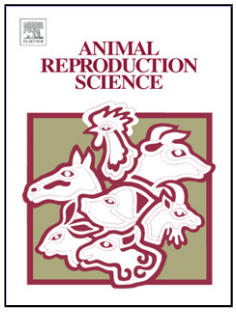

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Effects of a commercial canine gonadotropin releasing hormone vaccine on estrus suppression and estrous behavior in mares

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14

15 **Contents**

16 We investigated the effect of immunization against gonadotropin releasing hormone
17 (GnRH) using a commercial canine GnRH vaccine on estrus suppression and unwanted estrous
18 behavior in mares. In experiment 1, mares were immunized (n=6) twice with vaccine (5mL)
19 given intramuscularly 4 weeks apart or received a control diluent (n=5). Transrectal
20 ultrasonographic examination of the reproductive tracts were performed three days a week for 40
21 weeks after initial vaccination. Blood samples were collected weekly for GnRH antibody titer
22 and progesterone concentration determination. In experiment 2, privately-owned mares (n=12)
23 were immunized twice with vaccine (1mL) given intramuscularly 4 weeks apart. Blood samples
24 were collected prior to each vaccination as well as 12 and 20 weeks after initial treatment, and
25 transrectal ultrasonographic examinations of the reproductive tracts were performed 12 weeks
26 after the first vaccination. Vaccinated mares in experiment 1 responded with a GnRH antibody
27 titer, progesterone concentrations significantly lower than controls, and cessation of ovarian
28 activity. Vaccinated mares in experiment 2 also responded with a GnRH antibody titer,
29 progesterone concentrations that remained basal for the duration of the study, and cessation of
30 ovarian activity. Owners of vaccinated mares in experiment 2 reported that the number of
31 unwanted estrous behaviors present before vaccination significantly decreased following
32 vaccination. In conclusion, GnRH immunization using a canine GnRH vaccine is an effective
33 method for suppressing estrus and unwanted estrous behavior.

34

35 Keywords: antibody; estrous cycle; GnRH immunization; horse; immunocontraception;
36 progesterone

Introduction

Unwanted behavioral changes in mares during estrus affect handling and performance. Reproductive estrous behaviors exhibited by the mare in the presence of a stallion include raising the tail, clitoral eversion, and urinating (Ginther, 1992). However, mares also exhibit non-reproductive estrous behaviors that limit their performance potential, which include hyperexcitability, oversensitivity, abdominal discomfort, and aggression (McDonnell, 1992). Non-surgical estrous behavior suppression is most commonly achieved via daily treatment with an oral progestin, altrenogest (Regu-Mate®, Intervet Inc., Millsboro, DE) (Pryor and Tibary, 2005). However, daily oral administration is costly and can be impractical for horse owners (Burger et al., 2008). In addition, altrenogest is readily absorbed through human skin, which is a potential human safety concern (Hazan, 2011). Also, since altrenogest does not inhibit follicular activity, some mares continue to display unwanted estrous behavior (Pryor and Tibary, 2005).

Gonadotropin releasing hormone (GnRH) controls ovarian activity by regulating the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). Therefore, preventing GnRH from stimulating the release of LH and FSH may be a viable approach for the suppression of estrus. Immunization against GnRH to subsequently cease ovarian activity has been investigated in the mare. Two commercial GnRH vaccines, Improvac® (Pfizer Animal Health Australia) and Equity® (Pfizer Animal Health Australia) have demonstrated suppression of reproductive cyclicity (Botha et al., 2008; Elhay et al., 2007; Imboden et al., 2006). Improvac®, labeled for the prevention of boar taint in swine, was shown to suppress ovarian activity in the mare for at least 23 weeks (Botha et al., 2008; Imboden et al., 2006). However, one study reported a high percentage of adverse vaccine reactions (Imboden et al., 2006), while

the other study reported a small number of transient side effects (Botha et al., 2008). Equity®, labeled for the control of estrus in mares, showed no adverse reactions, a significant decrease in estrous behavior, and ovarian activity suppression for at least three months (Elhay et al., 2007). However, neither of these products are commercially available in the United States, so there remains a need for a safe and cost-effective method for long-term estrus suppression in mares.

In 2004, a commercial GnRH vaccine was launched in the United States (Canine Gonadotropin Releasing Factor Immunotherapeutic®; Pfizer Animal Health USA). This vaccine was labeled for the treatment of benign prostatic hyperplasia in intact male dogs (Pfizer Animal Health, 2004). The vaccine decreases serum testosterone concentrations and testicular volume in intact male dogs (Donovan et al., 2012) and has been used for pregnancy termination in bitches as well (Chew and Purswell, 2010). Recently, this vaccine has also been shown to be effective in male llamas and alpacas, decreasing serum testosterone concentrations, testicular volume and intermale aggressive behavior (Donovan et al., 2013).

The objectives of the current study were to determine (a) the effect of this canine GnRH vaccine on equine ovarian cyclicity and (b) the effect of the canine GnRH vaccine on equine non-reproductive estrous behavior. It was hypothesized that the canine GnRH vaccine would safely and effectively suppress estrus (both ovarian cyclicity and subsequent estrous behavior) for a prolonged duration.

Materials and Methods

Animals

Eleven mares (mean age 13.36 years) were obtained for experiment 1 and twelve privately-owned mares (mean age 9.91 years) that had histories of displaying unwanted behaviors during the estrous period were recruited for experiment 2. All mares had histories of normal reproductive cyclicity.

Experimental Design

In experiment 1, which began in May (spring in the Northern hemisphere), reproductive tracts were monitored by transrectal palpation and ultrasonography three days a week for four weeks. After initial monitoring, mares were either given 5mL of the canine GnRH vaccine (5 times the labeled canine dose; n=6) or a placebo (n=5) twice four weeks apart. Reproductive tracts continued to be monitored three days a week for 40 weeks after initial vaccination, until spring of the following season, and venous blood samples were collected at each session.

Experiment 2 was a clinical study and began in January-April (winter-early spring in the Northern hemisphere). All mares received 1mL of the canine GnRH vaccine (a similar antigenic mass as the Equity® vaccine; n=12) twice four weeks apart. Venous blood samples were collected prior to vaccination (week 0 and 4) and at weeks 12 and 20. Transrectal ultrasonographic examinations of the reproductive tracts were also performed at week 12.

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Oregon State University (ACUP #3699). For the clinical study (experiment 2), the vaccine was used in an extra-label manner by the attending veterinarian and each owner signed a consent form to participate in the study.

Vaccine

The canine GnRH vaccine (Canine Gonadotropin Releasing Factor Immunotherapeutic®, Pfizer Animal Health, Exton, PA) consists of the GnRH peptide conjugated to diptheria toxoid as the protein carrier and combined with a proprietary adjuvant of plant-based origin (Hashimi et al., 2008; Russo, 2008). Each 1 mL dose contains 200μg peptide conjugate (Hashimi et al., 2008). The placebo was a sterile diluent provided by the vaccine manufacturer for use in this experiment. Vaccines were administered into the semimembranosus muscle with not more than 2.5 mL of vaccine or diluent injected at one site (experiment 1) or the middle of the neck (experiment 2).

Vaccine Reactions

In experiment 1, mares were monitored twice daily for two days then once daily for one week for adverse reactions after each injection. The injection site was observed visually and digitally for warmth and swelling, and gait was monitored for lameness. In addition, the general appearance, behavior, and appetite of each mare was observed. If any (even subtle) adverse reactions were present, they were noted by investigators. If no adverse reactions were present at the time of each examination, this was also noted by investigators. In experiment 2, mares were monitored for adverse reactions by their owners using the same criteria as experiment 1.

Hormone Analysis

Blood samples from both experiments were collected into Vacutainer® clot tubes (02-685-A, Fisher Scientific Co.) and centrifuged upon clotting. Sera were separated, aliquoted, and frozen at -20°C until analysis.

In experiment 1, GnRH antibody titers were measured monthly from the time of initial vaccination in June until all mares were seronegative. In experiment 2, titers were measured at the four time points blood samples were collected. Titers were determined by ELISA using a technique modified from Elhay et al. (2007). Pooled serum from unvaccinated horses served as the negative control and pooled serum from vaccinated mares with a known high antibody titer served as the positive control. Briefly, 96-well microtiter plates were coated with 100 µL of 5 µg/mL of LH-RH (71447-49-9, Sigma, St. Louis, MO, USA) in sodium bicarbonate buffer (pH 8.0) at 4ºC overnight. After incubation, plates were washed with phosphate-buffered saline containing 0.05% Tween-20 (TPBS) (pH 8.0). Plates were then incubated for 1 hour at 20ºC with serum samples diluted in a buffer containing 0.5% bovine serum albumin (9048-46-8, Sigma, St. Louis, MO, USA) to yield final serum dilutions ranging from 1:8 to 1:1024. After tapping dry, antibodies were detected using horseradish peroxidase protein G conjugate (HRP) (10-1223, Invitrogen, Camarillo) diluted at 1:2000 in serum dilution buffer for 1 hour at 37ºC. After a final wash with TPBS, HRP was visualized with 100 µL of ABTS peroxidase substrate (50-66-01, KPL, Gaithersburg, MD, USA). Absorbances were read at 405 nm using a spectrophotometer (FLUOstar Omega, BMG Labtech Inc., San Francisco, CA, USA) and each serum sample was measured in duplicate. The cutoff for seropositivity, defined in this study as the upper limit of a 99% confidence interval above the mean negative control level, was calculated using the methods of Frey et al. (1998). Serological results were expressed as the reciprocal of the highest

144 twofold serial dilution above the calculated cutoff and linearized using a base-2 logarithmic
145 scale.

146 In experiment 1, progesterone concentrations were measured weekly from the time of
147 initial vaccination in June until the end of the breeding season in October for a total of 17 weeks.
148 In experiment 2, progesterone concentrations were measured at the four time points blood
149 samples were collected. Serum samples were analyzed for progesterone using a commercially
150 available kit (Immulite® Progesterone, Siemens) designed for an enzyme-amplified
151 chemiluminescence assay system (Immulite® 1000, Diagnostic Products Corporation) and
152 performed according to the manufacturer's protocol. The interassay coefficient of variation
153 ranged from 5.8% at 7.2 ng/mL to 16% at 0.81 ng/mL; the intraassay coefficient of variation
154 ranged from 6.3% at 7.9 ng/mL to 16% at 0.81 ng/mL, respectively. The detection limit was 0.2
155 ng/mL.

156

157 *Ovarian Activity*

158 Ovarian activity was monitored in experiment 1 mares three days a week for 44 weeks by
159 ultrasonography and transrectal palpation. In experiment 2, ovarian activity was observed by
160 ultrasonography at week 12, during the spring. At each observation, follicle diameter on both
161 ovaries were measured and the presence of a corpus luteum was noted. In experiment 1, each
162 mare was given a weekly score to reflect cyclicity:

163 Score 0: Anestrus-like. Follicles remain < 20 mm in diameter with no corpus luteum
164 present.

Score 1: Diestrus or estrus-like. Follicles reach > 20mm in diameter, corpus luteum or dominant follicle may be present.

Behavioral Analysis

Mares in experiment 2 were evaluated for the presence of estrous behavior. Owners scored their mare's estrous behavior (pre-vaccination score) reflecting the specific number of behaviors each mare exhibited during estrus. At the end of the study, owners again scored their mare's estrous behavior (post-vaccination score) reflecting the specific number of behaviors that were still present through the duration of the study.

Statistical Analysis

In experiment 1, the presence of a GnRH antibody titer was compared between the vaccination and control group using Fisher's exact test (GraphPad QuickCalcs Software, La Jolla, CA, USA). Ovarian activity scores were analyzed using the non-parametric Wilcoxon rank sum test in SAS (Version 9.2, SAS Institute Inc., Cary, NC, USA). Progesterone concentrations were analyzed as a repeated measure in time design using PROC MIXED in SAS. Fixed effects in the model were whether the animal was vaccinated, time after first vaccination, and the interactions between vaccination and time. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals.

In experiment 2, estrous behavior was analyzed using the non-parametric sign test in SAS. The presence of a GnRH antibody titer and progesterone concentrations were analyzed as a

repeated measure in time design using PROC MIXED in SAS. The fixed effect in the model was time after first vaccination. A first order heterogeneous autoregressive variance-covariance structure was fitted for repeated measurements within animals.

Results

Experiment 1

There were no local or systemic vaccine reactions experienced in either the vaccinated or control mares following the first or second injection. All eleven mares demonstrated normal ovarian cyclicity during the four weeks of monitoring pre-vaccination, and control mares continued to cycle normally for the duration of the breeding season (17 weeks). Circulating progesterone fluctuated throughout the season in control mares as expected. Within days after receiving the booster injection, largest mean follicle size began to decrease in the treated mares (Figure 1). By three weeks post-booster, the largest mean follicle size was consistently < 20mm. In addition, no corpora lutea were present based upon ultrasonographic evaluation and serum progesterone concentrations (Figure 2) for all six vaccinated mares throughout the breeding season. Ovarian activity scores for vaccinated mares were significantly less than controls ($p < 0.0001$).

Of the vaccinated mares, 50% (3/6) resumed normal cyclicity in the following spring, 40 weeks after the initial vaccination. The remaining mares displayed continued cessation of ovarian cyclicity. Control mares resumed cyclicity in the following spring.

All mares were seronegative for antibodies against GnRH prior to the first vaccination and all control mares remained seronegative for the duration of the study. Compared to control mares, antibody titers of vaccinated mares were significantly greater post-vaccination (Figure 3). One mare had an antibody titer until week 20, three mares had an antibody titer until week 32, one mare had an antibody titer until week 36, and one mare maintained an antibody titer for the duration of the study (40 weeks). The three mares that did not resume normal cyclicity the following spring had antibody titers that lasted 32, 36, and 40 weeks, respectively.

Experiment 2

There were no local or systemic vaccine reactions experienced following the first or second injection. Of the 12 mares vaccinated, all but one was examined by transrectal ultrasonography 12 weeks after the initial vaccination, during the spring. All of these mares had follicles < 20mm and no corpora lutea were present based upon ultrasonographic evaluation.

As expected, progesterone concentrations were basal (<1.0 ng/mL) in all mares when they were vaccinated initially during the winter-early spring. However, progesterone concentrations remained basal through the late spring and early summer (weeks 12 and 20). All mares were also seronegative for antibodies against GnRH prior to the first vaccination and all developed a GnRH antibody titer post-vaccination that peaked at week 12 (Figure 4).

Owner pre-vaccination and post-vaccination estrous behavior scoring was available from nine mares (Table 1). Common behaviors reported included poor performance under saddle, distractability, irritability, aggression towards other horses or handler, and frequent attempts to evade work. Estrous behaviors diminished completely in all mares, a significant decrease

227 compared to pre-vaccination (p=0.004) (Table 1). Furthermore, all owners reported that they
228 would be interested in yearly revaccination.

229

230 **Discussion**

231 In the United States, there is a need for a safe, effective, and long-lasting method to
232 suppress estrous ovarian cyclicity and subsequent unwanted estrous behavior in mares. This
233 study demonstrated that immunizing mares against GnRH using a vaccine labeled for dogs safely
234 elicited GnRH antibody formation that suppressed estrus. The clinical study component also
235 demonstrated owner satisfaction with the product.

236 Forty weeks after initial vaccination, 50% of the vaccinated mares in experiment 1
237 returned to normal cyclicity whereas the remaining 50% of mares had sustained estrus
238 suppression. Differences in individual responses with regard to estrus suppression in mares was
239 also observed when using Equity® (Elhay et al., 2007) and Improvac® (Imboden et al., 2006) as
240 well as a non-commercial GnRH vaccine (Dalin et al., 2002). Variation in response to GnRH
241 immunization has also been reported in other female species, including the queen (Levy et al.,
242 2011), heifer (Prendiville et al., 1995), and deer (Miller et al., 2000). It has been speculated that
243 genetic differences among individual animals is responsible for variations in immune response
244 (Miller et al., 2000); however, what these differences are has not yet been elucidated.
245 Regardless, differing individual responses should be expected when immunizing against GnRH.

246 The reversibility and the effect of GnRH immunization on fertility is unknown. Equity®
247 does not recommend vaccinating mares later intended for breeding (Pfizer Animal Health
248 Australia, 2008) considering response to vaccine is variable. One study investigating the

reversibility of Improvac® in mares found that 47/51 mares returned to normal cyclicity by 103 weeks after initial vaccination with a mean of 60 weeks; of the 4 mares that were still not cycling, all were ≤ 4 years of age (Schulman et al., 2013). Another study observed the effect of Equity® on fertility and achieved high rates of pregnancy for the two seasons following the season the mares were vaccinated (Card et al., 2007). While the current study was unable to continue monitoring the vaccinated mares that did not resume cyclicity, the three vaccinated mares that did resume cycling the following spring were artificially inseminated to determine whether their fertility had been compromised. Pregnancy was achieved in all three mares on the first cycle, which was in agreement with the findings regarding fertility following use of Equity® (Card et al., 2007).

With regards to behavior, mares display estrous behaviors even when they are not in the vicinity of a stallion, and these behaviors adversely affect performance (Jorgensen et al., 1996; Spiker, 2009). While these non-reproductive estrous behaviors are variable between mares, individuals have a relatively consistent style of estrus from one cycle to the other (Pryor and Tibary, 2005), allowing for owners to make definitive differentiations between non-reproductive estrous behaviors and other unrelated behaviors. The most frequently reported unwanted estrous behaviors in this study are in concordance with other reported behaviors as a result of estrus in performance mares (Jorgensen et al., 1996). By demonstrating diminished non-reproductive estrous behaviors in multiple mares as recognized by the owners, we were able to establish high owner satisfaction with the product and a desire to continue yearly vaccination regimens.

In conclusion, immunization against GnRH using Canine Gonadotropin Releasing Factor Immunotherapeutic® is a safe, effective, and long-lasting method for suppressing estrus in mares. Variability in duration of suppressed estrus is to be expected.

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Conflict of interest statement

The authors have declared no conflicts of interest.

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343 State University, Manhattan, KS.
344

344 **Tables**

Mare	Number of Estrous Behaviors Present Pre-Vaccination	Number of Estrous Behaviors Present Post-Vaccination
1	3	0
2	2	0
3	1	0
4	3	0
5	2	0
6	4	0
7	2	0
8	2	0
9	3	0

345 Table 1. Number of estrous behaviors present pre- and post-vaccination. There was a significant
346 overall decrease in behavior score (p=0.004).

Figure Legends

Figure 1. Mean largest follicle diameter (±SD) in control (□) and vaccinated (●) mares, measured three days a week until 3 out of 6 vaccinated mares and all control mares resumed cyclicity (44 weeks total). Dotted lines indicate times of vaccination.

Figure 2. Progesterone concentrations (Mean±SD) in control (□) and vaccinated (●) mares, measured every week from time of initial vaccination (week 0) to the end of the breeding season (week 17). There was an overall significant difference in progesterone concentration between vaccinated and control mares ($p<0.0001$).

Figure 3. GnRH antibody titer (Mean±SD) in control (□) and vaccinated (●) mares prior to each injection (0 and 4 weeks) and at weeks 8-36 following initial treatment. $^{*}p<0.05$ compared to controls.

Figure 4. GnRH antibody titer (Mean±SEM) in experiment 2 mares, measured prior to each injection (0 and 4 weeks) and at weeks 12 and 20. $^{*}p<0.05$ compared to week 0.

Figure 1

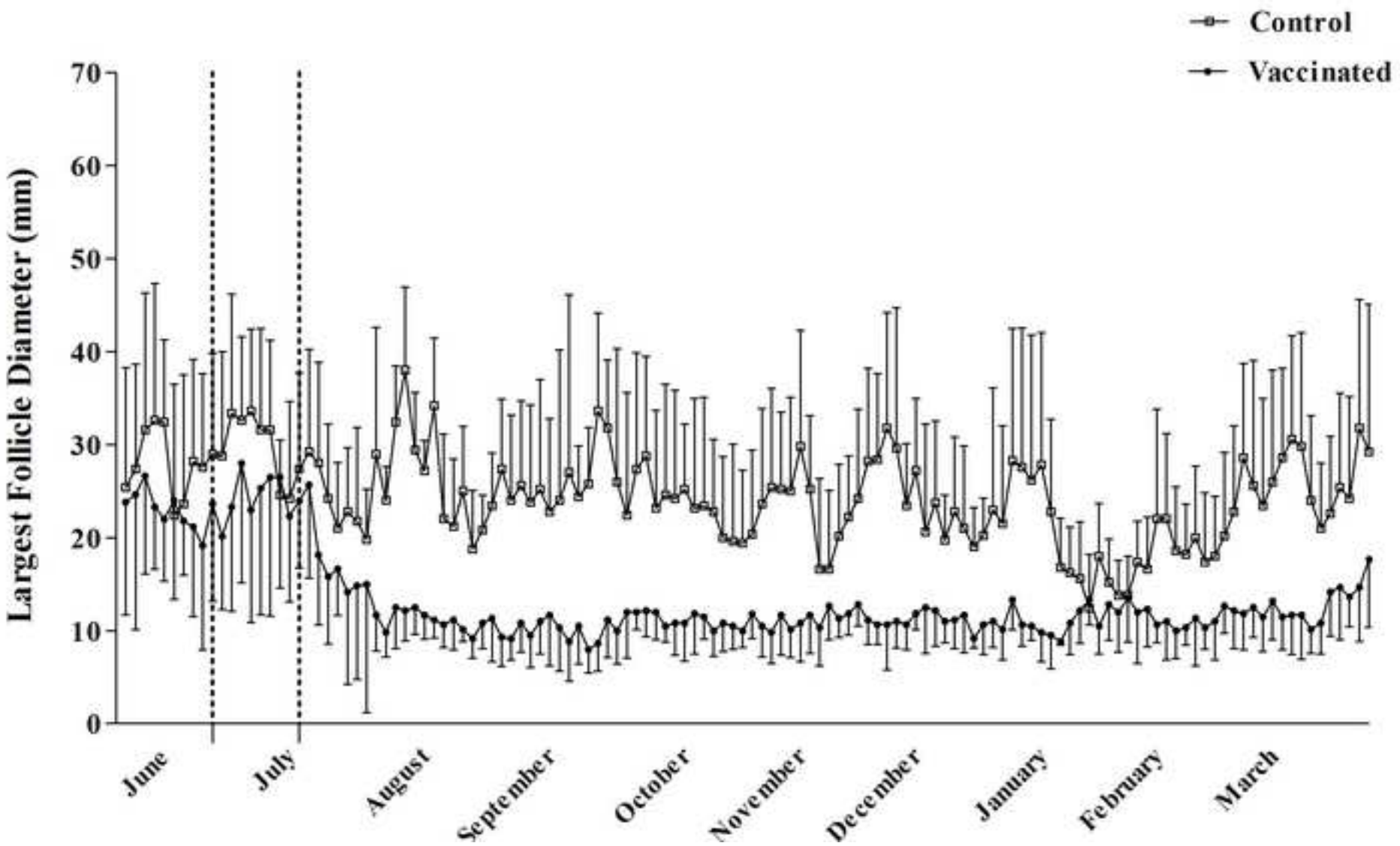

Control
Vaccinated
Largest Follicle Diameter (mm)
70
60
50
40
30
20
10
0
June
July
August
September
October
November
December
January
February
March

Figure 2

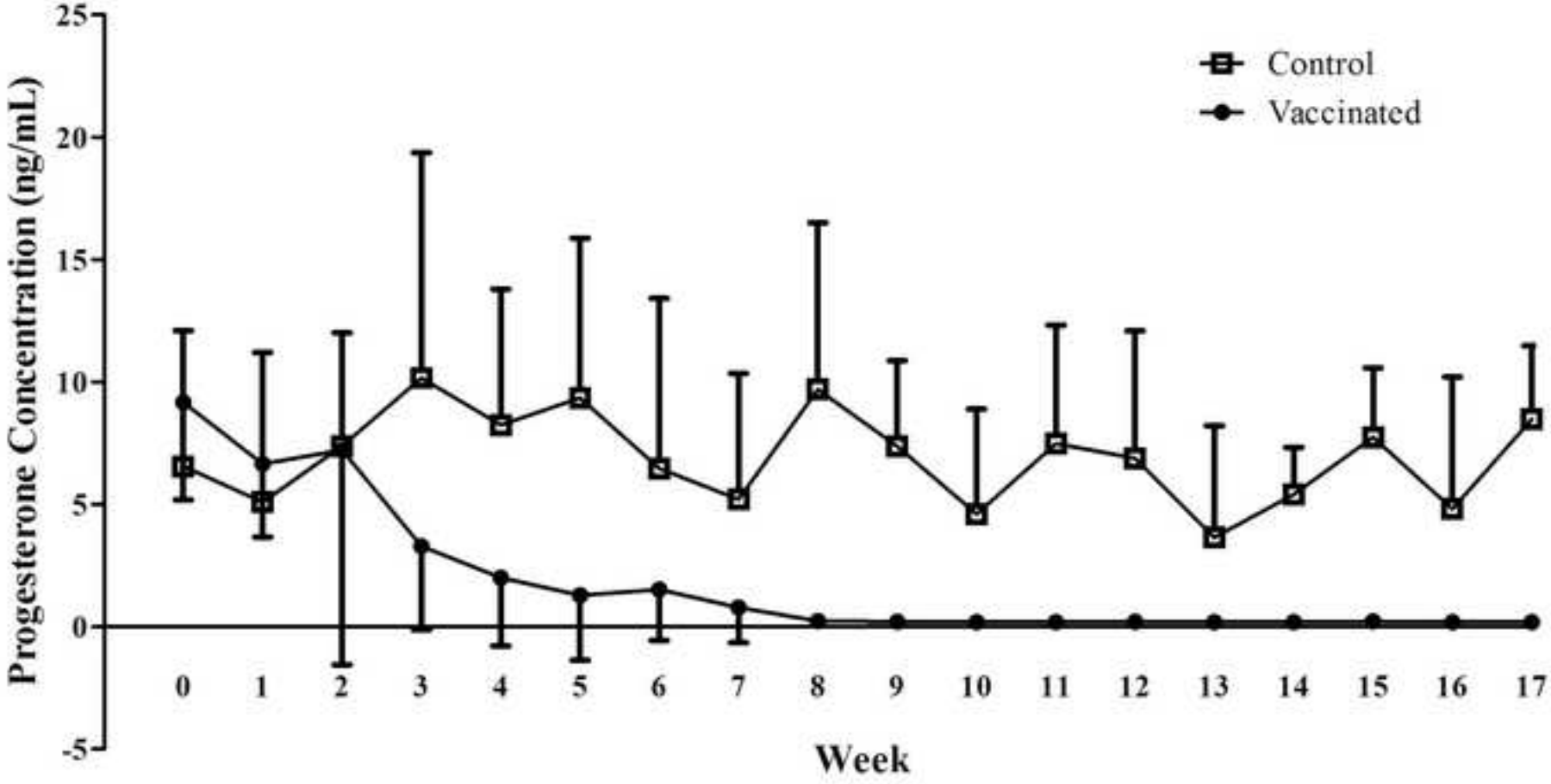

Control
Vaccinated
Progesterone Concentration (ng/mL)
25
20
15
10
5
0
-5
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
Week

Figure 3

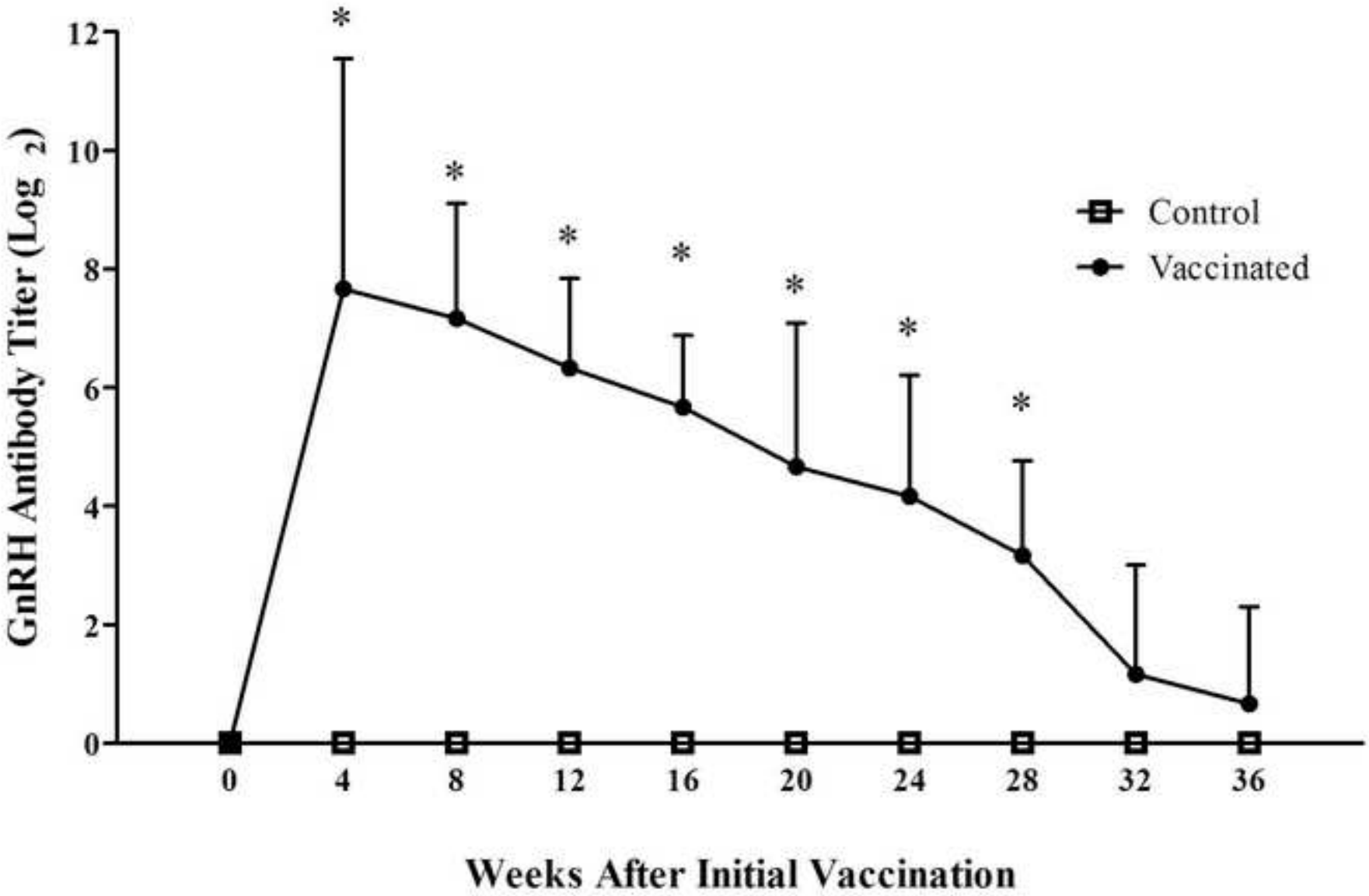
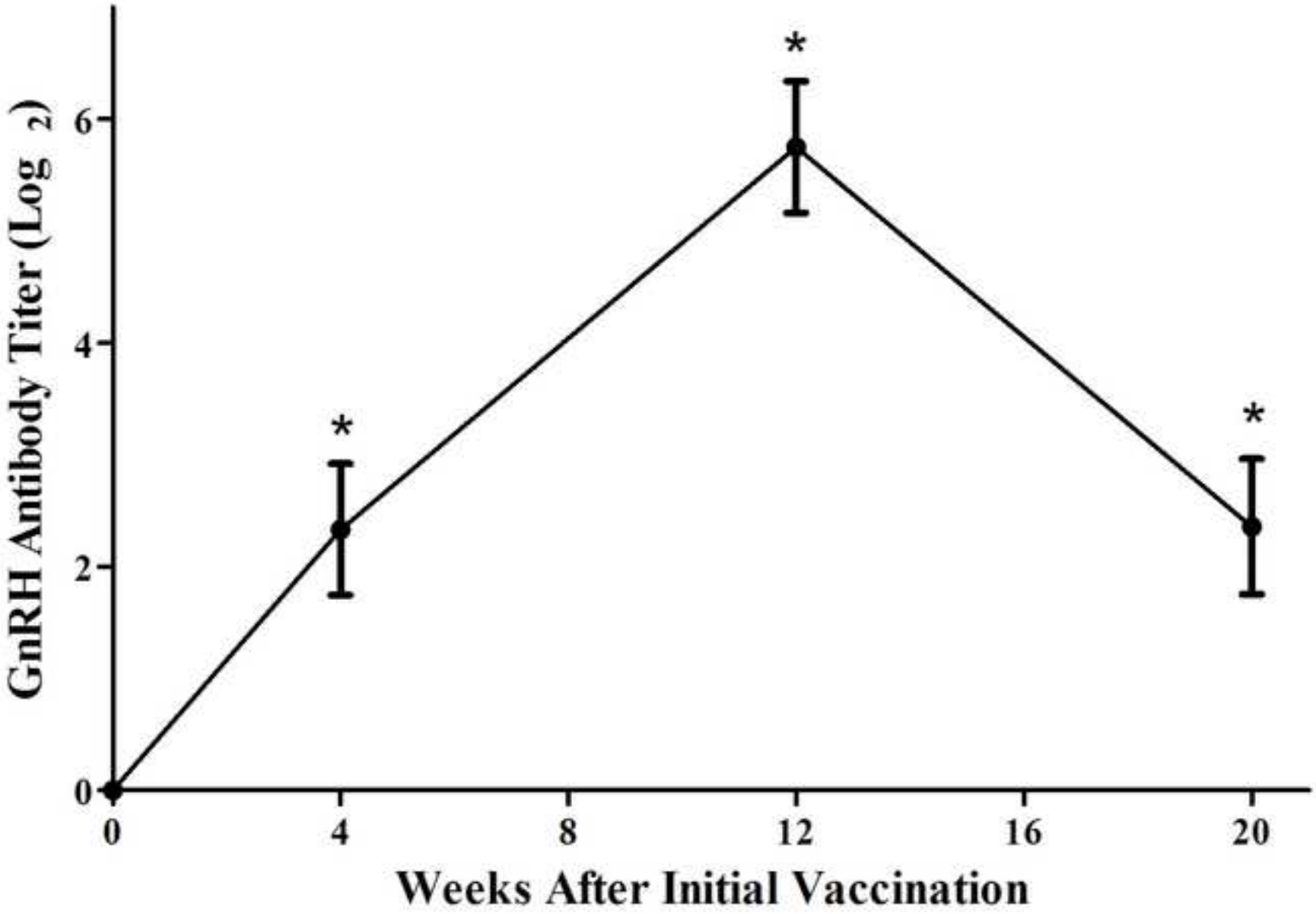

GnRH Antibody Titer (Log 2)
12
10
8
6
4
2
0
*
Control
Vaccinated
0
4
8
12
16
20
24
28
32
36
Weeks After Initial Vaccination

Figure 4


GnRH Antibody Titer (Log 2)
0
2
4
6
*
*
*
0
4
8
12
16
20
Weeks After Initial Vaccination