

Immunocontraception of male and female giraffes using the GnRH vaccine Improvac[®]

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Abstract

The aim of this study was to develop protocols for contraception in both sexes of giraffes (*Giraffa camelopardalis*) by using the GnRH vaccine Improvac[®]. We evaluated the success of immunization by analyzing fecal reproductive hormone metabolites in female ($n = 20$) and male ($n = 9$) giraffes. Endocrine analysis provided the basis for the successful immunization protocol, as well as for assessing long-term effects. Reliable reduction of fecal steroid metabolites to baseline levels in female giraffes was achieved with three, and in males with four or five injections at 4-week intervals. Effective booster injections were administered at 2-month intervals in the first year of treatment and at three to 4-month intervals in the following years. In addition to endocrine analysis, we determined vaccination efficacy in bulls by assessing testicular atrophy. Long-term (>2 years) use in females was often accompanied by prolonged periods of persistent corpus luteum activity, although normal cycles were not observed. Problems might occur with reversibility, because in a few males and females, even after more than 2 years since treatment had been stopped, fecal hormone metabolites have not returned to pretreatment levels. The results are somewhat ambiguous, as reproduction can be suppressed by use of Improvac[®], but the question of reversibility remains unsolved.

KEYWORDS

contraception efficacy and reversibility, endocrine monitoring, reproduction management

1 | INTRODUCTION

The giraffe has traditionally been recognized as one species (*Giraffa camelopardalis*) with commonly accepted nine subspecies (Dagg, 2014). However, recently published genetic analyses indicate four distinct giraffe clusters that should be granted species status (Burgin et al., 2020; Fennessy et al., 2016; Winter et al., 2018). Since 2016, the giraffe has been listed as vulnerable by the International Union for Conservation of Nature (IUCN, 2020). Some giraffe populations, i.e. in Niger, South Sudan/Ethiopia, and Zambia are estimated to be comprised of only 400–650 individuals (Fennessy et al., 2016).

For the population management of wildlife in human care comprehensive breeding management, focusing on reproductive health and the space available in a certain breeding program, is necessary (Penfold et al., 2014). Giraffes are breeding well in captivity. Without adequate breeding management, the population size would increase beyond the carrying capacity of the zoological institutions. Furthermore, genetic hybrids or over-represented captive giraffe genotypes need to be excluded from breeding (Jebam, 2015). Therefore, there is a need for effective, reversible and easily administrable contraceptive methods for giraffes kept in zoological facilities. Culling individual giraffes as a method of breeding management is considered to be ethically unacceptable in most societies. Castration is a possibility to exclude individual giraffe bulls permanently from breeding, but involves the risk of anesthesia and surgery related medical complications (Borkowski et al., 2009).

In general, reversible options of contraception used in zoological institutions include progestagen analogues, GnRH agonists (i.e., deslorelin) and immunocontraceptive vaccines directed against GnRH (gonadotropin-releasing hormone) or PZP (zona pellucida proteins). In female giraffes, progestagen analogues like medroxyprogesterone-acetate and chlormadinone-acetate have been used successfully. However, reports of continued male interest in progestagen treated females raise doubts about their efficacy (Patton et al., 2006). GnRH agonists like deslorelin or leuprolide acetate have been used in a variety of domestic and wild animal species (Herbert & Trigg, 2005; Lucas, 2014; Schoemaker, 2018; Stout & Colenbrander, 2004), including giraffes (Patton et al., 2006). In giraffes, the duration of action of deslorelin implants lasts for up to 2 years, but individual efficacy may vary. The necessary subcutaneous implantation is a drawback for the use of GnRH agonists in wildlife. Immunocontraceptive vaccines against GnRH or immunocontraception based on PZP-specific antigens have gained considerable attention over recent years, because they can be administered remotely using a blowpipe or dart gun (Bechert & Fraker, 2018; Kirkpatrick et al., 2011; Massei & Cowan, 2014; Naz & Saver, 2016). However, PZP based immunocontraception can only be used in females and because of a possible autoimmune reaction against the ovaries, their use is not recommended for zoo animals in breeding programs.

A major advantage of GnRH vaccines, apart from their administration via remote dart, is their applicability to both females and males. GnRH-vaccines block the ability of GnRH to stimulate pituitary

gonadotrophs. Thus follicle stimulating hormone (FSH) and luteinizing hormone (LH), and subsequently gonadal steroids are not stimulated. Immunizations against GnRH were developed in the 1970s, 1980s, and 1990s (D'Occhio, 1993; Thompson, 2000). Through the development of various GnRH-protein conjugates and the use of different adjuvants, in most domestic animal species only two immunizations are required for effective immunocontraception. The non-commercially available GnRH vaccine GonaCon[®] shows good and long-lasting efficacy in overabundant mammalian species after a single injection (Kirkpatrick et al., 2011; Massei & Cowan, 2014; Miller et al., 2004; Naz & Saver, 2016). GnRH vaccines like Improvac[®]/Improvast[®], GonaCon[®], or Equity[®] have been extensively tested for dosage, immunization intervals and potential reversibility in a large number of domestic animal species, such as male pigs (Claus et al., 2008; Einarsson et al., 2009), mares and stallions (Dalín et al., 2002; Donovan et al., 2013; Elhay et al., 2007; Imboden et al., 2006; Janett et al., 2009; Schulman et al., 2013; Stout & Colenbrander, 2004; Turkstra et al., 2005;), male goats (Lents et al., 2018), feral female cattle (Massei et al., 2018), male cats (Levy et al., 2004) or male dogs (Siel et al., 2020). These GnRH vaccines have also been tested in a variety of exotic companion animals (Schoemaker, 2018), or i.e. in female and male elephants (Benavides Valades et al., 2012; Lueders et al., 2017) or female elk (Powers et al., 2011). Results from other wild animal species of both sexes are summarized in the reviews by Kirkpatrick et al., 2011; Massei & Cowan, 2014; Miller et al., 2004; Naz & Saver, 2016; and Thompson, 2000.

We know from personal communication within the EEP (EAZA Ex-situ program), and veterinarians working in zoos that Improvac[®] is currently used for a large number of captive ungulate species. In very few cases, however, the efficacy is examined by endocrine analysis; instead, the absence of births is used as a proxy to determine effectiveness. The aim of this long-term study was to evaluate the contraceptive efficacy of the GnRH vaccine Improvac[®] on ovarian and testicular activity in female and male giraffes. For this purpose, we established an endocrine monitoring by fecal steroid analysis and used it to evaluate efficacy of immunization regimens and vaccination intervals in male and female giraffes. Finally, we assessed long-term effects and reversibility after vaccination termination.

2 | METHODS

2.1 | Study sites and animals

Starting in the year 2013, selected giraffes were treated with the GnRH vaccine Improvac[®] for contraceptive purposes. A total of 29 giraffes (female $n = 20$, male $n = 9$), aged between 2 and 17 years from ten European zoological institutions were included in this study (Tables 1 and 2). The giraffes were housed in mixed groups of both sexes and different ages. All treated animals were in reproductive age and in good body condition.

TABLE 1 Female study animals with description of individual results

Location	Animal ID (Studbook ID) Subspecies	Proven breeder	Age at first immunization (years)	Treatment interval (weeks)	Outcome
Berlin (DE)	Fem#01 (*-3817) hybrid	No	7	0-4-6-11-4-4-11-6-6-2-3-21-12-12-11-12-13-8-9-9-9-9	These four giraffes were kept together as a herd with an adult bull. Improvac [®] vaccinations commenced in 2014. As their efficacy was still uncertain after four injections, another basic immunization was conducted in early 2015, and subsequent immunizations followed at approximately 11-week intervals. In Fem#04 progesterone values were continuously below the threshold of 500 ng/g, while in the other three females progesterone peaks occurred and the bull showed sexual interest in them. Since 2016, the bull's interest in the females has ceased.
Berlin (DE)	Fem#02 (*-3818) hybrid	No	7	0-4-6-11-4-4-11-12-13-13-12-12-11-12-13-8-9-9-9-9	
Berlin (DE)	Fem#03 (*-3278) hybrid	No	12	0-4-6-11-4-4-9-15-13-13-12-12-11-12-13-8-9-9-9-9	
Berlin (DE)	Fem#04 (*-3291) hybrid	No	11	0-4-6-11-4-4-11-12-13-13-12-12-11-12-13-8-9-9-9-9	
Frankfurt (DE)	Fem#05 (4-2853) <i>G.c. reticulata</i>	Yes	15	0-5-18-18	Endocrine results depicted in Figure 1a.
Frankfurt (DE)	Fem#06 (4-2527) <i>G.c. reticulata</i>	No	21	0-4-13	After the 3rd immunization 20-oxo-pregnane values were below the threshold of 500 ng/g feces.
Gelsenkirchen (DE)	Fem#07 (5-2798) <i>G.c. rothschildi</i>	Yes	17	0-4-4-21-16-18-26-4-9-9-18-8-9-8-9-10-9-16-53-[56] [5 ml]	Conceived 5 days after completion of basic immunization; continuation of Improvac [®] at 4-5-month intervals during gestation; healthy calf born. After parturition vaccinations were continued at 2-month intervals for about 2 years; then at 1-year intervals. Displayed estrus behavior and was mated, before and some months after the last Improvac [®] treatment, although 20-oxo-pregnane values were below threshold of 500 ng/g feces.
Gelsenkirchen (DE)	Fem#08 (5-3778) <i>G.c. rothschildi</i>	Yes	6	0-4-5	After basic immunization, Improvac [®] treatment was stopped. The giraffe cow conceived 8 months later and gave birth to a healthy calf.
Gelsenkirchen (DE)	Fem#09 (5-3511) <i>G.c. rothschildi</i>	Yes	7	0-4-5-17-18-16-18-20-9-9-18-8-9-8-9-10-9-16-53-[56] [5 ml]	20-oxo-pregnane values below threshold of 500 ng/g feces. No signs of estrus even 2 years after last vaccination.
Karlsruhe (DE)	Fem#10 (4-3457) <i>G.c. reticulata</i>	Yes	10	0-4-11-9-12-9	Treatment during the postpartum period. The 20-oxo-pregnane pattern was similar to the results depicted in Figure 3, except for a lack of progesterone peaks. Three years after termination of Improvac [®] administration, 20-oxo-pregnane levels were above the 500 ng/g feces threshold, yet no regular estrous cycle activity was observed.
Karlsruhe (DE)	Fem#11 (4-3958) <i>G.c. reticulata</i>	Yes	7	0-4-11-9-13-10	Endocrine results depicted in Figure 3. Three years after termination of Improvac [®] administration, 20-oxo-pregnane levels were above the 500 ng/g feces threshold, yet no regular estrous cycle activity was observed.

TABLE 1 (Continued)

Location	Animal ID (Studbook ID) Subspecies	Proven breeder	Age at first immunization (years)	Treatment interval (weeks)	Outcome
Kerkrade (NL)	Fem#12 (5-3217) <i>G.c. rothschildi</i>	Yes	12	0-4	Comparable results as depicted in Figure 2b.
Kerkrade (NL)	Fem#13 (5-3399) <i>G.c. rothschildi</i>	Yes	10	0-5	Endocrine results depicted in Figure 2b.
Lyon (FR)	Fem#14 (*-3691) <i>G.c. antiquorum</i>	Yes	12	0-4-10-10-8-10-10-13-10-10-9-10-10	Endocrine results depicted in Figure 2a.
Lyon (FR)	Fem#15 (*-3632) <i>G.c. antiquorum</i>	Yes	12	0-4-4-10-10-8-10-10-13-10-10-9-10-10	Endocrine results depicted in Figure 2a.
Stuttgart (DE)	Fem#16 (4-4584) <i>G.c. reticulata</i>	No	2	[0-4-18-17-22-18-21-24-29] [4 ml]	Endocrine results depicted in Figure 7a. Two years after termination of Improvac® administration, 20-oxo-pregnane levels above the 500 ng/g feces threshold were measured, yet no regular estrous cycle activity was observed.
Stuttgart (DE)	Fem#17 (4-3436) <i>G.c. reticulata</i>	Yes	11	[0-4-18-17-22-18-21-24-29] [4 ml]	After the 3rd Improvac® administration, 20-oxo-pregnane values declined below the 500 ng/g fecal threshold and remained at this low level during the treatment except for some peaks. One year after the last immunization, 20-oxo-pregnane values were well below the 500 ng/g threshold.
Stuttgart (DE)	Fem#18 (4-3403) <i>G.c. reticulata</i>	Yes	11	0-5-4	Treatment during the post-partum period. The 20-oxo-pregnane pattern was similar to the results depicted in Figure 3 (including two progesterin peaks). The animal died 2 months after the 3rd Improvac® injection from a cause not related to the immunizations.
Vienna (AT)	Fem#19 (0-2937) <i>unknown subspecies</i>	No	14	0-4-4-18-17-16-20-17-17-17-24-16-15-18-17-17-17-17-17-17-17-17-17	Continuous long-term endocrine monitoring over more than 6 years. In both females, periods with 20-oxo-pregnane values above the 500 ng/g feces threshold were observed. However, extended periods of luteal activity did not indicate regular estrous cycles.
Vienna (AT)	Fem#20 (0-3506) <i>unknown subspecies</i>	No	8	0-4-4-18-17-16-20-17-17-17-24-16-15-18-17-17-17-17-17-17-17-17-17	

Note: Generally, the administered dose of the anti-GnRH vaccine Improvac® was 3 ml per treatment, with a few exceptions. Deviating dosages are marked by square brackets and italics in the chronological sequence, i.e. [5 ml].

TABLE 2 Male study animals with description of individual results

Location	Animal (Studbook ID) Subspecies	Proven breeder	Age at first immunization (years)	Treatment interval (weeks)	Outcome
Cologne (DE)	Male#01 (4-3143) <i>G.c. reticulata</i>	Yes	15	0-5-4-4-10-9-8-10-[28-4-4-8-8-8-8-8-8-7-8-8-8-8-8-8-8-8] 8-8] [5 ml]	This bull sired a calf after the third Improvac [®] injection. Once the calf was born, the immunization dose was increased to 5 ml and vaccinations were continued for about 2 years. One year after the end of the immunizations, androgen levels were below 20 ng/g feces and testicles were atrophic.
Frankfurt (DE)	Male#02 (4-2927) <i>G.c. reticulata</i>	Yes	14	0-5-18-18	Endocrine results depicted in Figure 1b.
Gelsenkirchen (DE)	Male#03 (5-4357) <i>G.c. rothschildi</i>	No	3	0-4-5-15-9-9-8-8-17-9-10-9-8-8-9-5-[95-7-5-11] [5 ml]	Testicular atrophy since the fourth month of the treatment (testicles depicted in Figure 6d). After a treatment pause of about 20 months, the bull mated with a giraffe cow in estrus, despite clearly atrophied testicles. The bull was then immunized four times with 5 ml of Improvac [®] per treatment. During a period of 15 months after the end of the immunizations, testis size did not visibly increase.
Gelsenkirchen (DE)	Male#04 (5-3223) <i>G.c. rothschildi</i>	Yes	12	0-4-5-9-9-9-8-9-9-10-9-8-8-9-5-[95-7-5-11] [5 ml]	Endocrine results depicted in Figure 5a. This bull mated with giraffe cows in estrus, despite clearly atrophied testicles. During a period of 15 months after the end of the immunizations, testis size did not visibly increase.
Lyon (FR)	Male#05 (2-3765) <i>G.c. antiquorum</i>	Yes	9	0-5-13-10-8-9-12-9-23-4-4-5-9-12-9-10-10-13-10-10-10-10	Endocrine results depicted in Figure 5b; testicles in Figure 6c.
Madrid (ES)	Male#06 (5-4634) <i>G.c. rothschildi</i>	No	2	[0-4-9-8-9-9-9-9-8-13-13-13-17-17-17-18] [2 ml]	Hormone levels at the beginning of the Improvac [®] treatment were between 400 and 450 ng/g feces. Levels declined below 100 ng/g after the third Improvac [®] injection.
Odense (DK)	Male#07 (4-2249) <i>G.c. reticulata</i>	Yes	7	0-4-4-8-8-10-4-4-7-9-4-5-7-8-14-13	This bull was treated with Improvac [®] for 2 years. 17-oxo-androstane values declined to basal values only after the 5th

TABLE 2 (Continued)

Location	Animal (Studbook ID) Subspecies	Proven breeder	Age at first immunization (years)	Treatment interval (weeks)	Outcome
Stuttgart (DE)	Male#08 (4-4699) G.c. <i>reticulata</i>	No	3	0-4-4	Improvac [®] injection. Currently, even 2 years after completion of Improvac [®] treatment, testicles are completely atrophic.
Stuttgart (DE)	Male#09(4-3048) G.c. <i>reticulata</i>	Yes	15	0-2-5-9-9-9-9-9	After the third Improvac [®] injection, androgen levels were below 100 ng/g. Endocrine results depicted in Figure 7b; testicles in Figure 6b.

Note: Like in the female study animals, the administered dose of the anti-GnRH vaccine Improvac[®] was 3 ml per treatment, with a few exceptions. Deviating dosages are marked by square brackets and italics in the chronological sequence, i.e. [5 ml].

2.2 | GnRH vaccination

The vaccine Improvac[®] (300 µg GnRH diphtheria toxoid protein conjugate/ml; Zoetis Inc.) was administered intramuscularly, using a blowpipe or dart gun for remote injection. Different zoos used slightly different vaccination protocols (Tables 1 and 2). In most cases the dose per administration was 3 ml, but in some cases, 2 or 5 ml of Improvac[®] were administered. Before this study had been conducted, no recommendations for a uniform immunization schedule were available. Recommendations were developed depending on hormone analyses. At an early stage, a vaccination schedule consisting of three consecutive monthly injections was recommended. We refer to this schedule as basic immunization, although during the course of this study, we found that the number of booster vaccinations did vary between female and male giraffes.

The application of Improvac[®] was classified as a medical procedure; immunizations were performed in accordance with relevant institutional guidelines and national veterinary regulations. Slight edematous swelling, which occurred at the injection site in a few animals, resolved without further treatment. A certain problem with the long-term use was the learning effect of the animals, which over time made them more alert and nervous about the coming booster immunizations.

2.3 | Fecal sample collection and steroid hormone metabolite assays

We evaluated the efficacy of the Improvac[®] immunizations in both, male and female giraffes by fecal hormone metabolite monitoring, and to some extent by photographic assessment of testicular atrophy in vaccinated bulls. However, because we noticed the occurrence of marked testicular atrophy only during the course of the study, we did not systematically collect photographs of the atrophied testes.

Fecal samples of the study animals were collected at different time periods and in different numbers. Samples from male giraffes were collected at one or 2-week intervals. Samples of female giraffes were mostly collected two times per week. However, periods with samples collected once per week were also included in this study, especially for animals in which the contraceptive effect was apparent from long-term hormone monitoring. The duration of the sexual cycle in giraffe cows is 15 days (Del Castillo et al., 2005; Dumonceaux et al., 2006; Lueders et al., 2009). Thus the impression might arise that a sampling frequency of 7 days is too long to diagnose cyclic ovarian activity. However, because samples were collected over several months, monitoring regular estrous cycle activity would have been possible with three samples per cycle.

Samples were collected from the floor under observation, and were stored at -20°C until analyzed. For endocrine analysis, samples were sent in batches to the University of Veterinary Medicine Vienna. For hormone metabolite extraction feces (0.5 g) was mixed with 4.5 ml of 80% methanol, vortexed for 30 min and after centrifugation, a portion of the supernatant was diluted and analyzed by using

enzyme immunoassays (EIAs). The methods used for analysis have been validated in a large spectrum of animal species (Schwarzenberger & Brown, 2013; Schwarzenberger, 2007).

Measurements of giraffe fecal progestagens and androgens by immune assays have been described previously (Del Castillo et al., 2005; Lueders et al., 2009; Patton et al., 2006; Seeber et al., 2013; Wolf et al., 2018). It was however necessary to validate our own protocols ahead of this study, as they differed from the previously published protocols. Only the 17-oxo-androstane assay was similar to the one described by Seeber et al. (2013) and Wolf et al. (2018).

To determine the efficacy of GnRH immunizations, two assays for progestagens and two assays for androgens were used. The assays for measuring progestagen metabolites included antibodies against 20-oxo-pregnanes (5 β -pregnane-3 α -ol-20-one, 3HS:BSA) and 20 α -OH-pregnanes (5 β -pregnane-3 α ,20 α -diol, 3HS:BSA; pregnanediol) as described by Flacke et al. (2017). The two EIAs for measuring androgen metabolites included antibodies against 17-oxo-androstanes (5 α -androstane-3 β -ol-17-one 3-HS:BSA; epian-drosterone) and 17 β -OH-androstanes (4-androstene-17 β -ol-3-one 3-CMO:BSA; testosterone) (Hoby et al., 2006). The reliability of these assays was evident from the biological data (estrous cycle pattern, decrease of hormones after birth, and in a male after castration). We evaluated the validity of the assays in the laboratory by matching dilutions of hormone metabolite concentrations with the standard curves. During the first years of the study, 53% of the samples from the female and 60% of the samples from the male animals were analyzed with two progestagen and two androgen assays, respectively. Thereafter, we carried out analyses using only the 20-oxo-pregnane assay and the 17-oxo-androstane assay. In the samples analyzed with both assays, we calculated the coefficients of correlation.

2.4 | Data analysis

We analyzed the results of this study with respect to the assays used, the efficacy of immunization protocols, the long-term effects of Improvac[®], and its reversibility. We continuously developed and adapted the immunization protocols during this study. To determine the efficacy of GnRH immunizations, we calculated sex specific threshold levels of fecal hormone metabolites and evaluated the graphed hormone results. For data management and data analysis, we used Microsoft Excel and SigmaPlot 13.0.

To evaluate the efficacy of GnRH vaccinations in downregulating fecal hormone metabolites, we calculated sex-specific threshold values. We pooled all measured 20-oxo-pregnane ($n = 1817$) or 17-oxo-androstane ($n = 671$) values separately, then sorted and plotted them in ascending order. For both female and male giraffes, the results showed an exponential increase. In these data series, we drew a cut-off line in the curvature at the transition from the slight to the steep increase. We calculated the threshold using the lower 66% of all data for the female and the male giraffes, respectively. We set the

threshold values as mean + 3 SDs. The calculated 20-oxo-pregnane threshold in the females was 508 ng/g feces (mean + SD = 185.5 + 107.5); for further use this was simplified to 500 ng/g feces. In males, the calculation resulted in 182.2 ng/g (43.3 + 46.3); for further use this value was set to 200 ng/g feces.

In most of the animals in this study, Improvac[®] was used for more than 2 years (Table 1). We grouped the results of long-term endocrine monitoring of eight females (individuals #01, #02, #03, #04, #07, #09, #19, #20) and of five males (#01, #03, #04, #06, #07) by years after treatment onset. We used results from the first year of treatment only from Day 150 on. For female and male giraffes, the study periods comprised 7 and 5 years, respectively. Because the grouped results were not normally distributed, we present them in boxplot diagrams.

3 | RESULTS

3.1 | EIAs and biological validation

Comparative patterns of fecal progesterone (20-oxo-pregnanes and 20 α -OH-pregnanes) and of androgen (17-oxo-androstanes and 17 β -OH-androstanes) metabolite concentrations showed an obvious correlation between the respective assays (Figure 1). The Pearson correlation coefficients between the two progestagen and the two androgen assays were $r = .73$ ($n = 966$; $p < .001$) and $r = .46$ ($n = 401$; $p < .001$), respectively. Because assay results between the progestagen and the androgen assays correlated significantly, we applied only the 20-oxo-pregnane EIA for female and the 17-oxo-androstane assay for male giraffes during the later stages of the study. Biological assay validation in female giraffes was evident from the estrous cycles before treatment (Figure 1a) and from the decline of 20-oxo-pregnane values around parturition (Figure 3). For males, the validation was obvious from the immediate reduction of fecal androgen metabolite levels after surgical castration in Male#02 (Figure 1b).

3.2 | Immunization protocol

Within the EEP, the immunization of giraffes with Improvac[®] started in 2013, when no recommendations for a uniform immunization schedule were available (Tables 1 and 2). These recommendations were developed based on the hormone analyzes. In this regard, results of the giraffes Fem#05 and Male#02 (Figure 1) were pivotal. In both animals, the primary immunization with two injections in a 1-month interval were followed by booster injections at 4-month intervals. A clear decrease in hormone metabolite concentrations was evident after four immunizations (~9 months) in Fem#05 and after three immunizations (~6 months) in Male#02. However, in both animals, the effect only lasted for about 2–3 months. Based on these results, an immunization protocol was developed, which included a basic immunization of three injections within 8 weeks, and then further immunizations carried out at 2–4-month intervals (Tables 1

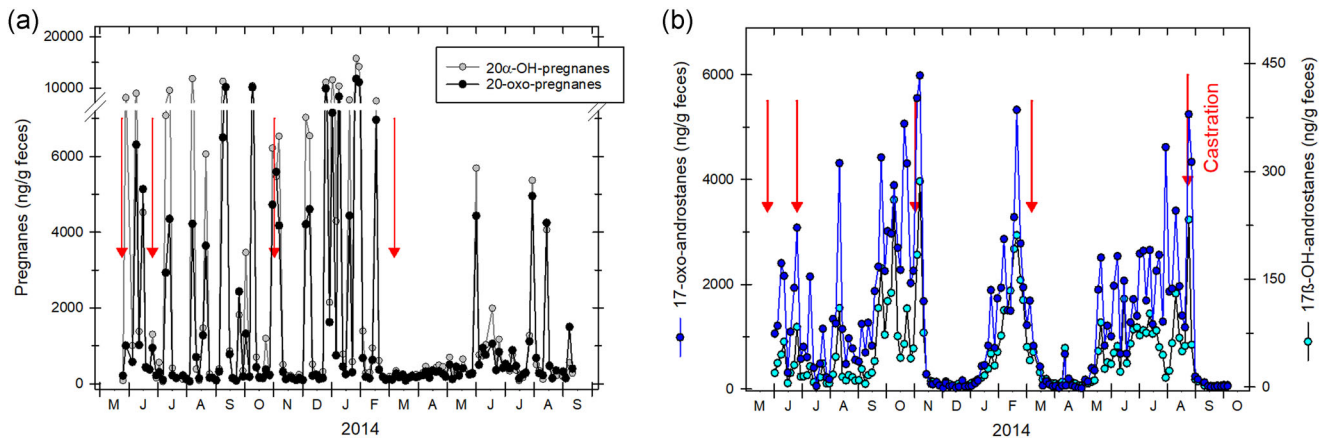


FIGURE 1 Endocrine profiles of (a) Fem#05, and (b) Male#02, after immunization with Improvac[®] (arrows mark individual treatments). Fecal hormone metabolites were analyzed with two hormone assays per individual

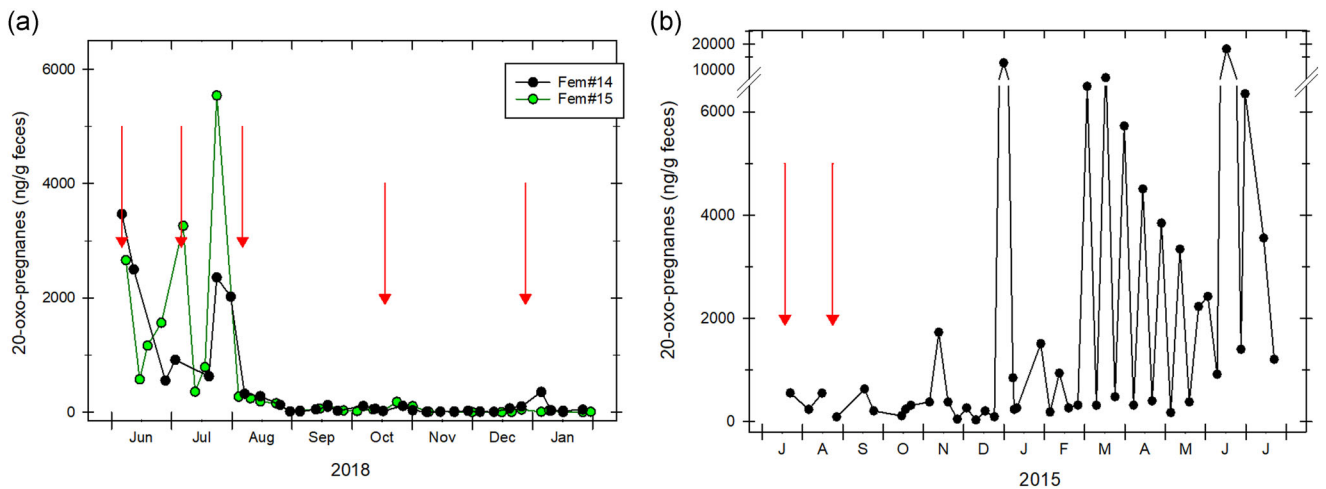


FIGURE 2 (a) Effect of immunizations with Improvac[®] (arrows in graphs) on the pattern of fecal progesterone metabolites. (a) Basic immunization in Fem#14 and Fem#15 was administered at 4-week intervals. (b) In contrast to treatments with three vaccinations, the administration of only two vaccinations in Fem#13 did not result in effective contraception

and 2). This scheme was largely applied for all immunizations carried out after 2014.

3.3 | Application examples and efficacy of immunizations

Data and results from female ($n = 20$) and male ($n = 9$) giraffes included in this study are summarized in Tables 1 and 2. To assess the efficacy of the GnRH immunizations to downregulate steroid hormone metabolites, we evaluated the sex-specific threshold and the graphical visualization of the hormone results. To a certain extent, we had to apply a subjective assessment, because a few individual values in giraffes with a “good” vaccination response exceeded the calculated thresholds of 500 and 200 ng/g feces for females and males respectively. In some animals, hormone levels were even elevated for an extended period of time, with 20-oxo-pregnane and

17-oxo-androstane levels above 4000 and 1000 ng/g feces, respectively (Figure 4). During these periods of elevated hormone metabolite levels, however, no offspring were sired, although the animals were kept in mixed-sex groups.

3.3.1 | Female giraffes

Representative results of hormone analyses demonstrating the efficacy of Improvac[®] vaccinations are shown in Figures 1–4. Sample collection did not always start at the beginning of Improvac[®] administration; for 11 of the 20 females involved in this study, sample collection began several months after the first administration of Improvac[®], thus we could not verify the onset of efficacy. Results on the onset of effective contraception, as suggested by baseline progesterone metabolite concentration, are available from Fem#05 (Figure 1a), Fem#14, Fem#15 (Figure 2a), and Fem#17 (graph not

shown). These results indicate that effective contraception with Improvac® in female giraffes was achieved within 2–3 weeks after basic immunization (three vaccinations within 2 months). Contrary to the effect of three immunizations, results of Fem#12 and Fem#13 indicate that two immunizations do not induce a successful suppression of ovarian activity (Figure 2b).

The contraceptive effect of Improvac® is not always achieved immediately after the third injection. In Fem#07 estrus behavior was noticed 5 days after basic immunization (three injections at 4-week intervals) was completed. Although mating that lead to conception in this female had not been observed, a healthy calf was born after 454 days. During gestation, Improvac® was further administered three times at approximately 4-month intervals. Two other cows kept in the same herd (Fem#08, Fem#09) were also given basic immunization at about the same time, but did not become pregnant. In one of these cows (Fem#08), the treatment with Improvac® was terminated after three injections of basic immunization. About 8 months later, this cow displayed estrus behavior and after a gestation of 460 days (2 years after basic immunization had been completed) a healthy calf was born.

Three lactating giraffe cows immunized with Improvac® during the postpartum period were included in this study (Fem#10, Fem#11, Fem#18; Figure 3). Fem#10 and Fem#11 were treated first with progestins (3 ml Depo-Clinovir® = 450 mg medroxyprogesterone-acetate) and Improvac® immunization commenced afterwards. During the 1-year monitoring period, progesterone metabolites in Fem#10 and Fem#11 were at low levels, although (despite progestin administration), two significant hormone peaks indicating ovulatory cycles occurred in Fem#11 (Figure 3). Three years after cessation of immunizations, fecal 20-oxo pregnane concentrations in both animals were analyzed over a 7-week period. Median levels of these cows were approximately 350 and 560 ng/g feces and maximum levels were approximately 975 and 1175 ng/g feces. These maximum values exceeded the threshold of 500 ng/g feces, however, the endocrine pattern did not indicate regular estrous cycle activity.

We grouped the long-term endocrine monitoring results of eight females by years after onset of treatment and presented them in a boxplot graphic, as the results are not normally distributed (Figure 4a). Throughout the years of long-term monitoring, we observed periods with 20-oxo-pregnane values exceeding the 500 ng/g feces threshold. However, these periods of luteal activity did not indicate regular estrous cycles.

3.3.2 | Male giraffes

We used the results of the 17-oxo-androstane assay to evaluate the efficacy of Improvac® in bulls. Like in cows, efficacy correlated with the mode of basic immunization. The administration of at least three injections at monthly intervals seemed to be a crucial factor. Bull#02 is an example where the basic immunization was not carried out at monthly intervals (Figure 1). In young bulls (#06, #08) hormone levels during Improvac® treatment declined after the third Improvac®

injection. In those bulls older than 3 years, immunization success was achieved after four (bulls #03 and #04; example shown in Figure 5a) or five injections (bulls #01, #05, #07, #09). We present the long-term endocrine monitoring results of five males, grouped by years after the onset of treatment, in a boxplot in Figure 4b.

Bulls may still be fertile after three or four immunizations. Male#01 was treated with four Improvac® injections at monthly intervals, and then at 2-month intervals. Despite this basic immunization, Male#01 sired a calf between the third and fourth injection. Unfortunately, there are no samples available for hormone analysis from before the birth of this calf. Due to this unexpected offspring, basic immunization was once again administered to this bull and he was subsequently treated at 2-month intervals for 2 more years.

In Male#05 the decline in hormone concentration only occurred with a very long delay; even after five injections of Improvac®, androgen metabolite concentrations above the threshold of 200 ng/g feces were observed (Figure 5b). Because of these hormone results, basic immunization with four injections in monthly intervals was repeated, 2 years after vaccinations had commenced. After the second basic immunization, 17-oxo-androstane levels had decreased significantly, although even during this phase a clear androgen peak was present. This bull is currently still under treatment. He has considerably reduced testes size and a shriveled scrotum (Figure 6c).

In addition to endocrine analysis, testicle and scrotum size is a good indicator of vaccination success in bulls. In many Improvac® treated bulls, scrotum size is reduced to a minimum, whereas e.g. in Male#09, in 2017, the scrotum was still medium in size despite the proven suppression of androgen production (Figure 6b). Despite atrophic testicles, some bulls show sexual arousal (courting, mounting) in the presence of females in estrus (e.g., Male#03 and Male#04 mated several times with cows in estrus).

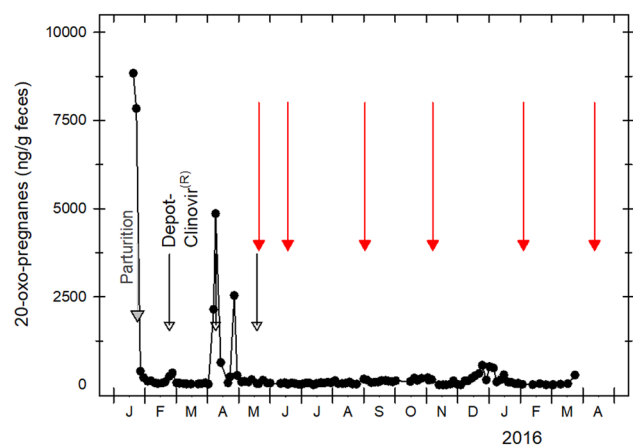


FIGURE 3 Effect of Improvac® immunization on fecal progesterone metabolites in the postpartum, lactating Fem#11. Initially, this female was treated three times with progestins (Depo-Clinovir®; indicated by arrows with an open symbol); 4 months after parturition this was followed by a series of immunization with Improvac® (arrows with filled symbols). Depo-Clinovir® may not have been effective, as the giraffe showed two estrous cycles. With the GnRH vaccine, endocrine activity was suppressed

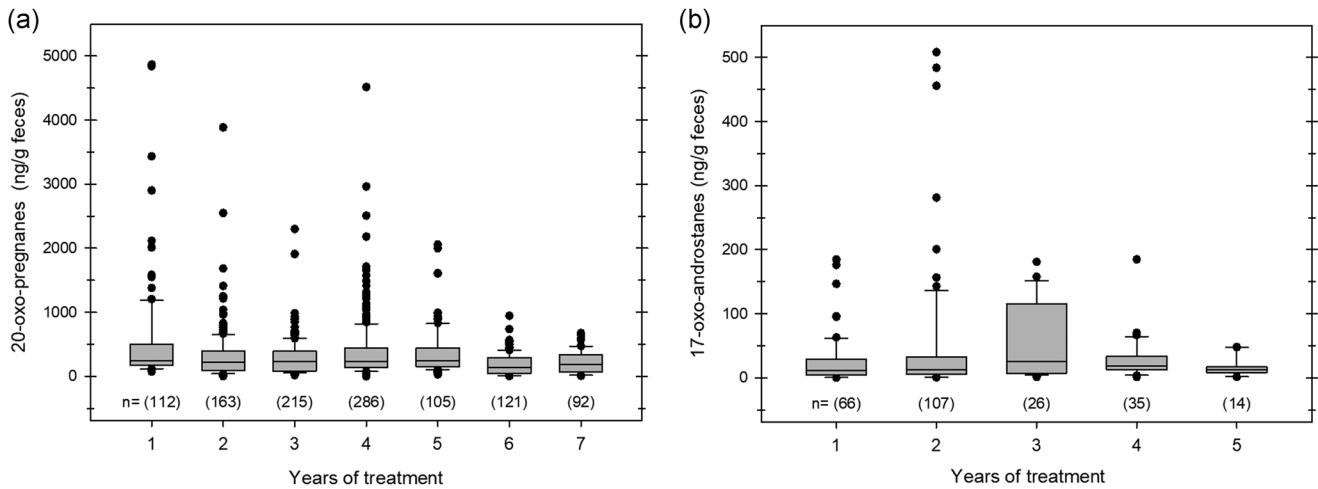


FIGURE 4 Results of long-term Improvac[®] immunizations and endocrine monitoring from (a) eight females and (b) five males. Results were grouped by years after onset of treatment; during the first year of treatment, results were used only from Day 150 on. The number of samples per year is given in brackets. The threshold values of progestagen or androgen concentration for the evaluation of a contraceptive effect were 500 and 200 ng/g feces, respectively. These values were exceeded in some cases, however all 75 percentile values were below the sex-specific threshold values

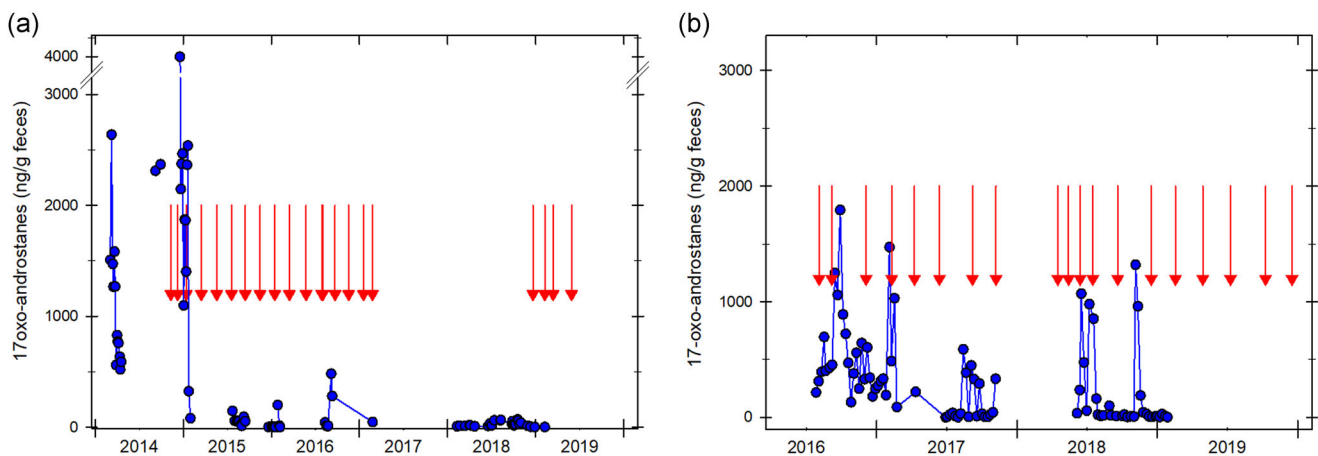


FIGURE 5 Individual results of male giraffes treated with Improvac[®]. (a) In the 12-year-old Male#04, values from before treatment were up to ten times higher. Despite significant testicular atrophy since the fourth month after the start of treatment, the sexual behavior of this bull was not suppressed completely and he mated cows several times even though his androgen levels were below the 200 ng/g feces threshold value. (b) The onset of the effect of Improvac[®] in the 9-year-old Male#05 took nearly 2 years. Therefore, about 2 years after the initial immunization, basic immunization was repeated. In this bull Improvac[®] treatment was continued during 2020 and testes size reduced considerably (Figure 6c)

3.4 | Reversibility of Improvac[®] immunizations

Within this long-term study it was possible to a certain extent to monitor and evaluate the reversibility of the Improvac[®] vaccination. As described above, Fem#08 received a primary immunization consisting of three injections within 8 weeks. Eight months after treatment had been stopped, Fem#08 conceived and later gave birth to a healthy calf.

Results of hormone analyses after termination of Improvac[®] immunizations are available from Fem#10, Fem#11, Fem#16, and Fem#17, and from Male#01 and Male#09. In Fem#16, treatment was started at the age of 2 years and was carried out with nine injections

over a 3-year period (Figure 7a). Even 2 years after the end of the treatment no regular ovarian activity has been restored. The recurring hormone peaks in this female were largely due to persistent corpus luteum activity over periods of up to 5 weeks. In Fem#10 and Fem#11, 3 years after termination of Improvac[®] administration, 20-oxo-pregnane levels were above the 500 ng/g feces threshold, but there was no normal estrous cycle activity.

The hormone profile of Male#09 (Figure 7b) is an illustrative example for both, evaluating the contraceptive efficacy (androgens declined after the fourth Improvac[®] injection), as well as monitoring the reversibility of the Improvac[®] treatment. Even 4 years after the termination of the Improvac[®] treatment, hormone levels and



FIGURE 6 Images of different stages of testicular size as a result of Improvac[®] immunization: (a) fully developed testicles before treatment (Male#08), testicles fill the scrotum and epididymal tails are clearly visible. (b) Male#09 with atrophic testicles in 2017, 18 months after the end of the treatment. (c) Male#05 4 years after the start of Improvac[®] treatment. (d) pronounced testicular atrophy during treatment in Male#03. Picture credits: (a) and (b): Annika Weigold, Stuttgart; (c): Gwendoline Anfray; (d) Franz Schwarzenberger

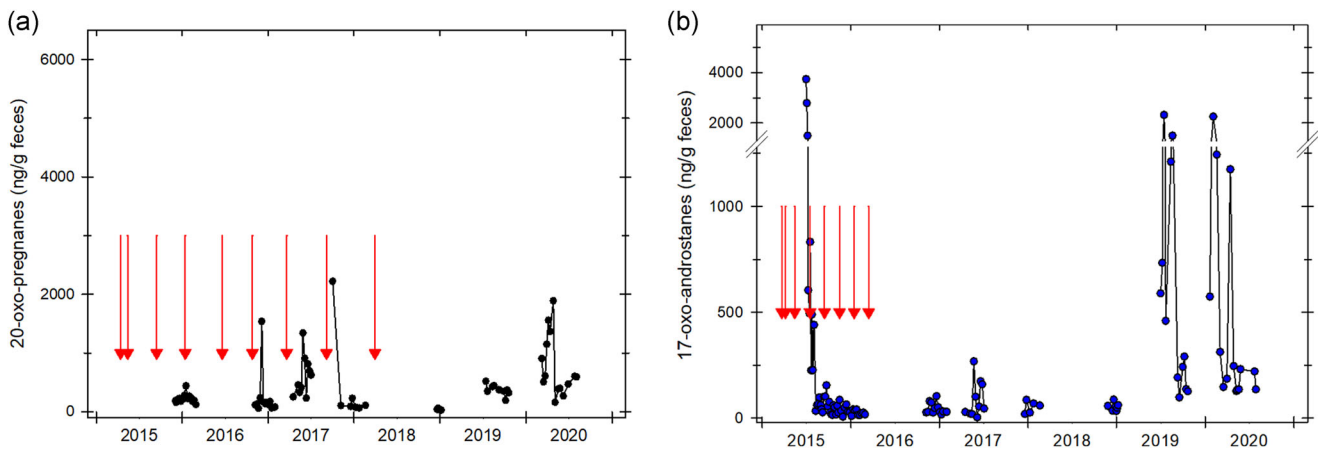


FIGURE 7 Reversibility after termination of Improvac[®] treatments: Endocrine results in (a) Fem#16 and (b) Male#09 indicate a good initial response to Improvac[®] vaccination. However, even more than 2 years after the female's last vaccination, and 4 years after that of the male, hormone metabolite concentrations had not returned to pretreatment levels

testicular size in this bull have not returned to pretreatment levels (Figure 6b). In Male#01, 1 year after immunization had been completed, androgen levels were below 20 ng/g feces and thus well below the 200 ng/g threshold.

4 | DISCUSSION

The aim of this multilocus and multiyear study was to develop an immunization protocol for the contraceptive use of the GnRH vaccine Improvac[®] (Zoetis Inc.) in female and male giraffes (*Giraffa camelopardalis*). Before this study commenced, no information on the efficacy of Improvac[®] in giraffes and no uniform immunization protocol was available. We monitored the effects of the vaccination by fecal hormone metabolite analysis, which provided the basis for the successful development of the immunization protocol, as well as for

assessing the long-term effects. We achieved the goal of developing an effective contraceptive method applicable to both sexes. This method can easily be administered through remote injections. However, we could not conclusively answer the question of reversibility, despite the long study period of 7 years.

We evaluated the efficacy of the GnRH vaccine Improvac[®] in female ($n = 20$) and male ($n = 9$) giraffes by analyzing fecal steroid metabolites. Initially, we analyzed fecal samples using two assays each for pregnane and for androgen metabolites; results of the respective methods were significantly correlated. Finally, we based the evaluation of the results of this study on the 20-oxo-pregnane and the 17-oxo-androstane assay. The characteristics of the 20-oxo-pregnane assay used are comparable to three different progesterone assays described in previous studies on reproduction in female giraffes (Del Castillo et al., 2005; and Dumonceaux et al., 2006; Lueders et al., 2009; Patton et al., 2006). However, at the beginning of this

study, we also obtained physiologically meaningful results with the additional use of the pregnanediol assay. Thus, in addition to the repertoire of three assays already described in the literature, we validated two further assays for the analysis of progesterone metabolites in fecal samples from giraffes as a side-result of the present study. The pregnanediol assay tested in giraffes was also used for the analysis of okapi fecal samples (Schwarzenberger et al., 1999). The 17-oxo-androstane assay for androgen analysis was likewise used in the studies of Seeber et al. (2013) and Wolf et al. (2018).

At the beginning of this study, no experience on the necessary immunization intervals for the use of Improvac[®] in giraffes was available. Thus, e.g. in Frankfurt, we applied a protocol common in horses. In horses of both sexes, effective immunization for about 5 months is achieved with two immunizations at 4-week intervals (Dalín et al., 2002; Donovan et al., 2013; Elhay et al., 2007; Imboden et al., 2006; Janett et al., 2009; Schulman et al., 2013; Stout & Colenbrander, 2004; Turkstra et al., 2005). In giraffes, however, through the use of hormone analyses it soon became clear that three immunizations in females, and four or five in males, are necessary to induce a contraceptive effect. As with horses, in other domestic animal species such as pigs, cattle, dogs, cats, goats, etc. a good immune response to GnRH vaccines is achieved with two injections 1 month apart (Claus et al., 2008; D'Occhio, 1993; Donovan et al., 2013; Einarsson et al., 2009; Lents et al., 2018; Levy et al., 2004; Siel et al., 2020). The use of the GnRH vaccine Gonacon[®] in a number of problematic wildlife species produced a long-lasting effect of 1–3 years even with a single injection (Kirkpatrick et al., 2011; Massei & Cowan, 2014; Massei et al., 2018; Miller et al., 2004; Naz & Saver, 2016; Powers et al., 2011).

In contrast to domestic animal species, in giraffes two Improvac[®] injections do not sufficiently downregulate steroid production, and thus do not induce a contraceptive effect. This is clearly evident from results of Fem#12 and Fem#13. These two females were treated with only two Improvac[®] injections and cyclic ovarian activity recovered within 4 months after the second administration. Likewise, hormone profiles from the giraffes of Frankfurt Zoo demonstrate that after three Improvac[®] injections immunity is not sufficiently achieved, if booster injections are only given at 4–5-month intervals. Insufficient immunity after the third Improvac[®] injection was also seen in Male#01, who sired a calf, or in Fem#07, who conceived at this early stage of Improvac[®] treatment. It is important to consider that a complete contraceptive effect is not to be expected immediately after the completion of a full basic immunization. In addition, despite suppressed androgen production, information on the ability of viable sperm production was not obtained. In female giraffes, the problem of immunity not starting immediately at the beginning of immune contraception can be circumvented by the administration of synthetic progestins.

The onset of the contraceptive effect of Improvac[®], as suggested by baseline reproductive hormone metabolite concentration, started in female giraffes after three and in males after four or five vaccinations, although the onset varies individually. In elephant cows, contraception could not be achieved with two injections of

Improvac[®] (Benavides Valades et al., 2012). In elephant bulls, with a protocol of two Improvac[®] injections at 6-week intervals and further injections at 6-month intervals, atrophy of the accessory sexual glands and suppression of sperm production was evident after a total of four applications. A complete suppression of sexual function was achieved after approximately 6–8 Improvac[®] injections (Lueders et al., 2017). In our present study in giraffes, the suppression of sexual function was achieved at a clearly earlier stage, because the immunization intervals were significantly shorter than those in the elephant study.

Like in elephants and in contrast to domestic animals, more than two Improvac[®] injections were necessary to achieve a contraceptive effect in giraffes. This number of injections could possibly be reduced with an alternative GnRH carrier protein or an alternative adjuvant. Relevant experiments have been conducted in different animal species (D'Occhio, 1993; Donovan et al., 2013; Kirkpatrick et al., 2011; Massei & Cowan, 2014; Naz & Saver, 2016; Siel et al., 2020; Thompson, 2000). In zoos, however, there are few alternatives to commercially available and approved vaccines. Improvac[®]/Improvest[®] is the most widely distributed GnRH vaccine, as Zoetis Australia announced the discontinuation of Equity[®] in February 2020. The GnRH-carrier protein in the Improvac[®] vaccine is diphtheria toxoid, whereas keyhole limpet hemocyanin is used for the noncommercially available GonaCon[®] vaccine (Massei & Cowan, 2014; Miller et al., 2004; Naz & Saver, 2016).

An important aspect for the application of immune-contraception is the individual immune reaction, which influences both, the onset of the effect and its reversibility. In most individuals in this study, endocrine analyses in both sexes and testicular atrophy in males indicated a good response to the Improvac[®] administration, given that a narrow immunization interval was used. Male#05 initially responded poorly to Improvac[®], despite the administration of several injections within short intervals over a period of more than 1 year. For a comprehensive evaluation of the immunization success, it would be beneficial to evaluate anti-GnRH antibody titers. Anti-GnRH titers have been reported in a variety of domestic and wild animal species (Claus et al., 2008; Dalín et al., 2002; Donovan et al., 2013; Elhay et al., 2007; Imboden et al., 2006; Janett et al., 2009; Levy et al., 2004; Powers et al., 2011; Schulman et al., 2013; Siel et al., 2020; Turkstra et al., 2005). Unfortunately, blood sample collection from giraffes, and the development of an assay for evaluating antibody titers were not possible for this study.

Reversibility might be dependent on the number of Improvac[®] applications and the duration of its use. Unfortunately, we cannot fully answer this question for giraffes, as only samples from a few individuals after termination of treatment were available, and only Fem#08 had the opportunity to breed. Fem#08 became pregnant 8 months after completing a three-times basic immunization. In some individuals (Fem#10, Fem#11 and Fem#16, as well as in Male#01 and Male#09) hormone metabolite concentrations did not return to pretreatment levels even after more than 3 years. As mentioned before, the measurement of anti-GnRH titers would be beneficial to evaluate individual differences in the reversibility of GnRH

immunizations. For example in mares, the longest interval to reversibility of estrous cycle activity, indicated by progesterone levels and a decline of GnRH antibody titers, was observed in younger individuals (Schulman et al., 2013). In a study on domestic pigs of the same age, the time to return to pretreatment testosterone levels varied between 74 and 170 days; increasing testosterone levels correlated with a decrease in GnRH antibody titers and an increase in LH pulsatility (Claus et al., 2008).

Long-term profiles of 20-oxo-pregnane metabolites of up to 6 years in Fem#07, Fem#09, Fem#19 and Fem#20 indicate the occurrence of extended periods exceeding the 500 ng/g feces threshold. However, this luteal activity has likely not been caused by regular estrous cycles; it appears that follicle formation and spontaneous luteinization of these follicles can occur in GnRH-vaccinated giraffes. In mares, GnRH vaccination prevents ovulation by suppressing LH release, but the effect on inhibition of FSH secretion is less pronounced, thus follicle formation is not completely suppressed (Dalin et al., 2002; Garza F et al., 1986; Stout & Colenbrander, 2004).

5 | CONCLUSIONS

In summary, the contraceptive efficacy of Improvac[®] treatment was achieved in both female and male giraffes. Although the results of the present study came from individual animals, it was possible to derive recommendations for establishing an effective vaccination protocol. Fecal hormone metabolite analysis was essential to assess vaccination efficacy, as well as long-term effects. In males, vaccination success can also be monitored by testicular atrophy. Our recommended protocol for basic immunization consists of three injections into female, and four or five injections into male giraffes; these injections should be administered at 4-week intervals. Booster injections during the first year of treatment should be given at 2-month intervals. In the second year, injection intervals can be extended to 3- to 4-month intervals. During long-term use, females showed longer lasting phases of luteal activity. The greatest unresolved aspect is reversibility. In cases where we administered five or more Improvac[®] injections, reproductive hormone levels did not return to physiological ranges, even more than 4 years after the last vaccination.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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