



# Reversible downregulation of the hypothalamic-pituitary-gonadal axis in stallions with a novel GnRH antagonist

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## ABSTRACT

The GnRH antagonist, acyline, has not yet been investigated in the stallion. Our study aimed to: (1) evaluate the downregulation of the stallion hypothalamic-pituitary-gonadal axis by acyline through assessment of seminal parameters, testicular volume, and sexual behavior; (2) assess hormonal response of acyline-treated stallions to GnRH stimulation; and (3) verify reversibility after treatment. Stallions were assessed pretreatment and subsequently treated (every five days) for 50 days: acyline ( $n = 4$ ; 330  $\mu\text{g/kg}$  acyline) or control ( $n = 4$ , vehicle). The stallions were then monitored for 62 days after the last day of treatment. Treatment-induced declines ( $P < 0.05$ ) in FSH, LH, testosterone, and estrone sulfate. Gonadotropins and testosterone returned to control values within 9 days, and estrone sulfate by 14 days, after discontinuation of treatment. Acyline-treated stallions failed to respond with a rise in FSH, LH, and testosterone after exogenous GnRH stimulation (gonadorelin) at Day 46 of treatment compared to pretreatment stimulation and control stallions. Decreases ( $P < 0.05$ ) were observed in total sperm numbers and motility (week 2) in acyline-treated stallions, as well as total seminal plasma protein (week 2) and testicular volume (week 5). Over the course of the study, the time to erection, time to ejaculation, and number of mounts increased ( $P < 0.0001$ ) across both groups of stallions; however, there was no effect of treatment or treatment by time interactions on these parameters. Testicular volume, and most seminal parameters regained normal levels within 62 days after treatment ended; on follow-up, sperm output of acyline-treated stallions was regained within 7 months after the end of experiment. In conclusion, acyline reversibly suppresses the stallion hypothalamic-pituitary-gonadal axis.

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## 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 elimination of the carrier state of equine arteritis virus (EAV) and alteration of sexual/aggressive behavior. Over the last decade, equine viral arteritis outbreaks on different continents acted to reemphasize the important epidemiologic role that persistently infected carrier stallions play in the dissemination of EAV through infective semen [1–4]. Persistent infection with

EAV is androgen dependent [5–7], and removal of endogenous testosterone results in termination of the persistent carrier state [5]. Endocrine manipulations as an aid to behavior modulation of intact stallions with excessive/unruly sexual or aggressive behavior is also a potential application for downregulation of the HPG axis. Orchiectomy seems to solve most of the cases in which such behavior may compromise performance of colts during training or competition [8]. However, surgical castration precludes future use for breeding after retirement from a successful sports career. Both elimination of the persistent EAV carrier status and modulation of male behavior in stallions would benefit from reversible methods to suppress reproductive steroids.

Several approaches have been investigated to downregulate the HPG axis in the stallion, including treatment with altrenogest [9–11] and immunization against GnRH [12–16]. However, the studies to date have been unable to demonstrate that stallions fully recover from prolonged impairment of reproductive function by either method.

GnRH antagonists lead to direct inhibition rather than activation of the GnRH receptor through its sole occupancy [17–19]. A study by Hinojosa et al. [20] reported that a single injection of GnRH antagonist, Antarelix, in pony stallions induced a decrease of peripheral gonadotropins and estradiol concentrations; however, circulating testosterone or libido were not affected. The use of antarelix and a second GnRH antagonist, cetrorelix, daily for 35 to 38 days in EAV carrier stallions [21] achieved a reversible decrease in testosterone concentration and a transient reduction in sperm number and quality.

A potent third-generation GnRH antagonist, acyline, was described in 1995 [22]. Initial trials in men have shown that acyline decreases FSH and LH for 15 to 20 days, and peripheral testosterone concentration to baseline for 2 weeks after a single injection of 300 µg/kg [23]. In male dogs, a single dose of acyline (330 µg/kg) decreased FSH, LH, and testosterone concentrations for at least 9 days [24]. Stud dogs treated with acyline had reduced sperm numbers for 6 weeks and severely reduced libido and erectile dysfunction for 4 weeks. Although recovery from effects on semen parameters was not reported in the 8-week follow-up, libido, and erection capability recovered during this period [25]. A more recent study showed that male dogs under acyline treatment were refractory to repeated stimulations with the GnRH superagonist, buserelin, as indicated by a lack of testosterone response at 1, 2, and 3 hours after agonist injections [26].

Although there are no reports of acyline administration to stallions, acyline administered (~10 µg/kg) to cyclic pony mares resulted in decreased peripheral FSH concentrations and retarded growth of the dominant follicle [27,28]. We hypothesized that acyline administration would result in downregulation of the HPG axis in stallions and that 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 parameters, sexual behavior, and testicular volume 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.

## 2. Material and methods

### 2.1. Animals

All animal experiments were 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 years) were housed during most of the day and entire night in individual box stalls at the Maine Chance Farm (University of Kentucky, Lexington, KY, USA). Stallions received hay and water ad-libitum in addition to combined whole oats and commercial concentrate (0.4% BW) and had access to salt and mineral sources. After samples were taken for each given day, stallions were turned out into small-fenced paddocks (at least 5 days a week) with visual contact with mares in nearby pastures.

### 2.2. Acyline dose determination

A preliminary study was conducted to compare the effect of two different doses of acyline [Ac-D2Nal-D4Cpa-D3Pal-Ser-4Aph(Ac)-D4Aph(Ac)-Leu-Lys(Ipr)-Pro-DAla-NH<sub>2</sub>] on serum testosterone concentrations. Two stallions (not included in the main study) received 100 µg/kg and 330 µg/kg acyline in 5% mannitol as a single intramuscular administration, one stallion for each dose. Blood was collected daily for 10 days for determination of serum testosterone concentrations. The stallion treated with the higher dosage presented 5 days of testosterone suppression (<100 pg/mL) after a single administration. Based on this preliminary study, a dosage of 330 µg/kg at 5-day intervals was chosen to be used in the main study.

### 2.3. Experimental design

The experiment consisted of a 14 days pretreatment period, followed by treatment, which lasted 50 days, and a recovery period of 62 days after last treatment. To establish pretreatment baseline seminal parameters, semen was collected daily (Monday–Friday) for 14 days. Testis volume was measured weekly by ultrasonography [29]. Daily sperm output (DSO) was considered the total number of spermatozoa collected on the fifth collection day of each week as previously described [30]. Stallions were matched based on DSO (average of pretreatment weeks), testicular size and age, then were randomly assigned to treatment ( $n = 4$ ; acyline 330 µg/kg every five days [q5d], IM) or control ( $n = 4$ ; 10 mL vehicle, q5d, IM) groups. The experiment was conducted from mid-August through mid-December in the Northern Hemisphere. Detailed information on specific data collection follows below.

### 2.4. Endocrine evaluation

Jugular blood samples were collected in the morning (~7 AM) of sampling days (below) before feeding and before acyline treatment (when administered). Blood was collected in heparinized tubes (BD, Franklin Lakes, NJ, USA), and plasma was separated by centrifugation at  $2300 \times g$  for

10 minutes at 4 °C. Thereafter, plasma samples were stored at –20 °C until analysis.

Plasma testosterone and estrone sulfate concentrations were determined daily for the last 5 days pretreatment and the first 9 days of treatment; then, on the days of treatment administration (q5d), and 2 days following each treatment administration through the end of treatment. During the recovery period, plasma steroid concentrations were determined once in a week. 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 [31]. Estrone sulfate was analyzed using a previously described competitive ELISA [32]. Samples were run in triplicates. The intraassay and interassay coefficients of variation (CVs) for testosterone were 13% and 11% (low binding = 10%; high binding = 12%), respectively. Estrone sulfate intraassay CV was 11% and interassay CV was 11% (low binding = 7%; high binding = 14%).

Follicle-stimulating hormone (FSH), and LH assays were determined 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 LH were determined by a heterologous double-antibody RIA [33]. Sensitivity was 0.25 ng/mL for both gonadotropins, and the intraassay CVs were 2.72% for LH and 2.46% for FSH, each run in a single assay.

Plasma anti-Müllerian hormone (AMH) concentrations were determined using a commercially available ELISA (Equine AMH ELISA, MOFA Global, Verona, WI, USA) per the manufacturer's instructions. Plasma AMH concentrations were determined weekly from Day –6 to 87 relative to the beginning of treatment. Interassay and intraassay CVs were 5.6% and 6.5%, respectively. Limit of detection of the assay was 0.03 ng/mL.

Gonadotropin-releasing hormone stimulation tests were performed 9 days before the beginning of treatment and at the 46th day of treatment to evaluate the suppression of the HPG axis. The procedure consisted of 25 µg of GnRH (IV, gonadorelin diacetate tetrahydrate, Cystorelin, Merial LLC, Duluth, GA, USA) administered at 8 AM. Blood was collected 30 minutes before, immediately before and 30, 60, and 120 minutes post-GnRH administration [34].

## 2.5. Semen collection and evaluation

Semen collections were performed Monday through Friday for 2 weeks before (pretreatment) and 2 weeks after the onset of treatment, and then on alternate weeks until the end of the experiment. Only the fifth daily ejaculate for each week (i.e., DSO ejaculate) was used for semen data analysis. Stallion handlers rotated through the week, and a single person performed the semen collections throughout the study. Collections were performed with a Missouri model artificial vagina with an in-line filter.

The gel-free volume was determined by weight (1 g = 1 mL). Spermatozoal concentration was obtained with a densimeter (591B densimeter, Animal Reproduction Systems, Chino, CA, USA). Total motility, progressive motility, and other sperm motion characteristics were obtained with computer-assisted sperm analysis (SpermVision, MOFA Global, Verona, WI, USA). Preparation of samples for

motility analysis consisted of diluting a fraction of ejaculate with a skim-milk extender (EquiPRO, MOFA Global, Verona, WI, USA) to a final concentration of  $30 \times 10^6$  spermatozoa/mL. Eight microliters of extended sperm suspension were placed on a microscope slide (22 × 22–2 mm coverslip) at 37 °C and allowed to stabilize for at least 30 seconds before acquiring the data. The settings of the CASA were as follows. Level one cell classifications: immotile = AOC (average orientation change of the head) < 7; locally motile = distance straight line < 6. Level two classifications: hyperactive = velocity curved line > 8, linearity (LIN) < 0.65, and amplitude lateral head (ALH displacement) > 6.5; linear = straightness > 0.9 and LIN greater than 0.5; nonlinear = straightness less than 0.9 and LIN less than 0.5; curvilinear = distance average path/radius 3 or more and LIN less than 0.5. Cell identification was set for an area of 14 to 80 µm<sup>2</sup>. Motility assessment was repeated with a minimum of 7 fields or 500 cells.

Aliquots for assessing spermatozoal morphology consisted of 50 µL of raw semen fixed in 1 mL 10% buffered formalin. Subsequent evaluation was performed in wet mounts using differential interference contrast microscopy (Axio Imager 2, Carl Zeiss Microscopy LLC, Thornwood, NY, USA) at 900× magnification. Seminal plasma from ejaculates collected on Tuesdays and Thursdays was obtained by centrifugation at ×2000g for 10 minutes, and stored at –20 °C until analysis. Osmolality of seminal plasma was determined using an osmometer (Micro Osmette, Model 5004 Automatic Osmometer, Precision Systems, Natick, MA, USA). Protein concentration in seminal plasma was determined with coomassie (Bradford) protein assay kit (#23200, Thermo Scientific, Kalamazoo, MI, USA) as previously described [35].

## 2.6. Testicular volume

Testicular volume was determined once a week throughout the study as described by Love [29]. Weekly testicular volume determinations were made throughout the study by the same examiner. Stallions were placed in stocks, sedated (100–150-mg xylazine, IV; AnaSed, Lloyd, Shenandoah, IA, USA) and the height, width, and length of individual testes were determined using B-mode ultrasonography (Sonoscape S8, Universal Medical Systems Inc., Bedford Hills, NY, USA). Values were recorded as the average of three measurements.

## 2.7. Sexual behavior

Stallions were brought into the breeding shed and exposed to an ovariectomized mare primed with estrogen (estradiol cypionate, BET Pharmacy, Lexington, KY, USA; 10 mg, q7d, IM). When erection was obtained, the penis was washed with warm water, and the stallion was allowed to mount a phantom for semen collection. Data on reproductive behavior were recorded for each stallion included time from entrance into the breeding shed until erection and time to ejaculation as well as the total number of mounts per ejaculation. If a stallion failed to mount the phantom within 10 minutes, the mare was positioned beside the phantom for additional stimulation. A total time of

15 minutes 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; however, a continued attempt was still made to obtain the ejaculate for other data points. At least two different tease mares were available if needed for rotation throughout the experiment. For analysis of data related to sexual behavior, time to erection, time to ejaculation, and number of mounts were analyzed for all semen collection attempts during the study.

## 2.8. Statistical analysis

Continuous data were analyzed using a mixed model with treatment and time as fixed effects and stallion as a random effect (JMP 10.0; SAS Institute, Cary, NC, USA). Where necessary, data were log transformed, and model assumptions were evaluated using normal quantile plots of residuals. Data are presented as untransformed means  $\pm$  standard error of the mean. In the variables in which a time-by-treatment interaction existed, 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 mixed model with treatment and period (pretreatment 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. The proportion of semen collection attempts that resulted in a failure in

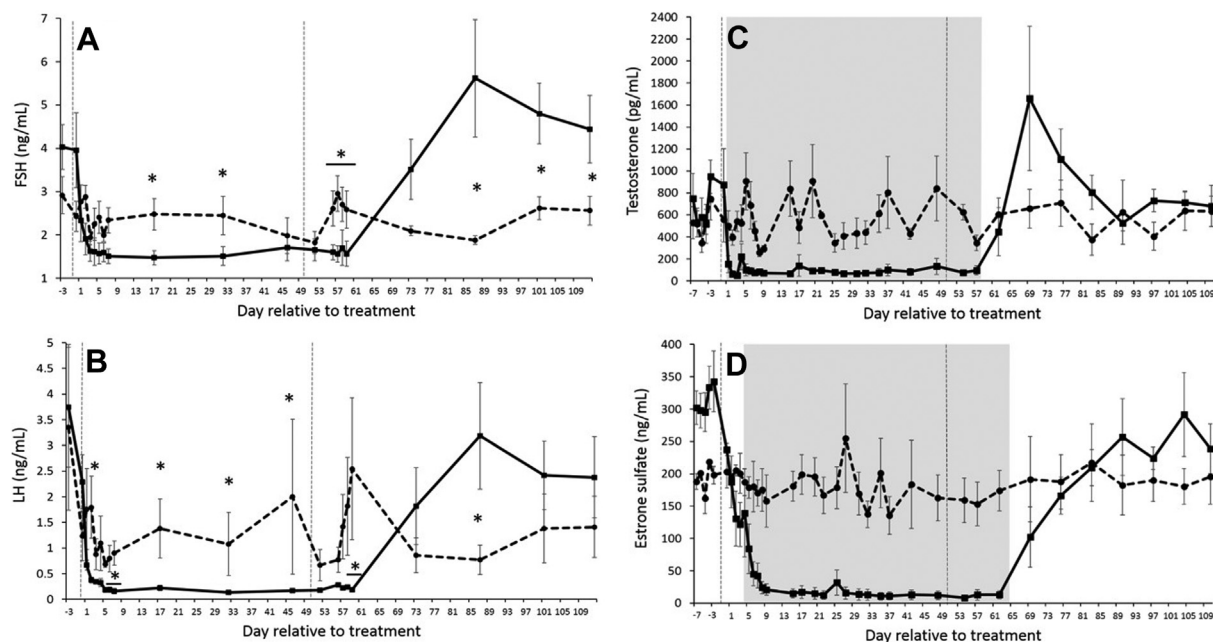
semen collection was compared between groups based on a chi-square test. Osmolality and protein content of seminal plasma (two observations per week of collection) were assessed based on the weekly mean value. Statistical significance was set at  $P < 0.05$ .

## 3. Results

For the data presented, days are shown relative to the initiation of treatment (Day 0). Plasma FSH concentrations in acyline-treated stallions were lower ( $P < 0.05$ ) than the control group at Day 17 and Day 32 of treatment, as well as at Days 6, 7, 8, and 9 after the cessation of treatment (Fig. 1). FSH concentrations were higher ( $P < 0.05$ ) in the acyline-treated stallions than in 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 ( $P < 0.05$ ) in acyline-treated stallions than in control stallions throughout the treatment for all measured time points except for Days 3 and 4. Luteinizing hormone values were not different between the control and acyline group by Day 2 and 6 after the end of treatment, although LH concentrations were lower in the acyline-treated stallions on Days 7, 8, and 9 after the end of treatment. Concentrations of LH displayed a rebound during the recovery period as shown by a higher ( $P < 0.05$ ) LH concentration in the acyline-treated stallions on Day 37 after the end of treatment (Fig. 1).

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



**Fig. 1.** Circulating FSH (A), LH (B), testosterone (C), and estrone sulfate (D) concentrations in acyline-treated and control stallions. Acyline-treated stallions (■, solid line) received acyline ( $n = 4$ ; 330  $\mu\text{g/kg}$  q5d, IM) and control (●, dashed line) received vehicle alone ( $n = 4$ ; 10 mL, q5d, IM). Vertical-dashed lines represent beginning and end of treatment period. Data expressed as mean  $\pm$  SEM (\*) or (\*\*) indicate time points or consecutive time points, respectively, where treatment and control values differ  $P < 0.05$ . SEM, standard error of the mean.

declined ( $P < 0.004$ ) by Day 1 of treatment, and testosterone concentrations remained suppressed relative to control stallions until the end of treatment (Fig. 1). After the last treatment, plasma testosterone concentrations in the acyline-treated stallions remained suppressed for 9 days before returning to concentrations comparable to the control stallions. The acyline-treated group showed a constant, steady suppression of peripheral testosterone, with a mean concentration of 129 pg/mL during the treatment period. 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 persisted 14 days after the end of treatment (Day 50; Fig. 1). Plasma AMH concentrations increased in both groups during the study ( $P < 0.0001$ ); however, there were no treatment effects or treatment by time interactions on AMH concentrations (data not shown).

Hormonal response to GnRH challenges (as AUC) was compared within groups before and during the treatment period, as well as between groups at each period. There were no differences in gonadotropin or testosterone responses between groups during the pretreatment period, or within the control group between the pretreatment to treatment period. 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 with the increase in those hormones during the pretreatment period or relative to control values (Table 1).

There was 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. 2).

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

Total sperm motility was lower in treated than control stallions by week 2 ( $P < 0.05$ ) and remained lower than the control group through the end of treatment. A similar effect was observed in progressive motility, which was lower in

treated than control stallions from week 4 ( $P < 0.003$ ) to week 8 ( $P < 0.003$ ). During the recovery period, both sperm motility parameters were similar in treated and control stallions (Fig. 2). 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).

Total seminal plasma protein was lower from week 2 of treatment until the fourth week after 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 recovery period (Fig. 2). Osmolality of seminal plasma samples was not changed due to treatment ( $P < 0.16$ )—data not shown. There was a time effect ( $P < 0.008$ ) on osmolality with an increase during the treatment period and the beginning of the posttreatment period. Since there was no treatment by time effect, osmolality was run 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.

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. 3). Testis volume increased in treated stallions during the recovery period and was not different from control stallions by week 12.

During the treatment and posttreatment periods, more ( $P < 0.05$ ) semen collections from acyline-treated stallions resulted in a failure of semen collection ( $>15$  minutes) than did semen collections from control stallions (14/176 vs. 5/176, respectively). Over the course of the study, the time to erection, time to ejaculation, and number of mounts increased ( $P < 0.0001$ ) across both groups of stallions; however, there was no effect of treatment or treatment by time interactions on these parameters.

#### 4. Discussion

Our findings clearly show a reversible suppression of the HPG axis in stallions treated with acyline. Down-regulation of the HPG axis was evident by a reduction in gonadotropins, testicular steroids, testis volume, DSO, total seminal plasma protein as well as sperm motility and, to some extent, behavioral changes, as well as diminished response to GnRH stimulation during treatment with acyline. Discontinuation of treatment enabled the axis to

**Table 1**

Total FSH, LH, and testosterone secretion (area under the curve; mean  $\pm$  SEM) during 150 min after GnRH challenge pretreatment and during treatment in acyline-treated and control stallions.

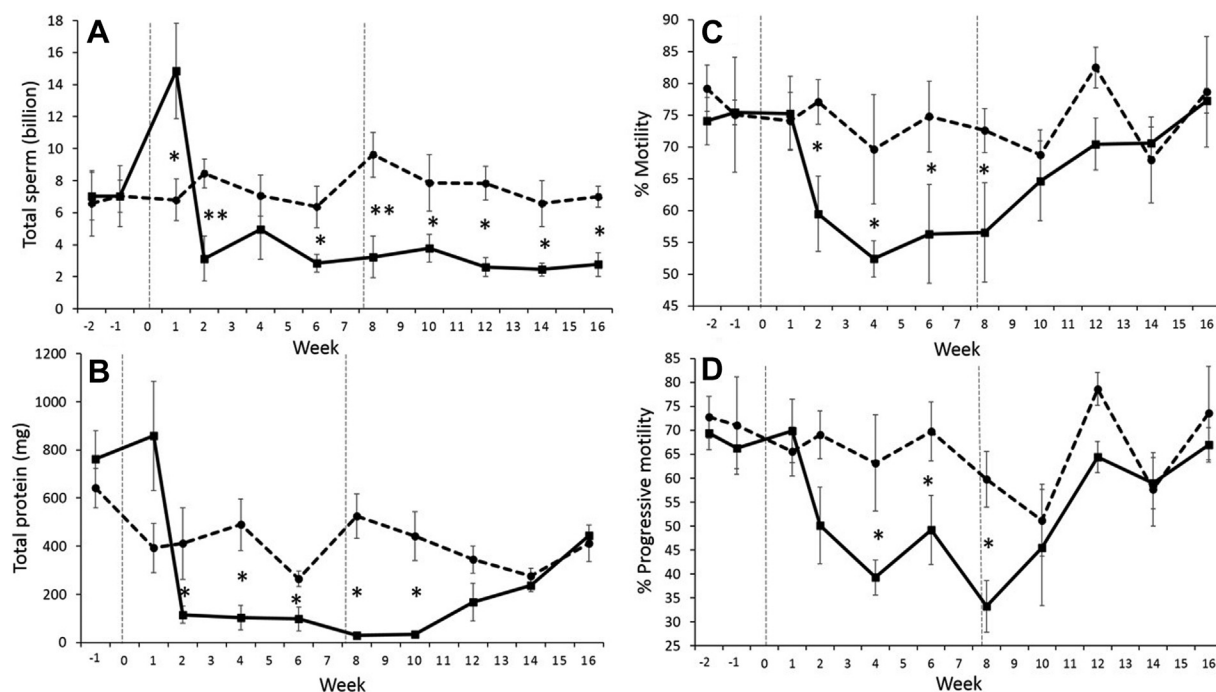
AUC	Pre-treatment		During treatment	
	Control	Acyline	Control	Acyline
FSH (ng/mL $\times$ min)	11.5 $\pm$ 0.7 <sup>a</sup>	19.4 $\pm$ 2.6 <sup>a</sup>	12.0 $\pm$ 0.8 <sup>a</sup>	6.8 $\pm$ 1.2 <sup>b</sup>
LH (ng/mL $\times$ min)	10.3 $\pm$ 4.1 <sup>a</sup>	21.5 $\pm$ 6.1 <sup>a</sup>	11.5 $\pm$ 6.6 <sup>a</sup>	0.7 $\pm$ 0.1 <sup>b</sup>
Testosterone (pg/mL $\times$ min)	3364.6 $\pm$ 1045.3 <sup>a</sup>	5393.3 $\pm$ 1302.2 <sup>a</sup>	4143.2 $\pm$ 1303.7 <sup>a</sup>	373.7 $\pm$ 171.6 <sup>b</sup>

Acyline-treated stallions received acyline ( $n = 4$ ; 330  $\mu$ g/kg q5d, IM) and control received vehicle alone ( $n = 4$ ; 10 mL, q5d, IM).

Different letters indicate statistical difference ( $P < 0.05$ ) across rows.

Abbreviations: AUC, area under the curve; SEM, standard error of the mean.





**Fig. 2.** Total sperm number (A), total seminal plasma protein content (B), total sperm motility (C), and progressive motility (D) in acyline-treated and control stallions. Acyline-treated stallions (■, solid line) received acyline ( $n = 4$ ; 330  $\mu\text{g/kg}$  q5d, IM) and control (●, dashed line) received vehicle alone ( $n = 4$ ; 10 mL, q5d, IM). Sperm count for DSO ejaculate shown as mean  $\pm$  SEM. Vertical-dashed lines represent beginning and end of treatment period. (\*) indicates time points where treatment and control values differ  $P < 0.05$ ; (\*\*) indicates  $P < 0.005$ . DSO, daily sperm output; SEM, standard error of the mean.

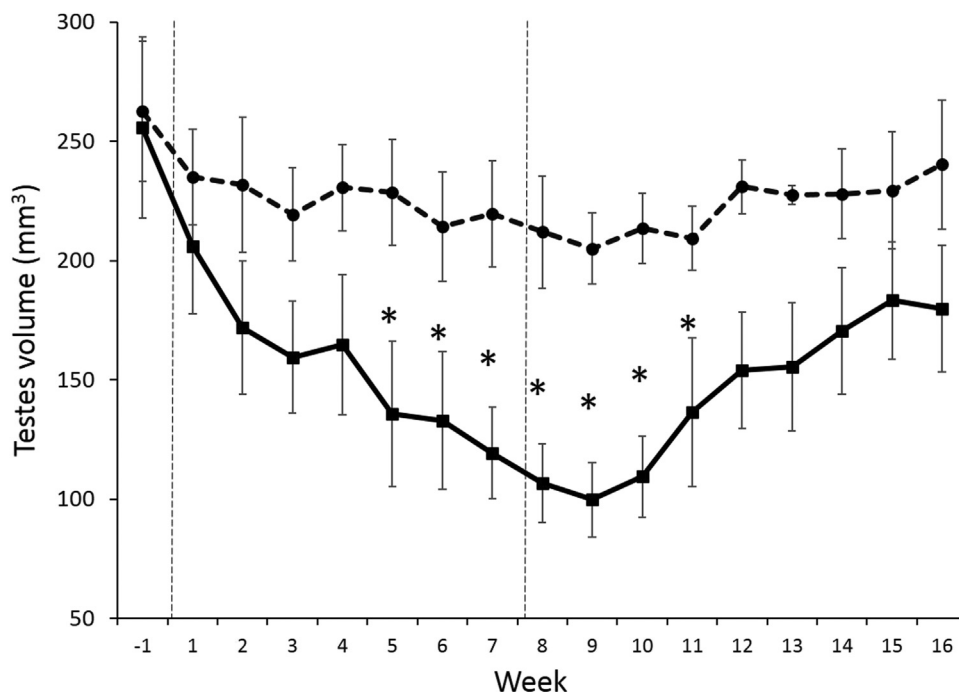
resume functionality based on return of FSH, LH, estrone sulfate, and testosterone to normal circulating concentrations, followed by recovery of the majority of the seminal and testicular parameters.

Administration of other GnRH antagonists has been used to examine downregulation of the HPG axis in the stallion. 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 [20]; however, when antarelix or cetorelix were administered twice a day for 5 weeks [21], testosterone decreased until a week after the end of treatment. The suppression of testosterone during acyline treatment in stallions is consistent with findings in men [23,36] and dogs [24]. In our study, concentrations of testosterone in treated stallions were consistent with those of geldings—lower than 130 to 200 pg/mL [12,13]. Even though circulating testosterone was not determined for every day of the experiment, the assayed group of samples included those collected immediately before treatment administration and on the second day following, which are presumably the lowest and the highest acyline suppression time points. In other words, the testosterone concentrations of acyline-treated stallions during the duration of the experiment are well represented within the range displayed in the results. 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 with control stallions or with response of treated stallions in the stimulation pretreatment. These results are similar to

those reported in dogs [26]. Furthermore, when compared to different approaches to downregulate the HPG axis in stallions, acyline appeared to result in a more rapid and consistent downregulation than altrenogest [9,10,37–39] and GnRH vaccination [12–16,40].

Although gonadotropins and testicular steroids were downregulated with acyline treatment, AMH, a product of the Sertoli cells, was not significantly reduced with acyline administration. Peripheral AMH concentrations decrease with sexual maturity, and AMH concentrations vary seasonally along with testosterone [41]. Not much is known about the regulation of AMH in the male, though the lack of effect of HPG axis suppression on AMH concentrations may suggest that regulatory mechanisms for AMH secretion in the adult stallion are independent of the HPG axis.

The reduction in sperm output due to acyline treatment is in accordance with previous work using GnRH antagonists in the stallion where a drop of almost 50% in sperm output was noted after a single injection of antarelix [20]. Whereas in the study by Fortier et al. [21], there was a near absence of sperm in the ejaculate at the end of a 35- to 38-day long treatment with twice daily antagonists antarelix and cetorelix. In dogs, a single acyline administration was sufficient to cause azoospermia in 4 of 7 treated dogs [25]. From the perspective of sperm motility, a 10% to 15% loss in total motility and a 10% to 25% drop in progressive motility were observed, with total motility reaching a nadir of about 35%. Put in contrast with the very low steroid hormone concentrations in treated stallions, it is intriguing that the



**Fig. 3.** Total testicular volume in acyline-treated and control stallions. Acyline-treated stallions (■, solid line) received acyline ( $n = 4$ ; 330  $\mu\text{g/kg}$  q5d, IM) and controls (●, dashed line) received vehicle alone ( $n = 4$ ; 10 mL, q5d, IM). Weekly values shown as mean  $\pm$  SEM. Vertical-dashed lines represent beginning and end of treatment period. (\*) indicates time points where treatment and control values differ  $P < 0.05$ . SEM, standard error of the mean.

sperm numbers and motility were not suppressed to almost complete azoospermia and asthenozoospermia. Although we have not performed further fertility assessment of the sperm of stallions under treatment, the partial persistence of these attributes may preclude the use of acyline as a contraceptive in the male equine, while it has been cited as a potential use for acyline in humans [23]. In addition to these findings, a side observation was the increase in sperm output of stallions in the first week of acyline treatment before it dropped on the week following. We lack a proper explanation for this observed phenomenon.

Disruption of normal spermatogenesis after down-regulation of the HPG axis may also be reflected by an increase in round spermatogenic cells in the ejaculate. The appearance of round spermatogenic cells was also noticed in dogs receiving acyline [25], and in stallions given antarelix and cetrorelix, in which testicular histology exhibited loss of round spermatogenic cells into the lumen 8 weeks after administration [20]. The increased number of round cells in acyline-treated stallions at weeks 2 and 8, in particular, seems to agree with previous reports.

Normal function of the accessory sex glands of stallions [42,43] and the epididymis of various species [44], depends on testosterone and estrogen [43,45]. The accessory sex glands are the major contributors to the protein content of the seminal plasma [44,46,47]. Downregulation of accessory sex gland function secondary to reduced testicular steroids likely accounts in part for the reduction in total protein content in the ejaculate.

Suppression of testosterone for at least 9 days after last treatment showed that the effect of serial administrations of the GnRH antagonist led to a suppression of the HPG axis for almost double the time it did after a single administration—5 days. A more extended suppression in FSH and LH following a series of GnRH antagonist injections compared with single injection was also observed in humans [48]. It was demonstrated in rats that, in fact, the amount of GnRH receptors [49–52] and GnRHR mRNA [52,53] 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, intervals of acyline administration could be progressively increased during treatment with repeated doses, or low doses given after a high, loading dose, as has been suggested for GnRH antagonists in humans [48], thus possibly reducing the cost of treating a stallion.

As peripheral gonadotropin concentrations recovered in treated animals, a rebound release of FSH and LH was noted in the present study and a similar rebound in gonadotropins was reported after discontinuation of GnRH antagonists in men [48]. After a single injection of acyline in dogs, a rebound was noticed in FSH, but not LH [24]; a testosterone peak was also noticed after recovery of the suppression in dogs [24,26], but no significant rebound occurred in testosterone concentration in acyline-treated stallions.

Both estradiol and testosterone—by itself or through aromatization at the encephalic tissue [43,54,55]—are considered to be implicated in sexual behavior of the

stallion. However, 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 increased number of collection failures (>15 minutes required for semen collection) in the acyline-treated stallions. In a previous report [43], stallions retained the desire to mount for longer than the ability to ejaculate after castration, which may partially explain this finding. It is possible that if the acyline treatment had been longer, clearer results would have been seen in the different aspects of sexual behavior observed in the study. Nevertheless, the results of a 50-day long treatment do not provide support to recommend acyline for modification of behavior in stallions. In consideration of clinical applications, studies with colts and stallions under training would be required to translate the effects of acyline into behavior modulation in a practical setting. These investigations should also aim to assess the impact of GnRH antagonists on body and gonadal development.

While sperm output was the only parameter in the present study 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 et al. [56] in 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 (62 days) was long enough for production of new sperm and epididymal transit [44], it may not have been sufficiently long if one takes into consideration the delay in the return of gonadotropins and steroids to normality. Nevertheless, follow-up semen collections, as part of another experiment 7 months after the end of the recovery period, showed that all of the sperm numbers obtained after every-other-day collections for 14 days, had returned to normal pretreatment results, though we did not determine when complete 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 [21], and by evidence that sperm germ cell differentiation, stalled due to GnRH antagonist in rats, had fully recovered 90 days after cessation of treatment [50].

In conclusion, acyline treatment rapidly downregulates 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, whereas there was an increase in round spermatogenic cells present in the ejaculate of treated stallions. Finally, sexual behavior was affected as reflected by an increased number of semen collection failures 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. On cessation of treatment, most of the affected parameters returned to control values within 62 days; though sperm output required longer to recover. Further experiments are necessary to determine if acyline would be efficacious as a treatment for EAV carrier stallions.

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## Competing interests

The authors declare no conflicts of interest.

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