., 2002), and by evidence that sperm germ cell differentiation, stalled due to GnRH antagonist in rats, had fully recovered 90 days after cessation of treatment (Bokser et al., 1991).

Change in behavior due to acyline, though slighter, recovered more readily than with anti-GnRH vaccination (Burger et al., 2006; Janett et al., 2009). A survey published by Line and others (Line et al., 1985) indicated that after surgical castration at least 30% of stallions continued to display some kind of aggressive behavior after castration. That agrees with the notion that aggressive behavior in stallions is not solely caused by reproductive behavior (McDonnell, 1986, 2000). If gonadal removal itself cannot resolve all of the behavioral issues in stallions, it seems that a pharmacological approach with reversible effects on libido, might be preferable to castration in certain situations. Other experiments are necessary to investigate if the effects of acyline on sexual behavior are sufficient for the desired application in resolving aggressive behavior in a training or competition environment.

Surgical castration of EAV shedding stallions resulted in cessation of virus shedding in semen as early as 4 days, though other stallions required over 25 days (Little et al., 1991). In our study, the fifth day after acyline injections, when the next dose was to be administered, was part of the assay schedule. Taking aside physiological fluctuations in circulating testosterone during the day, those time-points are presumably the lowest suppression by the GnRH antagonist and, consequently, the highest concentrations of testosterone during acyline treatment. Therefore, we believe that the gelding-like testosterone concentrations shown as result of acyline treatment truthfully represent the levels of this steroid throughout the entire treatment period. While, unfortunately, we had no opportunity to use EAV stallions for this trial, it can be speculated, based on immuno-castration (Burger et al., 2006) and GnRH antagonists (Fortier et al., 2002) that the reduction on testosterone levels brought by acyline should be adequate to promote virus clearance in the semen of EAV persistent carriers.

In conclusion, acyline treatment rapidly down regulates the HPG axis in stallions. Reduction in gonadotropins, testosterone and estrone sulfate was followed by a decline in sperm output and motility, seminal plasma proteins and testicular volume, while there was an increase in round spermatogenic cells present in the ejaculate of treated stallions. Lastly, sexual behavior was affected by an increase in time for ejaculation in acyline-treated stallions. Suppression of the HPG axis by acyline was also demonstrated by failure of stallions under treatment to respond to exogenous GnRH stimulus. Upon cessation of treatment, most of the affected parameters returned to control values within 72 d; though sperm output may require longer to recover. Further experiments are necessary to

determine the applicability of acyline as treatment for EAV carrier stallions and for behavior modulation.



## **Chapter Four: Summary and conclusions**

The administration of 330µg/kg acyline every five days for 50 days suppressed the hypothalamic-pituitary-gonadal (HPG) axis in stallions. Down-regulation of the HPG axis was reflected by a reduction in FSH, LH, testosterone and estrone sulfate. The lack of responsiveness to GnRH stimulation during treatment with acyline illustrates the strength of the inhibition of the HPG axis due to acyline treatment. This GnRH antagonist treatment also caused a decrease in testis volume, total sperm output, sperm motility and total seminal plasma protein content. That indicates the impairment of the activity of the testes as well as epididymides and accessory sex glands. Finally, to some extent, certain sexual behavioral traits seemed to be affected by acyline administration. A transient increase in round spermatogenic cells in semen was observed, which supports the presumed arrest on spermatogenic meiosis. Discontinuation of treatment enabled the HPG axis to resume its activity based on a return of FSH, LH, estrone sulfate and testosterone to normal circulating concentrations, followed by resumption of the testicular and most of seminal parameters investigated within a 72-day recovery period.

From the day following the first acyline injection, testosterone was reduced to gelding levels throughout the treatment period in acyline-treated stallions. Data derived from investigations regarding castration, immuno-castration and GnRH antagonists in EAV carrier stallions serve as reference on the suppression of androgens to induce clearance from EAV persistently infective state in stallions. Even though the stallions used in this experiment were not infected with EAV to testify this hypothesis, the reduction on testosterone brought by acyline might be adequate to promote clearance of virus presence in the semen of EAV persistent carriers. Despite the marked suppression in hormonal

profiles, spermatogenesis was not completely interrupted and sperm motility was only partially lost. Changes seen in libido, as measured by latency for ejaculation, seem to agree with the idea that there are at least two aspects of sexual behavior displayed by stallions: desire to mount and thrust, and ability to ejaculate. Since physical castration appears not to resolve all of the cases in which exacerbated sexual behavior is an issue, a chemical approach for castration cannot be expected to have better results and a treatment with GnRH antagonists may be appropriate as a substitute. Upon cessation of treatment, testosterone required longer to return to baseline than after a single injection, which may be explained by the down-regulation of GnRH receptors on the gonadotrophs. From a practical perspective, that may indicate a potential for decreasing the dosage of GnRH antagonist as treatment progresses while same suppressive effect is achieved.

In conclusion, acyline provided a more rapid and consistent downregulation of the HPG than various previous approaches used in stallions. The transient suppression was characterized by decline in gonadotropins and steroid hormones, which resulted in decrease of testicular volume and seminal parameters – including sperm output and motility, increase in round germ cells and decrease in seminal plasma proteins. Sexual behavior was also affected by acyline. After discontinuation of treatment, all of the parameters returned to their pretreatment levels. Further studies with acyline are necessary to determine the length of treatment that would be sufficient to reliably clear EAV from shedding stallions without returning to shedding state after treatment discontinuation. As a treatment for behavior modification future studies would include stallions and young colts at training or competition treated with acyline to verify the extent of its effects and usefulness in those settings.

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## VITA

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#### **Place of Birth:**

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#### **Academic Degrees:**

2010                      Medico Veterinario (DVM equivalent), granted by Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

#### **Professional Experience:**

Aug 2012 – Feb 2015    Gluck Equine Research Center, Department of Veterinary Science  
University of Kentucky, Lexington  
*Graduate student /Research assistant*  
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Oct 2011 - 2012        Private practice: services on equine field care and reproduction, in Porto Alegre (and surrounding area), RS, Brazil.

Dec 2011 - Mar 2012    Research project conduction at Reprolab, UFRGS, Porto Alegre, with Dr. Rodrigo Mattos.

Sep2010 - Jan 2011     Services in equine field care and reproduction partially associated with Dr. Gustavo H. Z. Winter.

#### **Awarded Research Scholarships**

2009                      Study of Endometritis  
Scholarship in Scientific Initiation Program  
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2007                      Study of *In Utero* semen transportation.  
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2005                      Study of Reproductive parameters of pony mares  
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## Abstracts Published in Congress Proceedings or presentations

**Effects of a third generation GnRH antagonist on reproductive parameters in the stallion.** Havemeyer Workshop – Second R. M. Kenney Symposium, September 2014, Winterthur, DE/West Chester, PA

GM Davolli, BA Ball, A Esteller-Vico, A Claes, I Canisso, CE Fedorka, EM Woodward, MH Troedsson, E L Squires. **Effects of a third generation GnRH antagonist on reproductive parameters in the stallion.** In: Proceedings of the 2014 Society for Theriogenology annual conference, Portland, OR.

G.M.Davolli, G.C.Camozzato, M.M.Figueiredo, H.B.A.Bastos,N. Cazales,R.C. Mattos. **Effect of different sperm concentration methods on sperm quality and recovery rate (Poster).** Sixth International Symposium on Stallion Reproduction, Vienna, Austria, 2012.

Neves, A. P.; Bustamante, I. C.; Wolf, C. A.; Castro, F. S.; Richter, G.; Motta, F. F.; Davolli, G. M., Camozzato, G. C.; Agne, G. F.; Mattos, R. C. **Alguns aspectos sobre o periparto, neonato e cio do potro em éguas miniatura.** In: XI Conferência Anual da ABRAVEQ, 2010, São Paulo, Revista Brasileira de Medicina Veterinária Equina (suplemento). São Paulo: ABRAVEQ, 2010. v. 29, p. 301.

Davolli, G. M.; Wolf, C.; Malschitzky, E.; Agne, G. F.; Jobim, M. I. M.; Mattos, R. C.. **Endometrite persistente pós-cobertura: efeito do tratamento com corticosteróides sobre o número de bandas protéicas da secreção uterina de éguas suscetíveis infectadas com *Streptococcus Zooepidemicus*.** In: XXI Salão de Iniciação Científica - UFRGS, 2009, Porto Alegre.

Wolf, C.; Malschitzky, E.; Davolli, G. M.; Jobim, M. I. M.; Mattos, R. C.. **Endometrite persistente pós-cobertura: efeito do tratamento com corticosteroids sobre o número de bandas protéicas da secreção uterina de éguas suscetíveis.** In: Congresso Brasileiro de Reprodução Animal,, 2009, Belo Horizonte. Anais do Congresso Brasileiro de Reprodução Animal, 18. Belo Horizonte : CBRA, 2009. p. 313-313.

Davolli, G. M.; Agne, G. F.; fiala, S. M.; Katila; T., Jobim, M. I. M.; Gregory, R. M.; Mattos, R. C.. **Distribuição espermática no oviduto e útero de éguas em diferentes momentos após inseminação artificial..** In: XX Salão de Iniciação Científica - UFRGS, 2008, Porto Alegre. Livro de Resumos do XX Salão de Iniciação Científica, 2008. p. 181-181.

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Davolli, G. M.; canibal, M. C.; Petrucci, B.; Neves, A. P.; Castro, F. S.; Borba, E. V. C.; Rocha, E. S.; gregory, R.M.; Mattos, r. C.. **Caracterização da dinâmica folicular durante o ciclo estral de éguas mini-pôneis.** In: XVIII Salão de Iniciação Científica - UFRGS, 2006, Porto Alegre.

Davolli, G. M.; Möller, G.; Trein, C. R.; Brito, E. L. R.; Gregory, R. M.; Mattos, R. C.. **Seleção de sêmen equino através de centrifugação e gradiente de densidade.** In: XVII Salão de Iniciação Científica - UFRGS, 2005, Porto Alegre.

### **Peer-reviewed Publications**

I. F. Canisso, B. A. Ball, C. Cray, N. M. Williams, K. E. Scoggin, G. M. Davolli, E. L. Squires, M. H. Troedsson. **Serum Amyloid A and Haptoglobin Concentrations are Increased in Plasma of Mares with Ascending Placentitis in the Absence of Changes in Peripheral Leukocyte Counts or Fibrinogen Concentration.** *American Journal of Reproductive Immunology*, 2014; 72:376-385.

I. F. Canisso, B. A. Ball, M. H. Troedsson, E. S. M. Silva, G. M. Davolli. **Decreasing pH of mammary gland secretions is associated with parturition and is correlated with electrolyte concentrations in prefoaling mares** *Veterinary Record*, 2013; 173(9):218.

A. P. Neves, I. C. Bustamante, C. A. Wolf, F. S. Castro, G. Richter, F. F. Motta, G. M. Davolli, G. C. Camozzato, G. F. Agne, R. M. Gregory, R. C. Mattos. **Some aspects of peripartum, neonate and foal heat in mini pony mares.** *Pferdeheilkunde*, 2010; 26: 36-38.

C. A. Wolf, E. Malshitzky, G. M. Davolli, M. I. M. Jobim, R. C. Mattos. **Persistent post-breeding endometritis: effects of corticosteroid treatment on the number of protein bands from uterine endometrial fluid of susceptible mares.** *Pferdeheilkunde*, 2010; 26: 22-24

### **Publications in Preparation:**

1. **Davolli, G.D.;** Ball, B.A; Esteller-Vico, A, Claes, A.N.; Canisso, I.F. Fedorka, C.E; Woodward, E.M.; Troedsson, M.H.T.; Squires, E.L. Reversible downregulation of hypothalamic-pituitary-gonadal axis in the stallion with a GnRH antagonist