Vaccination against GnRH as a prelude to surgical castration of horses

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Authors' declaration of interests

No competing interests have been declared.

Ethical animal research

The study was approved by the Animal Use and Care Committee of the University of Pretoria (approval # V047-11).

Owner informed consent

Castrations of colts at the South African Police Services Mounted Academy are carried out annually and thus consent was not required. Dr Birrel was also in the employ as colonel during the period of the study.

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Data accessibility:

Data available if required

Abstract

Background

Surgical castration is one of the most frequently performed surgeries in horses and is associated with various post-surgical complications ranging in severity.

Objectives

We investigated the effects of administering an anti-GnRH vaccine to colts for reduction of testis size on the incidence of complications associated with their subsequent surgical castration.

Study design

Randomised open controlled trial.

Methods

Nineteen colts were randomly assigned to one of the three groups. Treatment Groups V1-57 and V2-100 received two treatments of Improvac® (Zoetis, South Africa) before their castration in the field on either Day 57 or Day 100 following first treatment, respectively. Controls similarly received placebo treatments followed by castration on Day 57. Serial measurements of testicular dimensions and serum samples for measuring serum testosterone concentrations (STCs) and anti-GnRH antibody titres (ABTs) were obtained pre- and post-vaccination and on the day of castration. Clinical data recorded for 10 days determined post-surgical complications.

Results

All vaccinated colts showed a baseline STC concentration at castration with a strong ABT response. Mean testicular volume of Groups V1-57 and V2-100 reduced by 49.7% and 30.8%, respectively, on Day 57 and this further reduced in Group V2-100 by 63.9% at castration on Day 100. Testis size was significantly correlated with post-surgical preputial ($P = 0.001$) and scrotal ($P = 0.025$) swelling.

Main limitations

A study population of young light horse breed colts and a relatively small sample size limited this pilot study.

Conclusions

Administering two doses of Improvac® in colts 28 days apart prior to castration effectively reduced their testicular volumes with associated significant post-surgical improvement in both the incidence and degree of local swelling. These results may inform mitigation of potential post-surgical complications associated with castration in the field.

Anti-GnRH vaccine as a prelude to castration in horses

Keywords: Anti-GnRH vaccine, colt, surgical castration

1. Introduction

Surgical castration is primarily indicated to eliminate undesired behaviours in stallions not intended for breeding and is one of the most commonly performed procedures in horses. Less-frequent indications include testicular neoplasia, orchitis or trauma, torsion of the spermatic cord, hydrocoele, varicocoele and inguinal herniae [1]**.** Surgical castration is irreversible and subsequently the genetic potential of the individual is lost. Reported post-surgical complications associated with castration include scrotal oedema, excessive haemorrhage, schirrous cord formation, peritonitis, omental herniation, incisional infections, hydrocoele, penile trauma, evisceration (eventration) and anaesthetic incidents [1,2,3,4,5,6,7]. In addition, undesired behaviours may persist in up to 30% of geldings [8]. The most commonly reported complication is scrotal oedema, affecting 27.6% of 23,229 horses castrated [1]. Rates of 0.2-2.96% of non-fatal and fatal surgical complications are reported [1,9,10]. Evisceration of omental tissue or intestinal loops reportedly had an apparently high prevalence in Belgian (2.8%) and Percheron (4.8%) draft horses [6]. However, another study reported a 0.2 % prevalence of evisceration in Standardbreds, Arabs and draft horse breeds [1]. Current best practice guidelines for equine welfare dictate the importance of minimizing pain and suffering resulting from surgical castration [11].

The dependence of normal testicular function on the hypothalamic-pituitary-gonadal (HPG) axis, which involves classic feedback control mechanisms is well established in mammals [12,13]. The major HPG hormones involved are GnRH, LH, FSH,

testosterone (T), estrogens and inhibin. Active immunization of male mammals against hypothalamic GnRH has been recognized as an effective immunocastration method since the 1970s [14]. Antibodies formed in response to these vaccines neutralize endogenous GnRH in the hypothalamo-pituitary portal system, decrease FSH and LH secretion and subsequently down-regulate gonadal function. This is reported to reduce androgen-dependent aggressive behaviour in males of various species including cattle [15,16], pigs [17], sheep, rats, dogs [18,19,20], cats [21,22], feral swine [23], camels [24] and African elephants [25,26].

Anti-GnRH vaccines provide an alternative to surgical castration of stallions that includes inhibition of undesirable sexual behaviour with circumvention of surgical risk and facilitation of owner preference. Furthermore, additional benefits include the potential reversibility of GnRH immunization avoiding the loss of future breeding potential [27].

Most reported studies in stallions have focused on the effect of GnRH vaccines on serum testosterone concentration (STC), androgen-mediated behaviours, libido, testis morphology and semen quality [8,27,28,29,30,31,32]. Although observing variable responses to different formulations, all these studies reported a 13.4-33% decrease in testicular size following immunization. The effect of GnRH vaccination prior to castration on the incidence and severity of intra- and post-surgical risks and complications has not been reported.

This study aimed primarily to determine the effects of the administration in colts of a commercially available porcine anti-GnRH-vaccine (Improvac®, Zoetis, South Africa) on their testicular dimensions and post-operative complications associated with their

subsequent surgical castration performed under field conditions. In addition, the effects of vaccination on STC and anti-GnRH antibody titres were determined.

2. Materials and methods

2.1 Study population and data collection

Nineteen intact, mixed-breed light-horse colts between 15-20 months of age were included in the study. The colts were allocated from the pool of young stock bred by Mounted Academy of the South African Police Services for potential deployment as police mounts. The colts, individually identified and micro-chipped were maintained throughout the study in outside paddocks during the winter months of June-August in southern Africa. They were provided with *ad lib Eragrostis teff* grass hay and a winter lick[a] protein supplement formulated for antelopes). All colts received routine prophylactic primary health care including vaccinations against African horse sickness (September the previous year) and tetanus and rabies (January). Day 0 of the study was defined as the day of primary immunization with either the vaccine or a placebo.

Inclusion criteria: On the day before the first treatment (Day -1) with either the anti-GnRH vaccine or placebo, the clinical health status of colts was confirmed (routine clinical evaluation) and the bilateral presence of intra-scrotal testes and absence of inguinal herniae was confirmed. Thereafter, colts were assigned to one of the three experimental groups using an alternating allocation scheme starting with the first group.

Group V1-57: n = 7; mean age 18.8 months; mean body weight (Day-1) = 307.3 kg treated with GnRH vaccine on Days 0 and 28 and castration on Day 57;

Group V2-100: n = 6; mean age 18.4 months; mean body weight (Day -1) = 319.7 kg treated with GnRH vaccine on Days 0 and 28 and castration on Day 100; and

Group Control: n = 6; mean age 18.0 months; mean body weight (Day-1) = 301.7 kg treated with a placebo on Days 0 and 28 and castration on Day 57.

Castrating Group V2-100 approximately 6 weeks later than Groups V1-57 and Control colts was intended to observe if any further GnRH immunization-associated reduction in testicular dimensions would occur.

2.2 Procedures

2.2.1 Anti-GnRH vaccination

Two ml of the commercially-available porcine GnRH vaccine, (Improvac®)[b] complete with adjuvant and containing 400 µg GnRH-protein conjugate (2 mL) was delivered by deep intramuscular injection into the gluteal muscle mass [33] on each of treatment Days 0 and 28. Intramuscular route was selected instead of the subcutaneous route recommended for pigs because the former route was found to yield a better response in stallions (different anti-GnRH vaccine) [28] and, yielded excellent results following immunisation with Improvac in the mare [33]. The placebo treatment used for the Control group consisted of 2 mL of sterile 0.9% saline, similarly administered on Days 0 and 28. All colts were observed daily for signs of injection site swelling and overt lameness for the four days following each treatment. Rectal temperature was measured daily for the first five days following GnRH vaccine administration.

2.2.2 Anaesthetic protocol for castration

Premedication was a combination of detomidine hydrochloride (Domosedan®, [c]), at 20-40 µg/kg bwt i.v. and butorphenol tartrate[d] at 0.01-0.05 mg/kg bwt i.v., administered 20 min before induction of general anaesthesia using ketamine

hydrochloride (Ketalar®)[e] 2.2 mg/kg bwt i.v. and thiopentone sodium (Intraval®)[f] 48 mg/kg bwt i.v. Ketamine in a balanced electrolyte solution (lactated Ringer's) was first infused until signs of limb weakness were evident and followed by bolus injection of thiopentone sodium. Following induction, the colts were placed in right lateral recumbency.

2.2.3 Surgical procedure and post-operative management

The castrations were performed under field conditions using an open surgical technique with a Sera emasculator and surgical wounds were allowed to heal by second intention healing [5]. The surgical site was inspected for signs of haemorrhage and the horses observed closely for immediate post-operative recovery complications. No routine prophylactic antimicrobials or anti-inflammatory drugs were administered as we did not want to bias measured outcomes.

During the 10-day post-operative period, each colt was hand-walked daily to an adjacent clinical facility for clinical monitoring and management. Daily, topical cold tap water (temp 15-17 °C) therapy was applied via a hosepipe using gentle water pressure to the general area of the scrotum and prepuce for 10 min avoiding contamination of the scrotal wound. Following this, **e**ach colt was hand-lunged in a nearby enclosed exercise arena for 10 min to reduce development of any potential scrotal or preputial swelling. Additional post-operative care consisted of monitoring for clinical signs consistent with infection.

2.3 Observations and sample collection

2.3.1 Testicular and epididymal measurements

The length, width and height of each testis was measured using an adjustable spring calliper on Days 0, 28 and 57 in all groups and additionally on Day 100 (Group V2-

100). These measurements were used to calculate the testicular volume using the prolate ellipsoid formula (length x height x width x 0.523) [34].

Immediately following castration, each excised testis was weighed both with its adnexa (including the head, body and tail of the epididymis) and thereafter, without the adnexa using an electronic scale (Radwag PS 6000/C/2)[g]. Each excised epididymis was similarly weighed.

2.3.2 Post-operative clinical observations

All colts were monitored once daily for 10 days for clinical signs of post-surgical complications. Daily rectal temperature, resting heart and respiratory rates, appetite and habitus were recorded. The habitus was observed and scored subjectively as follows: 1 = very depressed, 2 = depressed, 3 = normal. This was based on a combination of subjectively assessed behavioural categories including the individual's activity, interactive behaviour, response to food and attention to the pain source [35]. In addition, both the scrotum and the prepuce were inspected for signs of swelling and scored (1 = no swelling; 2 = mild, 5 cm across; 3 = moderate, 10 cm across; 4 = severe, >10 cm) (A Dallas and R Parker, Personal communication, 2020). The surgical site was inspected for signs of discharge and scored (1 = none; 2 = mild, scanty serous to serohaemorrhgic discharge; 3 = moderate, obvious serous to serohaemorrhgic discharge; 4 = severe, copious serous to serohaemorrhgic or purulent).

2.3.3 Serum testosterone concentrations

Blood was collected by jugular venipuncture in plain evacuated 10 mL tubes (Vacutainer®)[h] on Days -4, 0, 2, 24, 28 and 57 from all three groups and additionally on Day 100 from Group V2-100. The samples were left to clot before centrifugation

(4000x*g,* 10 min), after which the serum was decanted into storage tubes and stored at -20 °C until analyzed for serum testosterone concentration (STC).

STC was analyzed using a validated [32] radioimmunoassay kit (Coat-a-Count testosterone)[i], in the Hormone Laboratory of the Section of Reproduction, Faculty of Veterinary Science, University of Pretoria.

2.3.4 Anti-GnRH antibody titres

Anti-GnRH antibody titre (ABT) assays were performed on serum samples collected on Days -4, 28, 57 and 100 from all horses. After clotting, serum was separated by centrifugation (4000x*g,* 10 min) and stored at -20°C until assayed. Anti-GnRH ABT were determined by enzyme immunoassay (EIA) using a modification of previously described methods [36,37]. Briefly, 96-well microtitre plates (MaxiSorp MTPs)[j] were incubated at 2-8°C for 16 h with 1µg GnRH in 100 µL coating buffer. (2.94% $NaHC0_{3;}$ 1.59% NA_2CO_3; pH 9.6) per well. Plates were washed with PBS containing 0.05% Tween 20 and then blocked with 0.03% bovine serum albumin in PBS for 16 h at 2-8°C. Plates were then incubated with serial dilutions (1:400-1:1600 for test samples and 1:200-1:6400 for positive reference serum) of standard and test serum samples at 37°C for 1 h. The positive reference serum consisted of pooled sera from the 13 anti-GnRH vaccine treated horses collected on Day 57. Blank wells were used as negative controls. After washing, antibodies were detected by incubating plates with recombinant anti-horse IgG (whole molecule)[k] at 37°C for 1 h. After further washing, plates were developed with trimethylene blue (Sure Blue^{TM})[l]. The reaction was stopped by adding 50µL 2M H_2SO_4 per well. Absorbance at 450mm was measured using a microplate photometer (Multiskan^{TM})[m].

Antibody response, measured as the mean sample absorbance (minus blank) was expressed as a proportion of the mean absorbance (minus blank) of the positive reference sample at the same dilution for each plate (1:400, 1:800, and 1:1600). The overall proportion positive was calculated as the average value over the three dilutions.

2.4 Data analysis

The normality assumption for quantitative and semi-quantitative data was assessed using available software (MINITAB Statistical Software, Release 13.32)[n] by plotting histograms, calculating descriptive statistics, and performing the Anderson-Darling test. Data violated the normality assumption and were subsequently described using the median and range and analysed using nonparametric statistical tests. Data were descriptively presented using line plots developed in the ggplot2 package [38] within R [39]. Changes in testicular volumes were calculated by subtracting baseline sizes from subsequent time periods to account for the repeated measures design. Surgical outcome data were averaged for the entire 10-day observation period prior to statistical analysis. Quantitative and semi-quantitative data were compared among treatment groups using Kruskal-Wallis tests followed by multiple pair wise Mann-Whitney U tests incorporating Bonferroni correction of P values. Pair wise correlations between quantitative data were estimated using Spearman's rho. Statistical analyses were performed using commercial software (IBM SPSS Statistics for Windows, Version 25)[o] and significance was set as alpha = 0.05.

3. RESULTS

3.1 Complications associated with immunization, anaesthesia and surgery

None of the 13 Improvac® immunized colts showed an increase in rectal temperature, swelling or overt signs of lameness post vaccination. Both induction

and recovery from anaesthesia was uneventful for all colts and no immediate intra- or post-operative complications were observed. The mean body weights of Groups V1-57, V2-100 and Control on the day of castration were 309.0, 331.0 and 308.3 kg, respectively. The mean weights gained from Day -1 to day of castration were 1.7, 11.3 and 6.6 kg for Groups V1-57, V2-100 and Control, respectively.

3.2 Testicular and epididymal dimensions

The effects of Improvac® treatment on testicular volume are shown in Table 1 and Figs 1 and 2. Groups V1-57 and V2-100 showed progressive decreases in testicular volume after the first (Day 0) and second immunizations (Day 28). In Group V2-100, a further decrease in volume was noted on Day 100. Group Control showed a decrease after Day 28 with group means of 59.1, 60.8 and 45.5 cm^3 on Days -6, 28 and 57, respectively. At castration, testicular volume was significantly correlated with testicular mass (Table 2; r_s= 0.970; P<0.001) in all 19 colts. The mean epididymal mass on the day of castration was 22.29 g, 30.15 g and 33.15 g for Groups V1-57, V2-100 and Control, respectively.

Table 1. Comparison of testicular volume (cm^3) changes over time in 13 anti-GnRH vaccinated (Groups V1-57 and V2-100) and 6 unvaccinated control colts (Group Control).

	Treatment group			
Time	**V1-57 Median (range)**	**V2-100 Median (range)**	**Control Median (range)**	**P value***
Baseline (day 0)	70.2 (12.0, 105.6)	65.1 (51.7, 107.2)	55.2 (23.9, 93.4)	0.714
Day 28 - baseline	-6.5 (-54.9, 15.8)	-9.6 (-41.6, 7.3)	0.6 (-2.5, 9.2)	0.101
Day 57 - baseline	-25.8 (-83.9, -0.8)	-15.4 (-44.9, -10.9)	-10.5 (-26.0, 12.3)	0.340
Day 100 - baseline	NA	-45.5 (-75.0, -26.5)	NA	NA
Surgery - baseline	-25.8[a,b] (-83.9, -0.8)	-45.5[a] (-75.0, -26.5)	-10.5[b] (-26.0, 12.3)	0.032

*Based on Krusakal-Wallis tests. Medians without superscripts in common were significantly different (P < 0.05) after post hoc pairwise comparisons incorporating Bonferroni correction of P values.

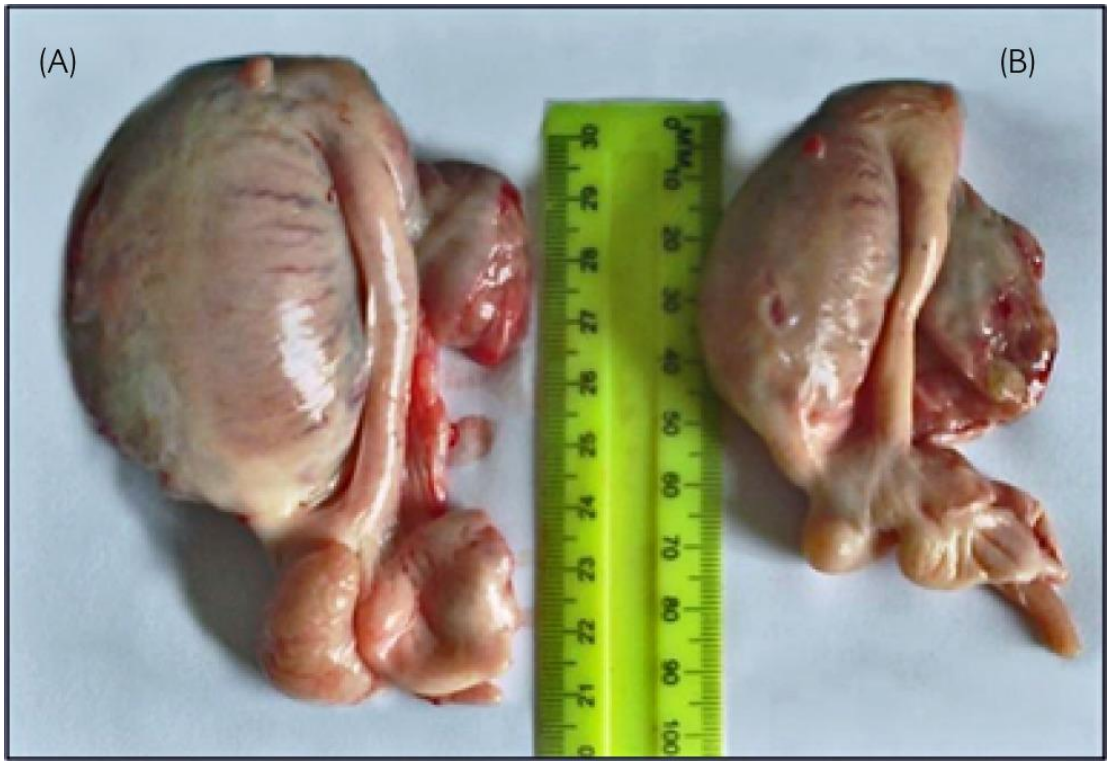

Figure 1. One excised testis intact with its epididymis of a representative control (A) and Improvac® -treated (B) colt.

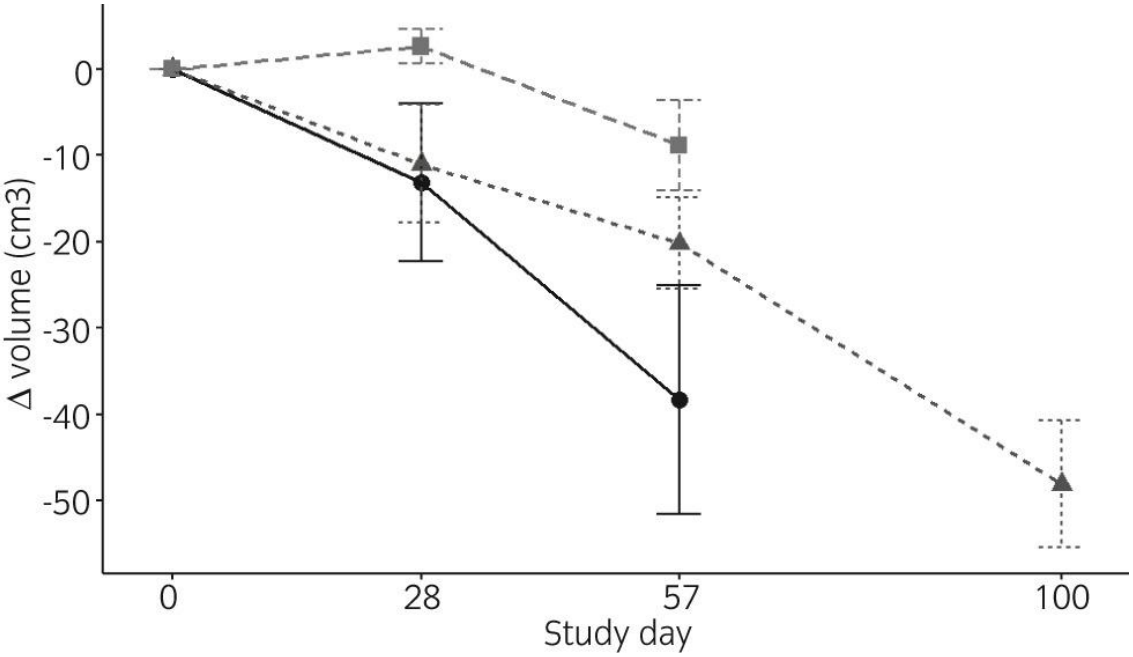

Figure 2. Changes in testicular volume compared to baseline (day -6) over time in 13 anti-GnRH vaccinated (Groups V1-57 & V2-100) and 6 unvaccinated control colts (Group Control). Testicular volumes from Group V1-57, Group V2-100, and Group Control are represented as the solid, short dash, and long dash lines, respectively. Points are mean values and error bars correspond to the standard error of the mean. Day 0 is the day of primary anti-GnRH vaccination.

Table 2. The correlation between anatomical sizes of structures at castration, clinical outcomes post-surgery, and antibody responses to GnRH vaccination in 13 anti-GnRH vaccinated (Groups V1-57&V2-100) and 6 unvaccinated control colts (Group Control).

Variable 1	Variable 2	Sub-group	n	Spearman's rho (P value)
Testis weight (g)	Testis volume (cm^3)	All groups	19	0.970 (<0.001)
	Preputial swelling (mean score)	All groups	19	0.720 (0.001)
	Scrotal swelling (mean score)	All groups	19	0.511 (0.025)
	Wound discharge (mean score)	All groups	19	-0.249 (0.305)
	Heart rate (/min)	All groups	19	0.378 (0.110)
	Respiratory rate (/min)	All groups	19	0.654 (0.002)
Adnexa weight (g)	Preputial swelling (mean score)	All groups	19	0.453 (0.052)
	Scrotal swelling (mean score)	All groups	19	0.526 (0.021)
	Wound discharge (mean score)	All groups	19	-0.008 (0.974)
	Heart rate (/min)	All groups	19	0.076 (0.756)
	Respiratory rate (/min)	All groups	19	0.286 (0.236)
Total weight (g)	Preputial swelling (mean score)	All groups	19	0.616 (0.005)
	Scrotal swelling (mean score)	All groups	19	0.622 (0.004)
	Wound discharge (mean score)	All groups	19	-0.078 (0.751)
	Heart rate (/min)	All groups	19	0.271 (0.261)
	Respiratory rate (/min)	All groups	19	0.513 (0.025)
Antibody (PP) †	Testis volume (cm^3)	Treatment groups	13	-0.148 (0.629)
	Testis weight (g)	Treatment groups	13	-0.181 (0.553)
	Adnexa weight (g)	Treatment groups	13	-0.621 (0.024)
	Epididymal weight (g)	Treatment groups	13	-0.621 (0.024)
	Total weight of structures (g)	Treatment groups	13	-0.533 (0.061)

†PP = proportion positive.

3.3 Post-castration clinical parameters

The median rectal temperature, habitus, preputial swelling, wound discharge and treatments did not differ significantly among the three groups during the 10-day post-surgical observation period (Table 3). The scrotal swelling observed in Group V1-57 was less than both V2-100 and Control groups (P<0.05). Both heart (P=0.029) and

respiratory (P=0.027) rates were significantly different between Groups V2-100 and Control, but not between Groups V1-57 and V2-100.

Table 3. Descriptive statistics and comparison of clinical parameters measured for 10 days after surgical castration in 13 anti-GnRH vaccinated (Groups V1-57 and V2-100) and 6 unvaccinated control colts (Group Control).

	Treatment group			
Parameters†	**V1-57 (vaccinated) Median (range)**	**V2-100 (vaccinated) Median (range)**	**Control Median (range)**	**P value***
Rectal temperature (°C)	37.3 (36.8, 38.1)	37.4 (37.1, 37.7)	37.5 (37.1, 38.0)	0.800
Heart rate (/min)	40.2[a,b] (35.8, 42.0)	37.2[a] (30.8, 37.6)	40.7[b] (36.6, 49.5)	0.029
Respiratory rate (/min)	17.6[a,b] (14.0, 20.4)	14.5[a] (14.0, 16.6)	17.7[b] (14.9, 19.8)	0.027
Habitus	3 (3, 3)	3 (3, 3)	3 (2.6, 3)	0.338
Treatments	0 (0, 0.5)	0 (0, 0.5)	0 (0 ,0.6)	0.973
Preputial swelling	1.4 (0.7, 2.0)	1.4 (0.8, 2.0)	1.6 (1.0, 2.7)	0.588
Scrotal swelling	0[a] (0, 0)	0.7[b] (0.1, 1.8)	1.6[b] (0, 2.8)	0.003
Wound discharge	0.3 (0.1, 0.5)	0.5 (0, 1.0)	0.3 (0, 0.4)	0.696

†Values averaged for the 10 day post-surgery observation period within each individual horse. Presented data describe the distribution of these means within each treatment group. *Based on Krusakal-Wallis tests comparing the 10-day means among groups. Medians without superscripts in common were significantly different ($P < 0.05$) after post hoc pair wise comparisons incorporating Bonferroni correction of P values.

A colt (#3) from Group V1-57 showed clinical signs consistent with colic accompanied by an elevated rectal temperature on Days 6 to 10 of the monitoring period. This was treated using a standard therapeutic protocol including antimicrobials and non-steroidal anti-inflammatory agents and the colt made an uneventful recovery. Another colt (#19), from the Control group showed a rise in both rectal temperature (Days 6 and 7) and respiratory rate (Day 1), but made an uneventful recovery although temporal changes in both appetite (Day 7) and habitus (Days 7 and 8) were registered and scored as severely depressed. Furthermore, this

was the only colt with severe wound discharge (Day 2) whereas all other colts showed only mild to moderate discharges during the observation period.

3.4 Anti-GnRH antibody titres

High ABT were observed 28 days after primary vaccination in all treated colts (Fig. 3). Mean ABT of the treatment groups were similar on Day 28 whereas on Days 57 and 100, Group V1-57 had higher ABT than Group V2-100 colts.

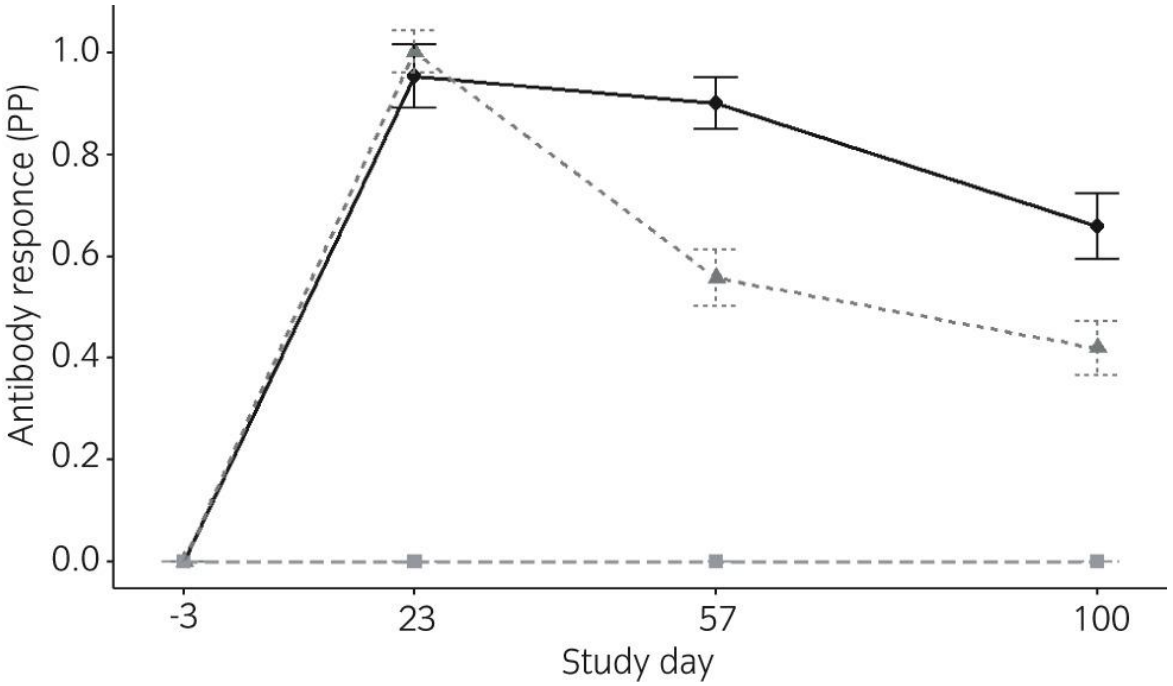

Figure 3. Changes in the proportion positive (PP; antibody response, measured as the mean sample absorbance (minus blank) expressed as a proportion of the mean absorbance (minus blank) of the positive reference sample at the same dilution (1:400, 1:800, and 1:1600)) antibody responses in 13 anti-GnRH vaccinated (Groups V1-57 & V2-100) and 6 unvaccinated control colts (Group Control). Antibody responses from Group V1-57, Group V2-100, and Group Control are represented as the solid, short dash, and long dash lines, respectively. Points are mean values and error bars correspond to the standard error of the mean. Day 0 is the day of primary anti-GnRH vaccination.

3.5 Serum testosterone concentration (STC)

The STC in Groups V1-57 and V2-100 started to decline after the first immunization and were undetectable by Day 28 (day of second immunization; Fig. 4). On Day 57 both groups were baseline, and Group V2-100 remained baseline until castration on Day 100. Group Control showed variable mean STC values on Days -4, 0 and 2 (2.9, 24.5 and 3.4 nmol/L) and then remained fairly consistent on Days 24, 30 and 57 (7.7, 7.7 and 5.3 nmol/L).

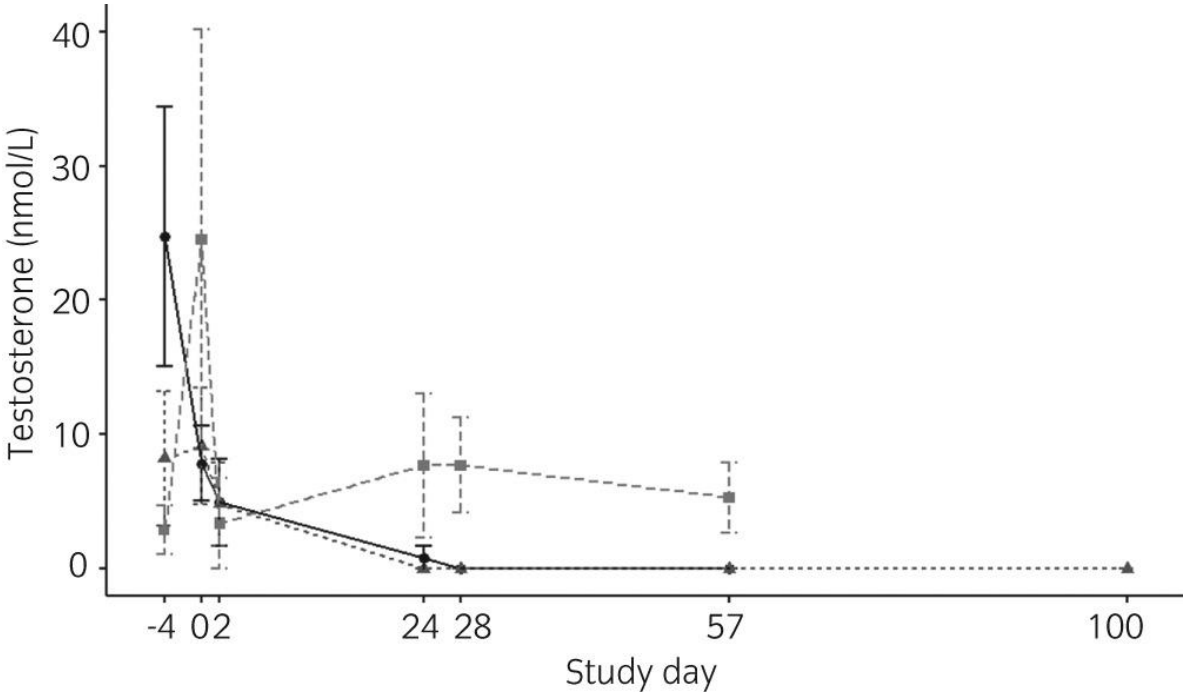

Figure 4. Changes in serum testosterone concentrations (nmol/L) over time in 13 anti-GnRH vaccinated (Groups V1-57&V2-100) and 6 unvaccinated control colts (Group Control). Testosterone concentrations from Group V1-57, Group V2-100, and Group Control are represented as the solid, short dash, and long dash lines, respectively. Points are mean values and error bars correspond to the standard error of the mean. Day 0 is the day of primary anti-GnRH vaccination.

3.6 Relationship between testicular mass and scrotal swelling, preputial swelling and wound discharge

Testis weight was positively correlated (Table 2) with both preputial (P=0.001) and scrotal (P=0.025) swelling and respiratory rate (P=0.002). Adnexa weight was only significantly correlated to scrotal swelling (P=0.021). Total combined weight of testis, epididymis and adnexa was significantly correlated with both preputial (P=0.005) and scrotal (P=0.004) swelling and respiratory rate (P=0.025). Antibody titre as proportion positive of the treated colts (n=13) was negatively correlated with weights of all structures but only adnexa (P=0.024) and epididymal (P=0.024) correlations were significant.

4. DISCUSSION

This study investigated the effects of pre-treatment of colts with the anti-GnRH vaccine Improvac® on testicular dimensions and subsequent post-surgical complications in colts subjected to castration in the field. Two treatment groups were immunized twice with Improvac® before surgical castration on Days 57 and 100, respectively. A similarly placebo-treated control group was castrated on Day 57.

Previous studies on anti-GnRH vaccination reported inconsistent decreases of between 13.4 - 33% in testicular dimensions [27,29,30,31,40,41]. In this study, mean testicular volume of all treated colts decreased by approximately 50% by Day 57 post-primary treatment. There was a further reduction with a resultant mean volume approximately one third of pre-treatment volumes in the group castrated nearly six weeks later. The decrease in testicular volume observed in the controls may well be attributed to a seasonal effect as the study commenced at the beginning of May (end of the summer season) and the controls were castrated in July (mid-winter) [45].

The Improvac®-treated colts all responded with a decline in STC by Day 24 and this continued to decline, to reach baseline concentrations by Day 28 and remaining baseline until their castration on either Day 57 or 100. The difference in response, which was either less consistent or not as rapid, could relate to different vaccine formulations administered in previous studies [32]. Mean STC of Group Control showed a spike on Day 0 where after it remained fairly consistent. While we cannot explain the spike observed, diurnal rhythms are reported in stallions with a peak approximately coinciding with sunrise and another during the night [42,43,44]. Times of sampling were, however, not recorded.

In the current study, the antibody response was rapid in both Improvac® treatment groups. The marked decline in ABT from Days 28 to 57 in Group V2-100 compared to Group V1-57 colts cannot be explained as both groups were subjected to the same vaccination procedure. This difference in ABT between the two treatment groups did not appear to affect the outcome on testis volume. Moreover, the mean testis volume of Group V2-100 colts continued to decline between Day 57 and 100. It was assumed that, although lower, the ABT were still sufficient to neutralize endogenous GnRH in Group V2-100 colts. The responses of testicular dimensions, STC and ABT to immunisation suggest that Improvac® is a more immunogenic than other vaccines investigated previously [27,30,32]. Poor or partial response to anti-GnRH vaccination in previous studies was attributed to vaccine adjuvant combinations and age differences with older stallions apparently being more resistant to down-regulation of testicular function [27,30,32]. The current study's experimental population was considerably younger than in previously reported studies and this might explain both the more marked and consistent responses.

The mean epididymis masses of the treatment groups were descriptively smaller in the treatment groups compared to the controls. It was anticipated that epididymis function, largely controlled by metabolites of testosterone including dihydrotestosterone, would be affected by down-regulation of Leydig cell activity. Arguably, the observed absence of any observed meaningful changes was ascribable to two factors. Firstly, the epididymis consists of a single continuous duct, unlike the testis with its numerous seminiferous tubules. A reduction in size of a single tubule will therefore have little effect on the overall size and weight of the epididymis. Secondly, the colts, only 15-20 months old were just starting to produce mature sperm. The size of especially the epididymis tail (where sperm are stored) may thus have been unaffected by anti-GnRH vaccination at this relatively early age.

In the present study no intra-surgical complications were observed and post-operative complications were limited to varying degrees of preputial and scrotal oedema, and increases in heart and respiratory rates. Supporting the study's hypothesis, significant positive correlations were observed between total weight of testes plus adnexa and testicular mass alone, and preputial and scrotal swelling and respiratory rate of all three groups. Thus, reducing the size of the testes and their adnexa resulting from Improvac® immunization had an apparently beneficial effect on both the incidence and degree of post-operative surgical complications. This study's observations also suggested that castration surgery following an interval of 100 days from primary Improvac® vaccination was associated with continued reduction in testicular size conferring further potential advantages.

This pilot study was limited by the experimental population consisting entirely of young light-horse breed colts and a relatively small sample size. Blocked randomization by weight (or age) could have been performed in an attempt to create more equal groups at the start. Further investigation of potential beneficial effects of pre-castration anti-GnRH immunization including both an older population and heavy-breed (draft) horses was indicated.

The administration of Improvac® in this population of young colts was not associated with any obvious side effects or systemic signs, which is consistent with a previous study in a population of mares using the same immunogen, dose and injection site [33].

In conclusion, the administration of Improvac® in a population of young colts effectively decreased their testicular dimensions. This outcome appeared to be associated with a reduction in the incidence and severity of complications commonly reported in association with surgical castration in stallions and supports best practice guidelines for equine welfare. An additional finding was that the vaccine effectively decreased STC and therefore has potential for the control of undesired androgen-related behaviour in stallions [27,30,31].

Manufacturers addresses:

[a]Voermol Feeds (Pty) Ltd, Maidstone, South Africa

[b, c] Zoetis South Africa (Pty) Ltd, Sandton, South Africa

[d,e]Kyron Laboratories (Pty) Ltd, Benrose, South Africa

[f] Merial South Africa (Pty) Ltd, Midrand, South Africa

[g]Radwag, Radom, Poland

[h]Becton Dickinson, Plymouth, UK

[i] Diagnostic Products Corporation, Los Angeles, California, USA

[j]Thermo Fisher Scientific, Roskilde, Denmark

[k]Sigma, Saint Louis, Missouri, USA

[l]Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA

[m]Thermo Fisher Scientific, Waltham, Massachusetts, USA

[n]Minitab Inc., State College, Pennsylvania, USA

[o] IBM Corp, Armonk, New York, USA

References

1. Moll, H.D., Pelzer, K.D., Pleasant, R.S., Modransky, P.D, and May, K.A. (1995) A survey of equine castration complications. *J. Equine Vet. Sci.***15**, 522-526.

2. Hunt, R.J. and Boles, C.L. (1989) Post castration eventration in eight horses. *Canadian Vet. J.* **30**, 961-963.

3. Hunt, R.J. (1991) Management of complications associated with equine castration. *Comp. Cont. Educ. Pract.* **13**, 1835-841.

4. Schumacher, J. (1996) Complications of castration. *Equine. Vet. Educ.* **8**, 254-259.

5. Searle, D., Dart, A.J. and Hodgson, D.R. (1999) Equine castration: review of anatomy, approaches, techniques and complications in normal, cryptorchid and monorchid horses. *Aust. Vet. J.* **77**, 428-434.

6. Shoemaker, R., Bailey, J., Janzen, E. and Wilson, D.G. (2004) Routine castration in 568 draught colts; incidence of evisceration and omental hernia. *Equine Vet. J.* **36**, 336-340.

7. Kummer, M., Gygax, D., Jackson, M., Bettschart-Wolfensberger, R. and Fürst, A. (2009) Results and complications of a novel technique for primary castration with an inguinal approach in horses. *Equine Vet. J.* **41**, 547-551.

8. Line, S.W., Hart, B.L. and Sanders, L. (1985) Effect of prepubertal versus postpubertal castration on sexual and aggressive behaviour in male horse. *J. Am. Vet. Med. Assoc.* **186 (3)**, 249-251.

9. Hutchins, D.R. and Rawlinson, R.J. (1972) Eventration as a sequel to castration of the horse. *Aust. Vet. J.* **48**, 288-291.

10. van der Velden, M.A. and Rutgers, L.J.E. (1990) Visceral prolapsed after castration in the horse: a review of 18 cases. *Equine Vet. J.* **22**, 9-12.

11. Price J, Eager RA, Welsh EM, Waran NK. (2005) Current practice relating to equine castration in the UK. *Res. Vet. Sci.* **78**, 277-280.

12. Roser, J.F. (1997) Endocrine basis for testicular function in the stallion. *Theriogenology* **48**, 883-892.

13. Roser, J.F. (2008) Regulation of testicular function in the stallion: an intricate network of endocrine, paracrine and autocrine systems. *Anim. Reprod. Sci.* **107**, 179-196.

14. Thompson, D.L. Jr. (2000) Immunization against GnRH in male species (comparative aspects). *Anim. Reprod. Sci.* **60-61**, 459-469.

15. Robertson, I.S., Fraser, H.M., Innes, G.M. and Jones, A.S. (1982) Effect of immunological castration on sexual and production characteristics in male cattle. *Vet. Rec.* **111**, 529-531.

16. Janett, F., Gerig, T., Tschuor, A.C., Amatayakul-Chantler, S., Walker, J., Howard, R., Bollwein, H. and Thun, R. (2012) Vaccination against gonadotropin-releasing factor (GnRF) with Bopriva significantly decreases testicular development, serum testosterone levels and physical activity in pubertal bulls. *Theriogenology* **78**, 182-188.

17. Dunshea, F.R., Colantoni, C., Howard, K., McCauley, I., Jackson, P., Long, K.A., Lopaticki, S., Nugent, E.A., Simons, J.A., Walker, J. and Hennessy, D.P. (2001) Vaccination of boars with a GnRH vaccine (Improvac) eliminates boar taint and increases growth performance. *J. Anim. Sci.* **79**, 2524-2535.

18. Janett, F., Lanker, U., Jorg, M., Hassig, M. and Thun, R. (2003) Castration of male lambs by immunisation against GnRH. *Schweiz. Arch. Tierhk.* **145**, 291-299.

19. Ferro, V.A., Khan, M.A.H., McAdam, D., Colston, A., Aughey, E., Mullen, A.B., Waterston, M.M. and Harvey, M.J.A. (2004) Efficacy of an anti-fertilty vaccine based on mammalian gonadotrophic releasing hormone (GnRH-1)-a histological comparison in male animals. *Vet. Immunol. Immunop.* **101**, 73-86.

20. Jung, M., Moon, Y., Cho, I., Kim, S., Chang, W., Park, S-Y., Song, C-S., Kim, H-Y., Park, K-K., McOrist, S., Choi, I-S. and Lee, J-B. (2004) Induction of castration by

immunisation of male dogs with recombinant gonadotropin-releasing hormone (GnRH) canine distemper virus (CDV) T helper cell epitope. *J. Vet. Sci.* **6**, 21-24.

21. Levy, J.K., Miller, L.A., Crawford, P.D., Ritchley, J.W., Ross, M.K. and Fagerstone, K.A. (2004) GnRH immunocontraception of male cats. **T***heriogenology* **4**, 62:1116-1130.

22. Robbins, S.C., Jelinski, M.D. and Stotish, R.L. (2004) Assessment of the immunological and biological efficacy of two different doses of a recombinant GnRH vaccine in domestic male and female cats (*Felis catus*). *J. Reprod. Immunol.* **64**, 107-111.

23. Killian, G., Miller, L., Rhyan, J. and Doten, H. (2006) Immunocontraception of Florida feral swine with a single dose GnRH vaccine. *Am. J Reprod. Immunol.* **55**, 378-384.

24. Ghoneim, I.M., Waheed, M.M., Al-Eknah, M.M. and El-Bahr, S.M. (2012) Immunization against GnRH in the male camel (*Camelus dromedaries*): Effects on sexual behaviour, testicular volume, semen characteristics and serum testosterone concentrations. *Theriogenology* **78**, 1102-1109.

25. De Nys, H.M., Bertschinger, H.J., Turkstra, J.A., Colenbrander, B., Palme, R. and Human, A.M. (2010) Vaccination against GnRH may suppress aggressive behaviour and musth in African elephant (*Loxodonta africana*) bulls - a pilot study. *J. S.A. Vet. Ass*. **81**, 8-15.

26. Lueders, I., Young, D., Maree, L., van der Horst, G., Luther, I., Botha, S., Tindall, B., Ganswindt, A. and Berstchinger, H.J. (2017) GnRH vaccination for contraception in wild and captive African elephant bulls (*Loxodonta africana*): effects on reproductive organs and semen quality. *PLoS ONE* **12(9)**, e0178270.

27. Janett, F., Stump, R., Burger, D. and Thun, R. (2009) Suppression of testicular function and sexual behaviour by vaccination against GnRH (Equity™) in the adult stallion. *Anim. Reprod. Sci.* **115**, 88-102.

28. Rabb, M.H., Thompson, D.L. Jr, Barry, B.E., Colborn, D.R., Hehnke, K.E. and Garza, F. Jr. (1990) Effects of active immunisation against GnRH on LH and FSH and prolactin storage, secretion and response to their secretagogues in pony geldings. *J. Anim. Sci.* **68**, 3322-3329.

29. Dowsett, K.F., Pattie, W.A., Knott, L.M., Jackson, A.E., Hoskinson, R.M., Rigby, R.P.G. and Moss, B.A. (1991) A preliminary study of immunological castration in colts. *J. Rep. Fert.* **44**, 183-190.

30. Malmgren, L., Andresen, Ø. and Dalin, A.M. (2001) Effect of GnRH immunization on hormonal levels, sexual behaviour, semen quality and testicular morphology in mature stallions. *Equine Vet. J.* **33**, 75-83.

31. Stout, T.A.E. and Colenbrander, B. (2004) Suppressing reproductive activity in horses using GnRH vaccine, antagonists or agonists. *Anim. Reprod. Sci.* **82-83**, 633-643.

32. Turkstra, J.A., van der Meer, F.J.U.M., Knaap, J., Rottier, P.J.M., Teerds, K.J., Colenbrander, B. and Meloen, R.H. (2005) Effects of GnRH immunization in sexually mature pony stallions. *Anim. Reprod. Sci.* **86**, 247-259.

33. Botha, A.E., Schulman, M.L., Bertschinger, H.J., Guthrie, A.J., Annandale, C.H. and Hughes, S.B. (2008) The use of GnRH vaccine to suppress mare ovarian activity in a large group of mares under field conditions. *Wildl. Res.* **35**, 548-554.

34. Pavlik, E.J., De Priest, P.D., Gallion, H.H., Ueland, F.R., Reedy, M.B., Kryscio, R.J. and van Nagell, J.R. (2000) Ovarian volume related to age. *Gynaecol. Oncol.* **77,** 410-412.

35. Gleerup, K.A. (2018) Assessing pain in horses. In Practice, 40: 457-463. http://dx.doi.org/10.11.36/inp.k4781.

36. Schulman, M.L., Botha, A.E., Muenscher, S.B., Guthrie, A.J. and Bertschinger, H.J. (2013) Reversibility of the effects of GnRH vaccination used to suppress reproductive function in mares. *Equine Vet. J.* **45,** 111-113.

37. Joonè, C.J., Bertschinger, H.J., Gupta, S.K., Fosgate, G.T., Arukha, A.P., Minhas, V., Dieterman, E. and Schulman, M.L. (2015) Ovarian function and pregnancy outcome in pony mares following immunocontraception with native and recombinant porcine zona pellucida vaccines. *Equine Vet. J*. **49**, 189-195.

38. Wickham, H. (2009) Gg plot2: elegant graphics for data analysis. Springer-Verlag New York. URL: http://ggplot2.org.

39. R Development Core Team. (2017) R A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.URL https://www.R-project.org/.

40. van der Meer, F.J.U.M., Turkstra, J.A., Knaap, J., Rotier, P., Meloen, R.H., Teerts, K.J., Stout, T.A.E. and Colenbrander, B. (2001) Immunocastartion of stallions: an investigation into the effects of anti-GnRH immunisation on reproductive parameters (preliminary results). In: Proc 3rd International Symposium on Stallion Reproduction, 45.

41. Burger, D., Janett, F., Vidament, M., Stump, R., Fortier, G., Imboden, I. and Thun, R. (2006) Immunization against GnRH in adult stallions: Effects on semen characteristics, behaviour and shedding of equine arteritis virus. *Anim. Reprod. Sci.* **96**, 107-111.

42. Sharma, O.P. (1976) Diurnal Variations of Plasma Testosterone in Stallions. *Biol. Reprod.* **15 (2)**, 158–162, https://doi.org/10.1095/biolreprod15.2.158

43. Kirkpatrick, J. F., Vail, R., Devous, S., Schwend, S., Baker, C. B. and Wiesner, L. (1976) Diurnal variation of plasma testosterone in wild stallions. *Biol. Reprod.*15, 98-101. DOI : 10.1095/biolreprod15.1.98

44. Kikuchi, M., Onuma, H., Kodaira, Y., Suzuki, A., Kawakami, T. and Nakanisi, S. (1993) Effects of Age and Season on Diurnal Variation and 24 Hour Mean Concentrations of Plasma Testosterone and Dihydrotestosterone in Stallions. *J. Reprod. Develop.* **39**, 17-21. DOI https://doi.org/10.1262/jrd.39.5_j17

45. Johnson, L. And Thompson, D.L. (1987) Effect of seasonal changes in Leydig cell number on the volume of smooth endoplasmic reticulum in Leydig cells and intratesticular testosterone content in stallions *J. Reprod. Fertil.* **81**, 227-232.