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Suppression of testicular function and sexual behavior by vaccination against GnRH (EquityTM) in the adult stallion

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ABSTRACT

The aim of this study was to evaluate the effects of an anti-GnRH vaccine on testosterone concentration, antibody titer, scrotal width, semen quality and sexual behavior in the stallion. Adverse reactions to the vaccine were also determined. Eight clinically healthy sexually experienced stallions aged between 6 and 15 years from the National Stud in Avenches (Switzerland) were used. Five stallions were immunized 3 times at an interval of 4 and 8 weeks, respectively, with 200 µg of a GnRH-protein conjugate (EquityTM, CSL Limited, Australia) intramuscularly in the neck and 3 control animals received an equivalent volume of saline solution. Plasma testosterone concentrations and GnRH antibody titers as well as semen quality and libido were determined weekly during 1 year (52 weeks). In addition, scrotal width was measured in all stallions before and 4, 8 as well as 12 months after first vaccination. Our results demonstrate that in 4 stallions plasma testosterone started to decrease after the second vaccination and remained suppressed for at least 6 months whereas one stallion showed no effect. Until the end of the experiment 2 stallions reached prevaccination testosterone values. Antibody titers varied individually in all 5 stallions and reached peak concentrations within 2 weeks after the third vaccination. Scrotal width was significantly ($P < 0.05$) lower in vaccinated than in control stallions 8 months after first vaccination. Semen quality started to decreased after the second vaccination and improved towards the end of the experiment. In 4 stallions libido was clearly reduced after the second immunization but normalized in 2 animals before the end of the study while 2 stallions continued to show poor libido. From

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our results we conclude that three immunizations with Equity™ are well tolerated in the stallion and may effectively suppress testosterone secretion and reduce semen quality as well as sexual behavior. The inhibiting activity of Equity™ on these parameters is individually different and may last for a minimum of 6 months.

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1. Introduction

The suppression of male reproductive function has long been an important issue in equine veterinary practice. In stallions, the main interest is primarily directed towards preventing aggressive and unwanted sexual behavior to make them easier to handle and to guarantee their level of performance in training or competition. Control of reproductive activity may either be achieved by surgical, hormonal or immunological castration (Stout and Colenbrander, 2004; Stout, 2005). Surgical castration which is widely used throughout the world irreversibly eliminates the source of testicular steroids and sperm resulting in loss of reproductive potential. Furthermore, castration carries surgical risks, especially in older animals and it has been shown (Line et al., 1985) that the behavioral problems will not be resolved in all cases. A noninvasive alternative to castration is the application of progestagens to suppress hypothalamic and anterior pituitary secretion of GnRH and LH, respectively thus decreasing testicular testosterone release (Brady et al., 1997; Squires et al., 1997). Since progestagens such as altrenogest must be given daily in high doses to control sexual behavior in older stallions (Miller et al., 1997) and because little is known about possible side effects, progestagen treatment has not been well accepted in practice. In addition, progestagen administration cannot be used in horses which are intended for human consumption and in many equine sporting events this treatment is considered as doping-offence.

Another possibility of reducing LH and testosterone secretion in the stallion is the application of GnRH antagonists (Hinojosa et al., 2001; Fortier et al., 2002). The effect on libido appears to be age-dependent being absent in mature stallions despite a dramatic fall in testosterone concentrations (Fortier et al., 2002). Treatment with high doses of a GnRH agonist can effectively desensitize the pituitary to GnRH (down-regulation) in various species. In the stallion, however, agonist treatment had either no (Brinsko et al., 1998) or only a limited suppressive effect (Montavon et al., 1990; Boyle et al., 1991) and occasionally even enhanced gonadotropin secretion (Roser and Hughes, 1991; Sieme et al., 2004). While results using hormones, antagonists or agonists are not satisfactory, a practical alternative for inhibiting male reproductive function is by active immunization against various hormones of the hypothalamic–pituitary–gonadal axis (D'Occhio, 1993; Thompson, 2000). Experiments using different GnRH vaccines were first performed in cattle (Robertson et al., 1979, 1982), sheep (Lincoln and Fraser, 1979; Jeffcoate et al., 1982) and swine (Falvo et al., 1986; Caraty and Bonneau, 1986). In horses, Schanbacher and Pratt (1985) were the first to describe the successful vaccination against GnRH in a cryptorchid stallion. Until now, several studies have been published (Dowsett et al., 1991, 1996; Malmgren et al., 2001; Dalin et al., 2002; Clement et al., 2005; Turkstra et al., 2005; Imboden et al., 2006; Elhay et al., 2007) not only investigating the anti-GnRH effect on reproductive activity in the horse but also for the treatment of equine arteritis virus (EVA) shedder stallions (Burger et al., 2004, 2006). In 2001, a GnRH vaccine (Equity™, Pfizer Animal Health, Australia) for specific use in horses was licensed in Australia and New Zealand for the control of estrus in the mare. As comprehensive studies in the stallion are lacking, the aim of the present investigation was to evaluate the safety and efficacy of Equity™ in the stallion with special emphasis on plasma testosterone and antibody concentrations as well as on semen quality and sexual behavior.

2. Materials and methods

2.1. Experimental design

Eight clinically healthy and sexually experienced warmblood stallions from the National Stud in Avenches (Switzerland) aged between 6 and 15 years were used for the experiment. The animals

were kept in box stalls bedded with straw and were fed hay or haylage, oats, barley, corn and pellets supplemented with minerals. Water was available *ad libitum*. All animals were regularly exercised and had daily access to a paddock. Before the onset of the experiment the stallions were trained to mount the phantom and extragonadal sperm reserves were minimized by five daily semen collections (Hurtgen, 1992). Five stallions (A–E) were randomly allocated to a treatment group and 3 animals served as controls (F–H). Beginning in May collections of blood and semen were performed weekly for a period of 1 year. Scrotal width was measured using a calliper at the beginning of the experiment (May) and 4 (September), 8 (January) as well as 12 months (May) later. Subsequent to semen collections in weeks 1, 5 and 13 the stallions A–E were immunized with 1 ml (200 µg GnRH-protein conjugate) of a commercially available anti-GnRH vaccine (EquityTM, Pfizer Animal Health, Australia). Control animals received an equivalent volume of saline solution. All injections were applied intramuscularly into the neck. Adverse effects were monitored for 3 days after injection by twice daily clinical examination with particular regard to body temperature, signs of tissue reaction at the site of injection and stiffness of the neck. All animal experimentation was performed following approval from the local Animal Ethics Committee (Etat de Vaud, service vétérinaire, protocol approval number 1498).

2.2. Semen collection and examination

Once the stallion had mounted the phantom, semen was collected with an artificial vagina (model “Avenches”). Volume of the ejaculate was measured after removal of the gel fraction. Sperm concentration, total sperm number and total motility were assessed in freshly diluted (INRA 96, IMV, Aîgle, France) semen with a sperm analyser (HTM-IVOS, Version 12, Beverly, MA, USA) using 20 µm standard count analysis chambers (Art. no. SC 20-01-C, Leja, Nieuw-Vennep, The Netherlands) and standardized threshold values for stallion semen. For morphological examination, three drops of fresh semen were fixed in 2 ml buffered formol saline solution (Na₂HPO₄ 4.93 g, KH₂PO₄ 2.54 g, 38% formaldehyde 125 ml, NaCl 5.41 g, distilled water qs 1000 ml) and smears prepared. At least 200 spermatozoa were subsequently evaluated by phase contrast microscopy (Olympus BX50, UplanF1 100×/1.30) and abnormal spermatozoa classified in major (acrosome defects, nuclear vacuoles, abnormal heads, loose abnormal heads, abnormal midpieces, proximal droplets) and minor (loose normal heads, abnormal tails, distal droplets) defects (Blom, 1973; Jasko et al., 1990).

2.3. Sexual behavior

The stallions were exposed to the phantom in the presence of an ovariectomized mare during maximal 10 min for each attempt to collect semen. Sexual behavior was measured as time to first erection and ejaculation.

2.4. Testosterone analysis and GnRH antibody titers

Blood samples (EDTA) were collected in the morning between 8 and 9 a.m. at each examination day by jugular venipuncture, immediately centrifuged (4000 × g, 10 min) and the plasma frozen (−20 °C) until analysis. Testosterone was determined by electrochemiluminescence immunoassay (Elecys 2010, Roche Diagnostics, Basel, Switzerland) as described earlier (Wang et al., 2004). The detection limit of the assay was 0.02 ng/ml. All samples were analyzed using a biotinylated monoclonal antibody against testosterone. Cross-reactivity with estrogens and progesterone was <0.01%, with androstenedione 0.91% and with 5-α-dihydrotestosterone 1.89%. For validation of between assay precision a pooled sample was analyzed 6 times a day for a total of 10 days (*n* = 60) and for within assay precision pooled samples were measured 20 times a day (*n* = 20). Inter- and intra-assay coefficients of variation were 2.2 and 1.4%, respectively.

Antibody titers against GnRH were determined using a radioligand binding assay as described previously (Finnerty et al., 1994). Results are presented as percentage of total ¹²⁵I-labelled GnRH bound at a plasma dilution of 1:40.

2.5. Data analysis

Data were analyzed using StatView 5.0 software program (SAS Institute, Wangen, Switzerland). A multivariate analysis of variance (ANOVA) was carried out to assess the effects of group allocation, time and vaccination (interaction between group allocation and time) on plasma testosterone concentrations and scrotal width. Between group differences were compared with unpaired *t*-test. Values were considered to be statistically significant at $P < 0.05$. Because of high individual variation antibody titers, plasma testosterone as well as total sperm number, motility and major sperm defects are shown for single animals.

Testicular function was considered to be suppressed when testosterone values were lower than 0.1 ng/ml for at least 2 consecutive weeks. After a period of testicular suppression stallions were judged as having resumed reproductive activity when they fulfilled the following criteria: (a) plasma testosterone concentrations >0.1 ng/ml for at least 2 consecutive weeks and (b) ejaculation of more than 500 million motile sperm within 10 min of exposure to the phantom and the ovariectomized mare.

3. Results

3.1. Adverse effects of the vaccine

In the 5 stallions immunized with 1 ml Equity™ neither apathy nor pyrexia could be diagnosed following the first or booster vaccinations. One animal (B) reacted with a small not painful swelling after the first and third immunization and another stallion (D) had a sore swelling after the third injection. All adverse effects resolved within 2–3 days after appearance. There were no injection site reactions in the control stallions.

3.2. GnRH antibody titer

Antibody titers against GnRH in all vaccinated stallions are shown in Fig. 1. Titers started to rise immediately after the second vaccination and reached peak values between 14.6 and 43.9% binding 2–4 weeks after the third vaccination. Thereafter the titers slowly decreased to values between 6.1 and 21.0% binding at week 51. The least antibody response with maximum values of only 15% binding occurred in stallion C.

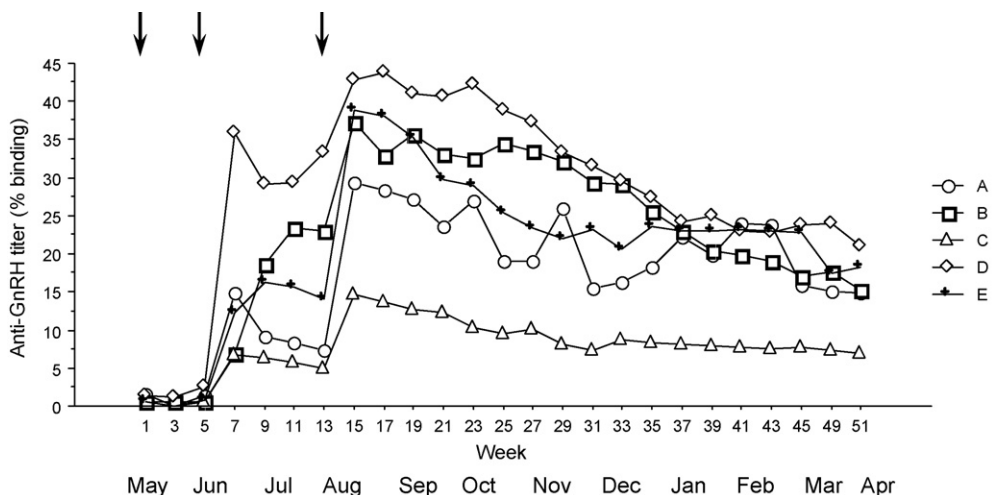


Fig. 1. Anti-GnRH antibody titers (% binding at a 1:40 plasma dilution) in 5 stallions (A–E) after vaccination with Equity™ (↓).

3.3. Testosterone

Results from ANOVA demonstrate that weekly measured plasma testosterone concentrations were significantly influenced by group allocation ($P<0.0001$), time of blood collection ($P<0.0001$) and by

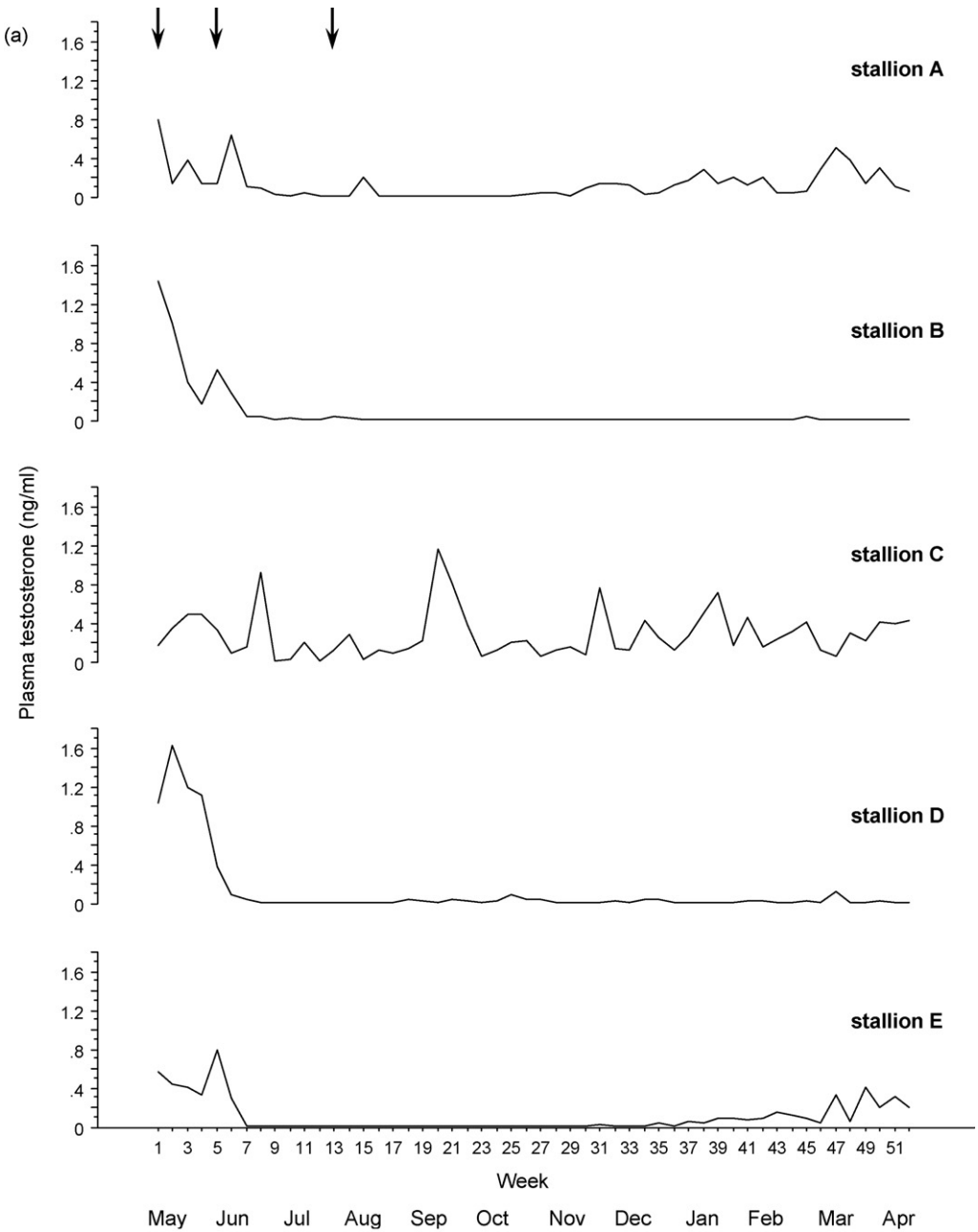


Fig. 2. (a) Individual blood testosterone concentrations of 5 stallions (A–E) after vaccination with Equity™ (↓). (b) Individual blood testosterone concentrations of the control stallions F, G and H.

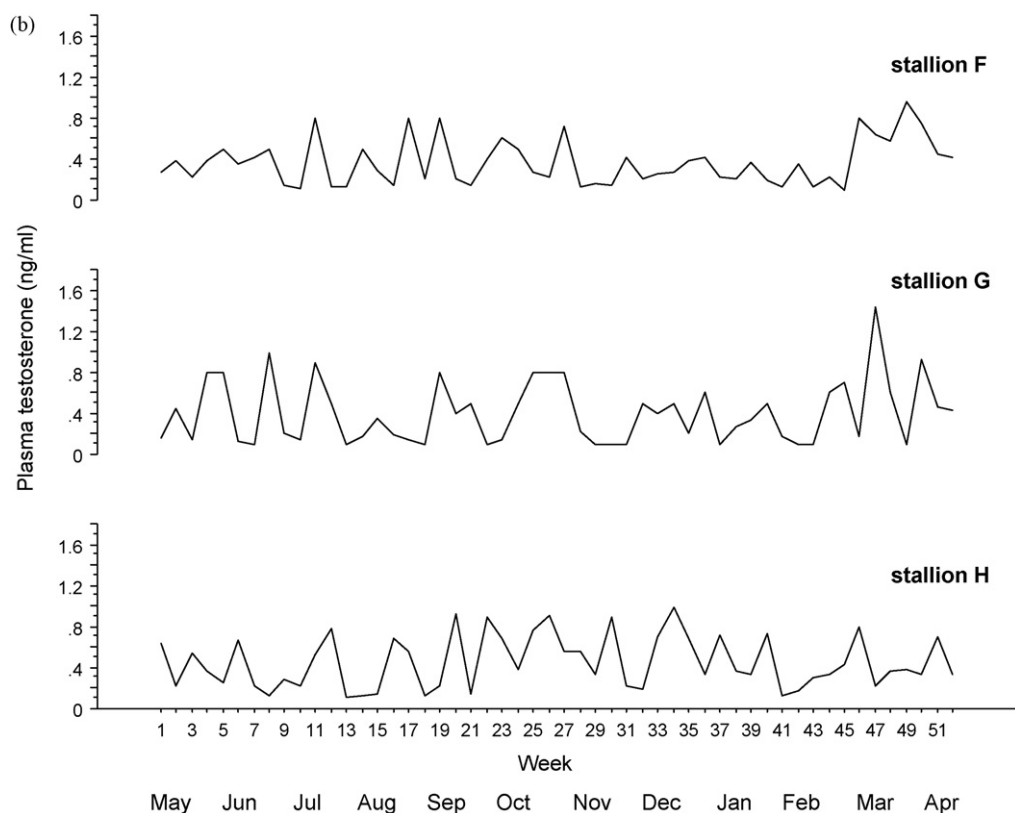


Fig. 2. (Continued).

vaccination (interaction between group and time, $P=0.0003$). The individual testosterone profiles in all vaccinated stallions are shown in Fig. 2a. Starting at 2 weeks after the second vaccination only low fluctuating concentrations (<0.1 – 0.5 ng/ml plasma) were observed in 4 stallions. In one animal (C) the range of values was higher (<0.1 – 1.2 ng/ml plasma) and testosterone not suppressed during the whole study. Regarding duration of suppression 2 stallions had low plasma testosterone (<0.1 ng/ml) during 45 weeks (B, 7–52) and 46 weeks (D, 6–52) while in the other two animals suppression lasted for 24 weeks (A, 7–31) and 36 weeks (E, 7–43), respectively. In control animals (Fig. 2b) testosterone secretion was episodic with fluctuations between 0.1 and 1.4 ng/ml plasma.

3.4. Sexual behavior

In comparison to control stallions exhibiting normal sexual behavior including erection within 1–3 min and ejaculation during the first mount in the presence of a mare, libido varied considerably in all vaccinated stallions. In all but one stallion (D) time to erection and ejaculation was negatively affected by GnRH vaccination and in 2 stallions (A and C) both parameters were temporarily (weeks 20–42) prolonged and occasionally no semen could be collected. During this period stallion A often needed several mounts until ejaculation and stallion C failed to ejaculate at all during weeks 26–42. However, in these two animals libido normalized towards the end of the experiment. In the remaining 2 stallions the interest for mares continuously decreased after the second vaccination and no ejaculation occurred in stallion B from week 29 until the end of the study (week 52) and in stallion E during weeks 12–42. In control animals semen collection was possible during the whole study.

3.5. Semen quality

In all vaccinated stallions total sperm number fluctuated between 2 and 18×10^9 and decreased after the third vaccination (Fig. 3a). In control animals total sperm number varied between 2 and 17×10^9 throughout the whole experiment (Fig. 3b). With the exception of stallion C sperm motility

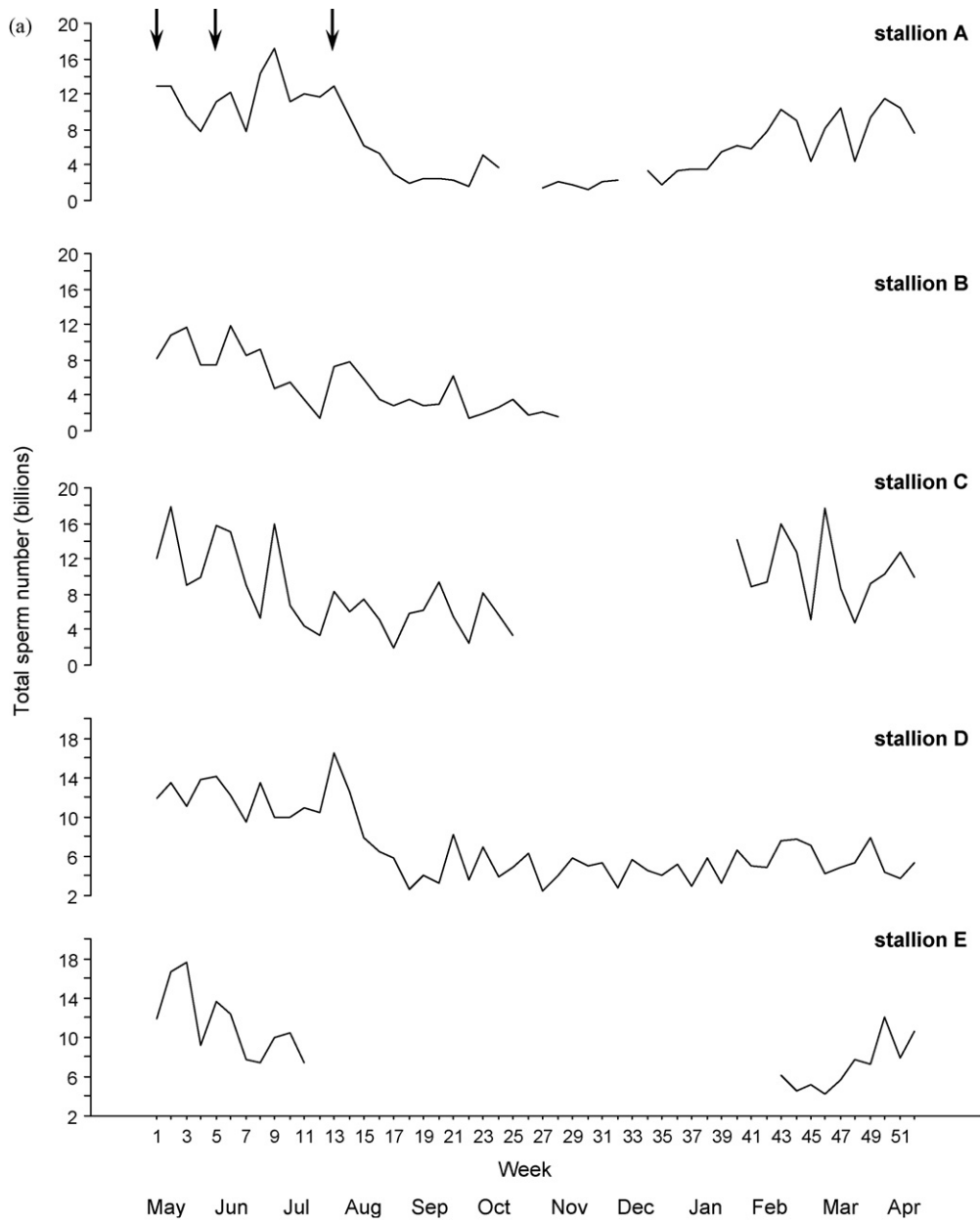


Fig. 3. (a) Total sperm number in weekly collected ejaculates of 5 stallions (A–E) after vaccination with EquiTM (↓). (b) Total sperm number in weekly collected ejaculates of the control stallions F, G and H.

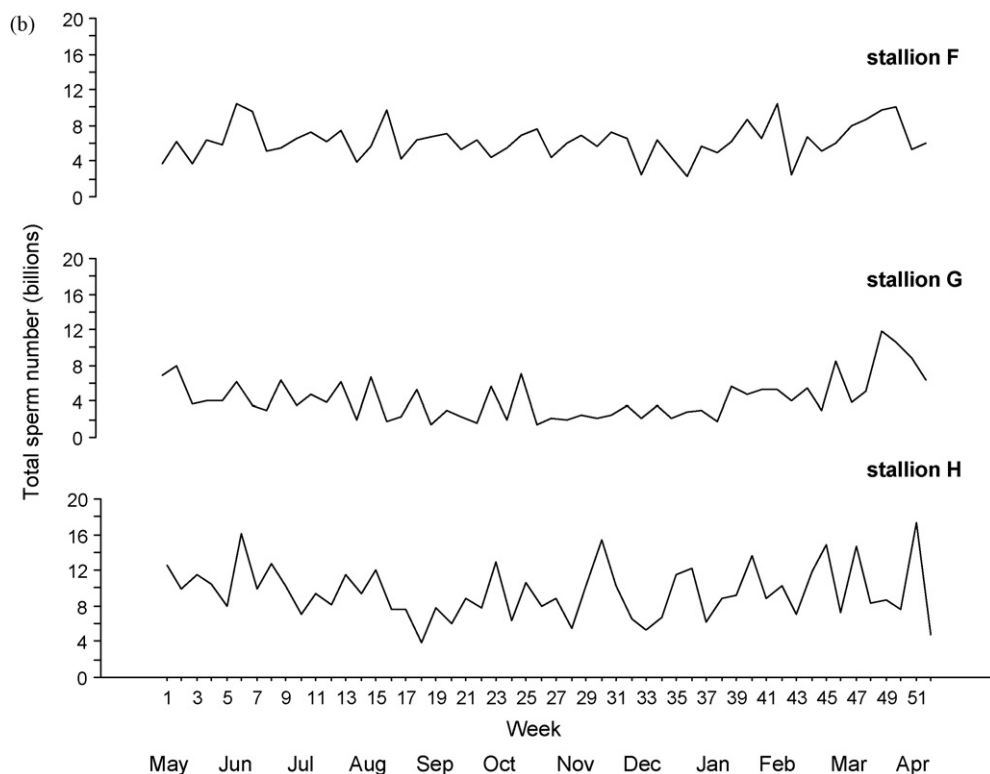


Fig. 3. (Continued).

began to decrease individually after the second and third vaccination and recovered to prevaccination values after week 40 (Fig. 4a). In control animals sperm motility fluctuated on an individually different but constant level during the whole study (Fig. 4b). Changes in the percentage of major sperm defects in vaccinated stallions are shown in Fig. 5a. After immunization, an increase of major sperm defects was noticed in 4 stallions (A, B, D and E), while in stallion C a high percentage of major sperm defects (80–90%) was already present before the experiment started and these values were not affected by vaccination with EquityTM. All control animals showed a high percentage of major sperm defects with only minor fluctuations on a constant level.

3.6. Scrotal width

Scrotal width was significantly ($P < 0.05$) influenced by vaccination against GnRH (interaction between time and group allocation, $P = 0.0073$) but not by group allocation ($P = 0.1061$) and time ($P = 0.0515$). Means ($m \pm \text{S.E.M.}$) of scrotal width in vaccinated animals decreased from 11.8 ± 0.6 cm before to 8.8 ± 1.0 cm and 8.0 ± 0.8 cm and increased to 10.6 ± 0.5 cm, 4, 8 and 12 months after first vaccination, respectively (Fig. 6). In control stallions mean scrotal width ranged between 11.0 ± 0.6 and 11.7 ± 0.9 cm. A significant ($P < 0.05$) group difference was present 8 months after first vaccination.

3.7. Resumption of reproductive activity

Based on criteria defined in Section 2 only two vaccinated stallions A and E regained reproductive activity at weeks 34 and 43, respectively. In stallions B and D testicular function remained suppressed until the end of the study.

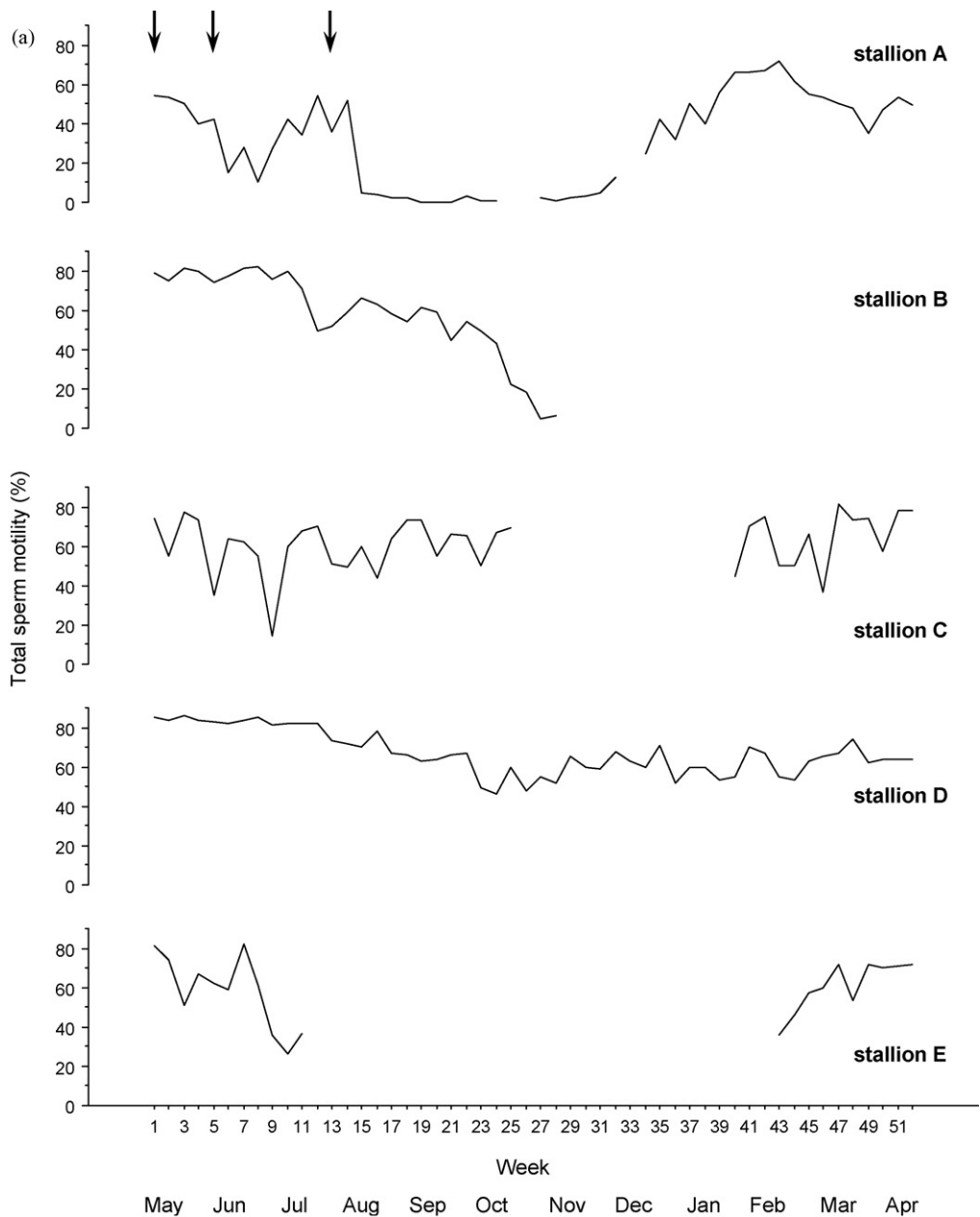


Fig. 4. (a) Total sperm motility in weekly collected ejaculates of 5 stallions (A–E) after vaccination with Equity™ (↓). (b) Total sperm motility in weekly collected ejaculates of the control stallions F, G and H.

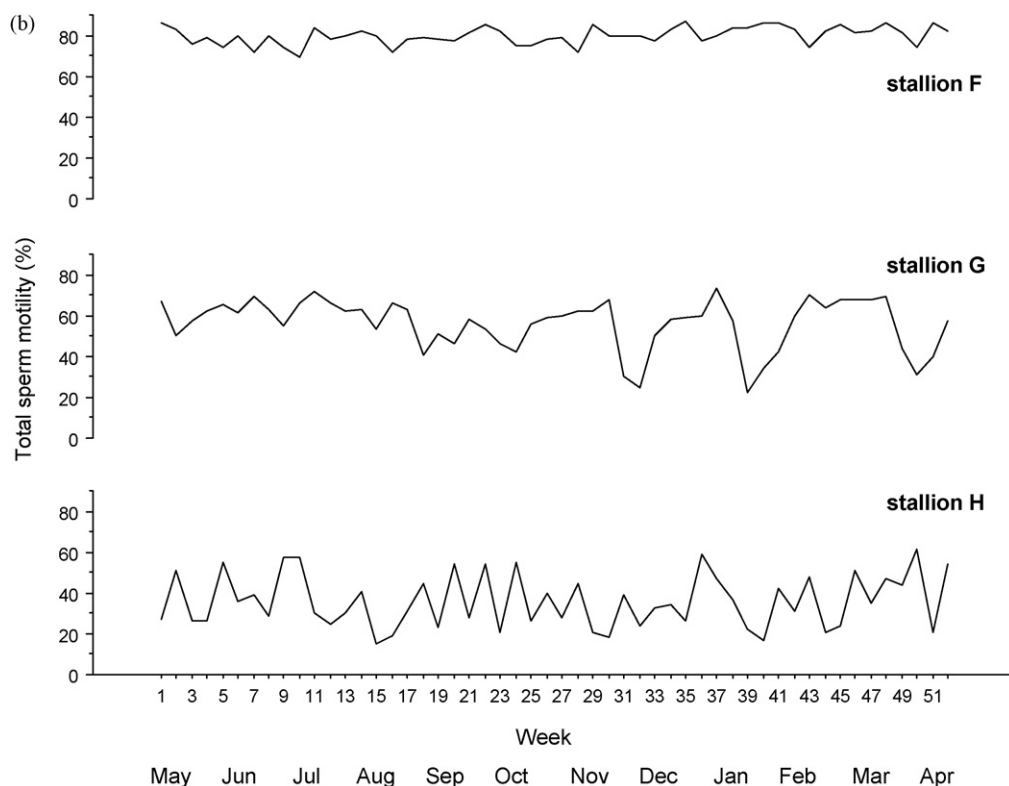


Fig. 4. (Continued).

4. Discussion

Results of our study demonstrate that administration of the GnRH vaccine Equity™ to adult stallions reduces testosterone secretion, scrotal width as well as semen quality and sexual behavior. The immune response to vaccination, however, was characterized by considerable variation as depicted in the profiles of the individual animals.

Unwanted side effects including minor swelling at the injection site lasting for only 2–3 days were observed in 2 stallions. The high degree of safety when using Equity™ in horses agrees with a recently published study in mares (Elhay et al., 2007) showing that only 10 of 24 vaccinated mares reacted with a transient slightly raised and flat swelling of the skin following first or second immunization. In contrast, the application of Improvac™ (Imboden et al., 2006) or other GnRH vaccine formulations (Dowsett et al., 1991, 1996; Dalin et al., 2002) in the horse may cause severe adverse effects as pyrexia, apathy and stiffness of the neck and should therefore not be used in horses.

First successful vaccination of a cryptorchid stallion against GnRH was reported by Schanbacher and Pratt (1985), but several booster injections were required to suppress testosterone secretion. This vaccine contained Freund's adjuvant and inhibition of testosterone lasted 4 months. Because of severe reactions at the site of injection other vaccine formulations containing a water-soluble adjuvant were subsequently developed. Using such a vaccine in 2-year-old colts (Dowsett et al., 1996) testicular function as well as sexual behavior and libido were individually suppressed for 12–26 weeks after two to three immunizations. In our study using the commercially available horse specific vaccine (Equity™) given three times, testosterone concentrations were suppressed (<0.1 ng/ml) for a minimum of 24 weeks in 4 of 5 adult stallions. In 2 animals (B and D) testosterone remained low for 45 and 46

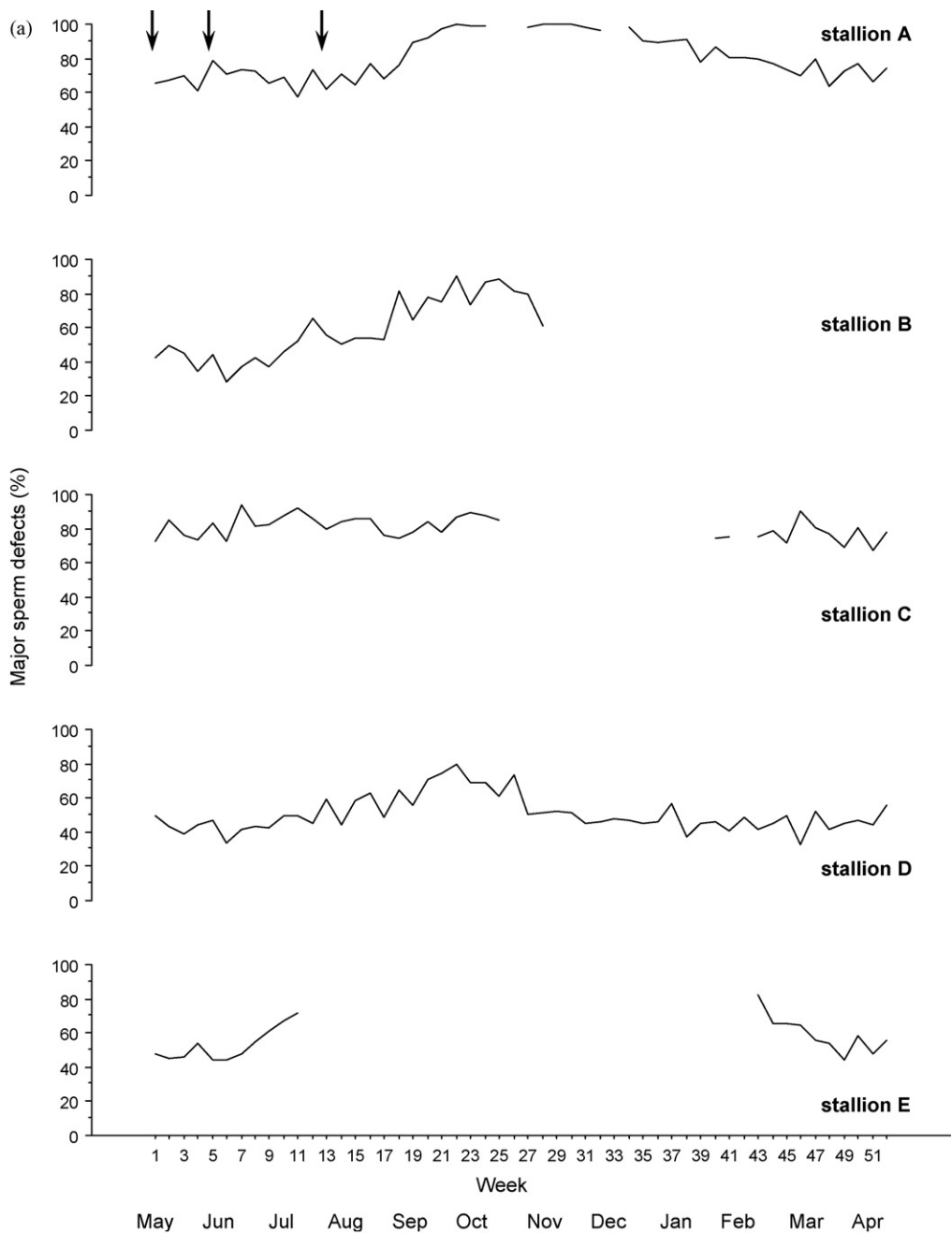


Fig. 5. (a) Major sperm defects (%) in weekly collected ejaculates of 5 stallions (A–E) after vaccination with Equity™ (↓). (b) Major sperm defects (%) in weekly collected ejaculates of the control stallions F, G and H.

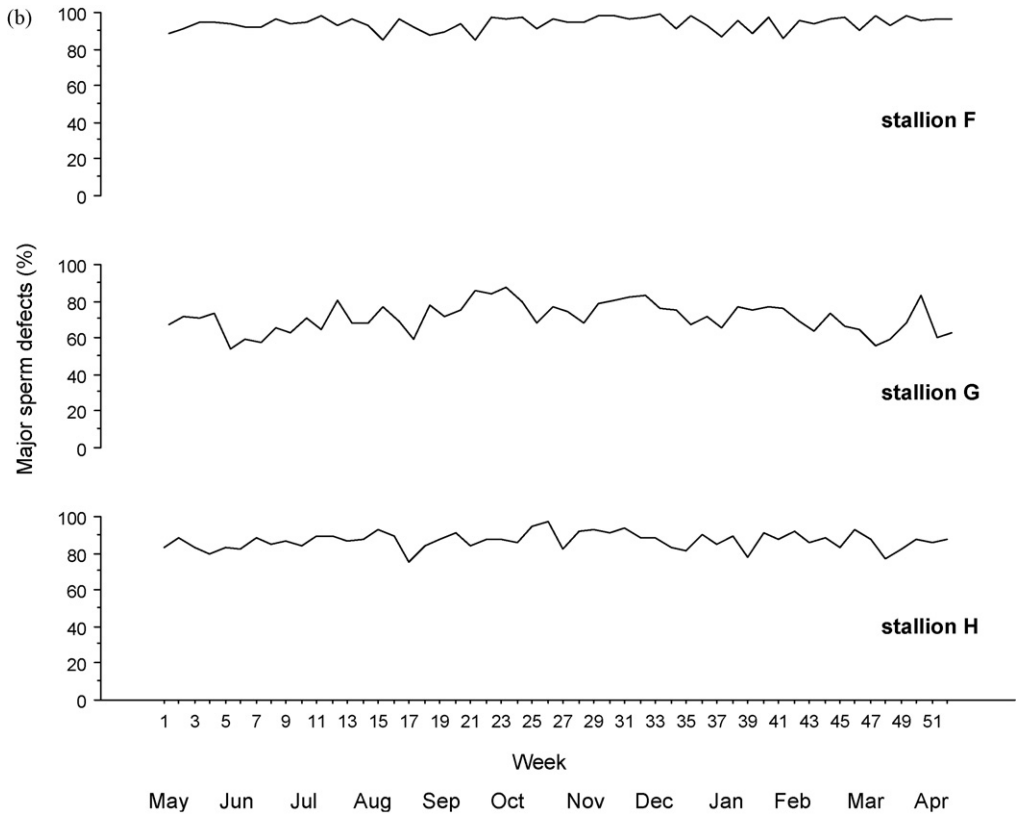


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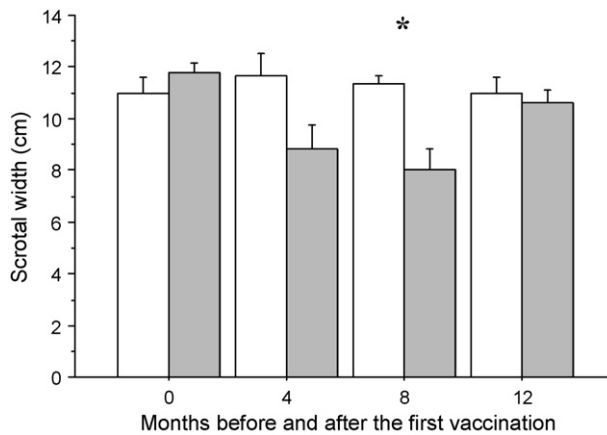


Fig. 6. Scrotal width ($m \pm \text{S.E.M.}$) in 5 vaccinated (■) and 3 control (□) stallions before, 4, 8 and 12 months after the first vaccination with EquiVTM. *Significant difference between groups ($P < 0.05$, t -test).

weeks, respectively. One stallion (C) showed a very poor rise in antibody titer without any visible effect on testosterone secretion. This clearly shows that the application of Equity™ in adult stallions may elicit variable immune responses with different antibody titers and different duration of testosterone suppression.

A negative influence of GnRH immunization on semen quality was observed in 4 of 5 stallions and the drop of total sperm number and sperm motility as well as the increase in major sperm defects varied considerably between single animals. These results agree with others studies (Dowsett et al., 1996; Malmgren et al., 2001; Clement et al., 2005; Turkstra et al., 2005) showing that good responders (high antibody titers) reacted with a reduced semen quality. In our experiment the most dramatic decrease in sperm motility from 79 to 3% within 6 months after the first vaccination occurred in stallion B and this animal also had a marked increase in major sperm defects. Lower sperm production, lower sperm motility and an increase in sperm abnormalities are in accordance with low testosterone concentrations caused by high circulating antibody titers. These results confirm the essential role of testosterone for spermatogenesis and epididymal function but leaves the question open why the changes in semen quality were highly variable between individual stallions despite constant low testosterone concentrations for more than 6 months. A possible explanation might be the different effect of GnRH immunization on pituitary LH and FSH secretion (Garza et al., 1986; Rabb et al., 1990). Because FSH is less affected by GnRH, circulating FSH together with low testosterone concentrations may be sufficient to maintain spermatogenesis.

Regarding sexual behavior the application of Equity™ reduced libido in 4 of 5 stallions. Interestingly enough, in stallion C which had the lowest antibody titers and highest testosterone concentrations, libido and mounting behavior were markedly reduced during weeks 26–39. Quite the opposite situation was observed in stallion D which exhibited strong sexual and mounting behavior during the whole experiment in spite of high antibody titers and low testosterone values. From these findings it is obvious that sexual behavior is not only testosterone dependent but may rather be influenced by various other factors as age of the animal and previous sexual experience (Stout, 2005). Looking at other studies in which GnRH vaccination had either no (Clement et al., 2005) or only a limited effect (Malmgren et al., 2001; Turkstra et al., 2005) on libido, the use of Equity™ in our experiment was more successful to control stallion behavior. However, because of differences in vaccine formulations and dosage, number of boosters as well as age, breed and number of vaccinated stallions comparison of results between these studies is difficult.

5. Conclusion

This study demonstrates that 3 immunizations with Equity™ are well tolerated and can reliably suppress testicular function and sexual behavior in adult sexually experienced stallions. The inhibitory effect, however, is highly variable and may last from a minimum of 24 weeks to more than 46 weeks.

Conflict of interest

None of the authors has any financial and personal relationships with other people or organizations that could inappropriately influence the study.

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