Effect of rabies booster vaccination on antibody levels in African wild dogs (*Lycaon pictus*)

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Word count = 4187

ABSTRACT: Rabies is a highly virulent viral disease that has been associated with large-scale population declines of the endangered African wild dog, *Lycaon pictus*. While rabies vaccination may be a valuable conservation tool in this species, studies indicate that a single dose does not always confer protective immunity. We examined 47 serum samples from 22 captive African wild dogs (sampled opportunistically for other purposes) to assess whether serum antibody levels after vaccination correlated with the number of doses received, and whether other factors affected outcomes. Results of the fluorescent antibody virus neutralization test showed that median antibody titers were 0.085 IU/ml pre-vaccination, 0.660 IU/ml after a single vaccination, and 22.150 IU/ml after a booster vaccination. Antibody titers above 0.5 IU/ml, internationally accepted as the threshold for seroconversion, were found in none of the samples taken pre-vaccination, 66.67 % of samples taken after primary vaccination, and in 90.90 % of samples collected after booster vaccination. This study illustrates the likely protective benefit a rabies booster vaccination can potentially provide in African wild dogs and serves as a basis for future research to improve vaccination protocols that contribute to the conservation of this endangered species.

Key words: African wild dog, booster, rabies, vaccination

INTRODUCTION

The African wild dog (*Lycaon pictus*) is an endangered canid species and the sole extant member of the Genus *Lycaon* (Creel and Creel 2002). With fewer than 7,000 individuals estimated to remain in the wild, these animals are among the most endangered mammals on the African continent (Woodroffe and Sillero-Zubiri 2020). Once a widespread species in sub-Saharan Africa, the African wild dog is now found in less than 10 % of its historic range (Fanshawe et al. 1991; Davies-Mostert et al. 2016). Numbers have continued to decline since the first International Union for Conservation of Nature (IUCN) Red List assessment of the African wild dog was published in 1986, leading to the species being currently classified as "endangered" (Woodroffe and Sillero-Zubiri 2020). Reasons for this decline mostly stem from the continued encroachment of human populations on African wild dog habitat. As a wide-ranging species living at low densities, the African wild dog is highly sensitive to habitat fragmentation (Courchamp et al. 2000; Courchamp and Macdonald 2001). This fragmentation and increased contact with humans in turn contributes to the exacerbation of human-wildlife conflicts, resulting in the persecution and retaliatory killing of African wild dogs due to predation on livestock and farmed game (Ginsberg and Woodroffe 1997). Higher rates of contact also facilitate transmission of pathogens from domestic dogs (*Canis familiaris*), which may be reservoirs of multiple pathogens to which African wild dogs are susceptible (Woodroffe et al. 2012). This includes rabies virus (RABV) (Rhodes et al. 1998; Prager et al. 2012), which is zoonotic and causes the fatal disease rabies in both wild dogs and people, and against which a single vaccination may not always elicit protective immunity in wild dogs (Gascoyne et al. 1993; Hofmeyr et al. 2000; Woodroffe et al. 2004).

This study investigated the association between rabies vaccination status and antibody response in African wild dogs, hypothesizing that a second vaccination would be correlated with higher neutralizing antibody titers. We also evaluated how other factors, namely, sex, age, vaccine brand, time between vaccination and serum collection, and time between vaccinations are associated with the results obtained.

Vaccinology

Vaccination serves to protect via the adaptive immune response, with the aim of reducing the impact of a pathogen or neutralizing its toxic components (Siegrist 2018). When a pathogen-associated antigen is introduced to the body via vaccination, it is taken up by antigen-presenting cells (APCs), mostly dendritic cells (Siegrist 2018). APCs then migrate to local lymph nodes, where they present the antigen to naïve B and T lymphocytes, inducing their differentiation and proliferation (Palucka et al. 2010). T cells may differentiate into several subtypes, including CD4+ T cells (T helper cells, THCs). THCs promote the function and clonal expansion of several other lymphocyte types (Luckheeram et al. 2012). Naïve B cells mostly differentiate into mature B cells and memory B cells (MBCs) (Siegrist 2018). While mature B cells immediately produce antibodies, MBCs instead circulate in the blood stream in a quiescent state and can persist for several years. MBCs can be re-activated by repeat antigen exposure (via infection or booster vaccination), rapidly secreting antibodies in large amounts and with high affinity to the pathogen involved (Spiegelberg 1974).

B cell-secreted antibodies are a vital part of immunity against RABV (Rupprecht et al. 2018). After differentiation, B cells will produce antibodies of the IgM subtype, which have been shown to largely remain within the blood circulation (Spiegelberg 1974; Turner 1978). THCs

eventually induce a class-switch in B cells, causing them to instead produce IgG antibodies, which can enter tissues by diffusion (Spiegelberg 1974). In contrast to IgM antibodies, IgG antibodies are therefore considered more effective in providing protection against rabies, as they can locally inhibit neuronal spread of RABV. Booster vaccinations have been shown to directly induce the production of large amounts of high-affinity IgG antibodies upon re-activation of MBCs (Spiegelberg 1974; Siegrist 2018).

Rabies in African wild dogs

Although rabies is a zoonosis, there are no known cases of African wild dogs infecting humans with RABV. Instead, this lethal generalist pathogen is endemic in domestic dog populations in Africa (e.g., Prager et al 2013) which act as a RABV reservoir, presenting a significant and pressing public health threat (World Health Organization 2018). The transmission of RABV from domestic dogs is thought to be the primary cause of rabies outbreaks observed in African wild dog packs (Kat et al. 1995; Prager et al. 2012; Flacke et al. 2013), including via intermediate hosts such as the black-backed jackal (*Canis mesomelas*) (Hofmeyr et al. 2000). African wild dog populations are highly vulnerable to the effects of a rabies outbreak. Their deeply social nature facilitates rapid intra-pack transmission of the pathogen once it has been introduced (Ginsberg and Woodroffe 1997, Woodroffe and Ginsberg 1997) and, if a small number of animals survives, they are unlikely to breed unless they can form a new pack with unrelated mates, as wild dogs very seldom join existing packs (Cozzi et al. 2020). A recent study summarizing data collected between 1989 and 2019 (Gold 2021) illustrates the devastating effect that rabies can have on wild dog packs: Six rabies-affected packs which received no human intervention (such as vaccination or removal of first symptomatic animals) experienced 100 %

mortality. Most notably, rabies was implicated in the complete extinction of African wild dogs in the Serengeti-Mara ecosystem between 1991 and 1992 (Gascoyne et al. 1993; Kat et al. 1995). Over the past few decades, the virus has also impacted populations in South Africa (Hofmeyr et al. 2000; Hofmeyr et al. 2004), Botswana (Woodroffe et al. 2004), Namibia (Scheepers and Venzke 1995), and Zimbabwe (Kat et al. 1995), with further outbreaks in the Central African Republic, Zambia (Woodroffe and Ginsberg 1997), and Kenya (Woodroffe 2011). As these countries hold some of the largest remaining populations of wild dogs on the African continent (Woodroffe and Sillero-Zubiri 2020), the conservation significance of addressing the threat posed by RABV becomes apparent.

Vaccination of African wild dogs

Effective rabies vaccination is the only method of protecting an individual at risk from the almost inevitably fatal disease of rabies (Rupprecht et al. 2006; World Health Organization 2018). Mass vaccination of the domestic dog reservoir has the potential to eliminate RABV entirely (Coleman and Dye 1996; Cleaveland et al. 2003). However, this is a challenging undertaking in many African countries (Lembo et al. 2010; Bitek et al. 2019). African wild dogs in a few managed populations are currently vaccinated opportunistically against rabies (Gold 2021), with approaches ranging from vaccinating animals once (Kat et al. 1995; Hofmeyr et al. 2000), to multiple times within the same year (Hofmeyr et al. 2004; Canning et al. 2019). Simulation models show that protecting a viable core population of African wild dogs should be sufficient to prevent local extinction during a rabies outbreak (Vial et al. 2006). Still, modelling results must be interpreted with caution, as they assume that vaccination of African wild dogs can provide 100 % immunity for a minimum of one year, which is not currently supported by

existing evidence. Vaccination has failed to protect individuals on several occasions (Gascoyne et al. 1993; Scheepers and Venzke 1995; Hofmeyr et al. 2000), raising concerns about the general utility of rabies vaccination in this endangered species (Woodroffe 2001). However, rabies vaccination does seem to have benefits in African wild dogs. One notable example of this was observed during a 2000 rabies outbreak in South Africa: individuals that had received multiple vaccinations prior to the event were much more likely to survive than those that had received no or only a single dose of rabies vaccination (Hofmeyr et al. 2004).

Depending on the context, African wild dogs may get vaccinated by hand (e.g., during translocation) or remotely via dart-injection. While the dose of RABV vaccine is 1 ml per individual, regardless of size (Connolly et al. 2015), which can easily be darted by most dart systems, there is currently no data regarding the consistent delivery of 100 % of this volume through remote inoculation in wild dogs. However, a recent study compared the efficiency of dart- versus hand-inoculation for canine parvovirus vaccination in African wild dogs (Anderson and Smith 2019). While titers were higher after hand-injection than remote injection following initial vaccination, titers were comparable after booster vaccination and both methods led to titers that are assumed to be seroprotective against canine parvovirus after single and booster vaccination.

It has been suggested that observed vaccine failures may be due to a single dose of inactivated rabies vaccine not sufficiently eliciting a protective immune response in African wild dogs (Woodroffe 2001). Current serological evidence on the protection of wild dogs after rabies vaccination is mixed: whereas a study by Connolly et al. (2015) found that all captive wild dogs which received a single dose of rabies vaccine seroconverted (i.e. developed titers above 0.5 IU/ml), and some could still be considered “seropositive” up to 36 months later, a study

conducted by van Heerden et al. (2002) showed that titers in most vaccinated individuals (n = 6 out of 8) decreased below the "seropositive" threshold 100 days post-vaccination. Additionally, van Herden et al. were able to show that booster vaccination five months after initial immunization induced seroconversion in all individuals receiving a second dose of rabies vaccine, while booster vaccination was not assessed in Connolly et al.'s study. However, van Heerden et al.'s work examined a very small sample size (n = 4), limiting the robustness of conclusions drawn from their work. The purpose of the current study was to better understand the serological response of African wild dogs to rabies booster vaccination, and to provide insights into some of the factors potentially influencing measured antibody titers.

MATERIALS AND METHODS

Samples

All samples were collected from African wild dogs held in captivity at Port Lympne Reserve, UK. All individuals included in this study were vaccinated and blood sampled under general anesthesia by a veterinarian prior to the start of this project. As all samples had been previously collected for health monitoring purposes unrelated to vaccine responses and as a component of routine zoo collection management, the authors' institutions did not require ethical approval for this work. A total of 47 serum samples were analyzed, which were collected from 22 captive African wild dogs between the years 2001 and 2019 (Table 1, further information in supplemental material, table S1). Animals were vaccinated intramuscularly with inactivated rabies virus vaccine, either Canigen® Rabies (2.0 IU/ml, 00973, Virbac Ltd., Sussex, UK), or Rabisin® (1.0 IU/ml, 01166, Boehringer Ingelheim Animal Health UK Ltd., Berkshire, UK). While protocols varied, all animals were vaccinated against rabies at least once within the study

period. Of these 22 individuals, seven received a rabies booster vaccination. Blood samples were collected at varying times after vaccination, with most samples collected within the first two years after vaccination (supplemental material, table S1). Blood was collected in a standard serum separator tube and centrifuged within 30 minutes of collection. The resulting serum was then decanted and stored at -18 °C at Port Lympne. In early 2022, samples were transported to the serology laboratory for analyses, where samples were maintained at -20 °C. Aliquots of 10 microliter (µl) each were analyzed by fluorescent antibody virus neutralization (FAVN) test. Prior to analysis, all aliquots were heat treated at 56 °C for 30 minutes.

Fluorescent Antibody Virus Neutralization (FAVN) testing

FAVN testing of all samples was carried out at the Animal and Plant Health Agency (Addlestone, Surrey, UK) as described by Cliquet et al. (1998). Briefly, rabies challenge virus (CVS strain) was incubated at 37 °C in microplate wells with serial dilutions of the test serum aliquots to be titrated. After 60 minutes, baby hamster kidney (BHK21-13s) cell suspension was added to each well. The wells were then again incubated at 37 °C for a period of 48 hours, in which any virus not neutralized by potentially present serum antibodies infected the BHK-cells. After incubation, the cells were fixed with acetone and stained with a fluorescein isothiocyanate conjugated (FITC) rabies nucleoprotein antibody. The plates were then read under a fluorescence microscope using an "all or nothing" approach, whereby wells in which fluorescence was observed in one or more cells were considered to be positive. The last dilution at which no fluorescence was observed was considered the "end point" used for titer calculation. To account for possible cytotoxicity, the integrity of the cells in each sample was assessed during each reading. Furthermore, control cell cultures were examined to which BHK growth medium was

added, but no challenge virus, which allowed for general assessment the viability of cells. Four replicates were analyzed for each serum sample. Final titers in international units (IU) per milliliter (ml) were then calculated using an Excel FAVN calculation package based on the Spearman-Kärber method, relating mean test serology results to the international World Organization for Animal Health (WOAH) standard reference serum (Spearman 1908; Kärber 1931). As per the definition provided by the World Health Organization, an individual whose antibody titer exceeds 0.5 IU/ml is typically considered to have seroconverted. This term indicates a strong probability that they have attained immunity against RABV (World Health Organization 2018).

Data Analysis

Data analysis was carried out using R software version 2022.02.3 (R Core team 2020). FAVN test titers were log-transformed to achieve normality of residuals. Residuals of all models were assessed for normality using the Shapiro-Wilk test and visual inspection of the histogram. The association of explanatory variables (listed in Table 2) with FAVN test results was assessed using generalized estimating equations (GEE), which allowed us to account for repeat measurements from the same individual (Ziegler et al. 1998). Each observation was grouped according to the unique animal ID, effectively treating observations from the same individual as their own distinct cluster. To construct the GEE models, we selected an exchangeable correlation structure due to its suitability for handling the correlated nature of our data. We chose this structure because it assumes that the correlation between repeated measurements within the same individual is the same for all individuals. We assumed a Gaussian distribution, as we were modelling log (FAVN) test results as a continuous outcome. Variables were first individually

examined using univariable analysis. All variables that correlated with antibody results at $p \leq 0.2$ were then further analyzed using multivariable analysis. To compare all three categories of "vaccination status" (Table S2), we used Tukey's multiple comparison of means test (Table S3).

All models were fitted using the *geepack* package in *R* (Bates et al. 2015). Rabies antibody titers are described by median and interquartile range (IQR, 25 %, 75 %). Results of model analyses are reported with coefficient, standard error (SE) and p-value. Type I error rate is set at 5%.

RESULTS

General observations

There was no cytotoxicity observed in any of the samples analyzed. None of the samples taken pre-vaccination (n = 12) showed a titer above 0.5 IU/ml (Figure 1). Out of all samples collected after the first vaccination (n= 24), 66.7 % (n = 16) showed a titer above 0.5 IU/ml, whereas this threshold was exceeded in 90.9 % of all samples collected after booster vaccination (n = 10 out of 11, Figure 1). The overall median of raw pre-vaccination titers was 0.085 IU/ml [IQR 0.070, 0.130], while raw median titers after the first and second vaccination were 0.660 IU/ml [IQR 0.290, 2.600], and 22.150 IU/ml [IQR 7.005, 50.120], respectively (Figure 1).

Multivariable analyses

All variables other than sex met criteria for inclusion in the multivariable analyses. Multivariable models were only constructed for FAVN titers measured in samples collected after vaccination, as all relevant predictor variables were obtained from data collected post-immunization. As the variable "booster interval" could only be analyzed for samples collected

after a second vaccination, two multivariable models were built (supplemental material, Figure S1). The results of the univariable analyses are not presented in this section for reasons of clarity but are available for further review in the supplementary materials (Table S2).

Model 1 – all samples collected after vaccination

Model 1 included all samples taken after vaccination. This model assessed the association of all explanatory variables except sex (following results of the univariable analysis) and booster interval (not recorded for pre-booster samples) with FAVN titers (supplemental material, Figure S1). Only a second vaccination and time since vaccination were significantly associated with antibody titers measured in vaccinated individuals (Table 3, supplemental material, table S4). Rabies antibody titers were significantly higher after a second vaccination than after a first (coefficient (SE) = 2.57 (1.11), p = 0.02); note that this analysis excluded the "booster interval" variable, which only applies to samples collected after the second vaccine dose and could therefore not be included in this model. Titers were significantly lower after longer intervals between vaccination and blood collection (coefficient (SE) = -0.02 (0.01), p = 0.05).

Model 2 – all samples collected after booster vaccination

Model 2 consisted of a subset of samples included in Model 1, specifically those taken after a booster vaccination (supplemental material, Figure S1). This model assessed the effect of all explanatory variables except sex (following results of the univariable analysis) and vaccination status (uniform across samples) on measured FAVN titers. Only time since vaccination was found to be a significant predictor of antibody titers (coefficient (SE) = -0.05 (0.01), p < 0.001): Again, longer intervals were associated with lower values (Figure 2, Table 3).

DISCUSSION

The aim of this study was to examine the relationship between rabies booster vaccination and RABV antibody levels in African wild dogs. Our findings support field evidence that a single vaccination does not consistently protect these endangered canids against RABV (Hofmeyr et al. 2000; Hofmeyr et al. 2004; Woodroffe et al. 2004; Canning et al. 2019).

Results of the FAVN tests showed a significant association between antibody titers and vaccination status, with a booster vaccination associated with titers above the 0.5 IU/ml threshold in nine out of ten samples examined. This pattern is consistent with studies in other canids (Fooks et al. 2002; Cliquet et al. 2003; Mansfield et al. 2004), in which seroconversion occurred in a significantly higher percentage of animals that received multiple doses of RABV vaccine. A study examining rabies antibody titers in a worldwide sample of over 17,000 domestic dog sera revealed that 14.5 % of animals which had received only a single vaccination exhibited titers below 0.5 IU/ml. This percentage notably decreased to 9.5 % in dogs that had received a booster vaccination (Cliquet et al. 2003).

Only one previous study has specifically examined rabies antibody titers in African wild dogs after booster vaccination, and their sample size was limited to four individuals (van Heerden et al. 2002). The results of our study confirm and expand upon these findings, showing a clear effect of RABV booster vaccination in African wild dogs. As no cytotoxicity was observed during the FAVN analyses, false-positive results are unlikely.

It is important to emphasize that the cutoff value of 0.5 IU/mL, while crucial for FAVN test interpretation, cannot be considered an absolute measure in an ecological context. Indeed, some animals with titers above 0.5 IU/ml will succumb to rabies, while others with lower values

may survive infection (Moore et al. 2017). This can be species-specific. In the small Indian mongoose (*Herpestes auropunctatus*), a titer of as low as 0.25 IU/ ml has been found to be associated with survival (Moore et al. 2017). This might be due to both humoral and cell-mediated immune responses being required to effectively combat RABV infection. The relative contribution of each of these mechanisms may vary across species, and FAVN tests can only capture the humoral aspect of this complex immune response. Without pathogen challenge, it is therefore impossible to confirm whether an antibody titer of over 0.5 IU/ml would be protective in African wild dogs. Nonetheless, while there is no threshold for RABV-neutralizing antibodies that can universally be described as "protective", empirical evidence from challenge studies in domestic dogs demonstrates that elevated levels of neutralizing antibodies at the moment of RABV exposure are critical for survival (Aubert 1992).

We also found that one individual did not seroconvert, despite receiving a booster vaccination. The existence of such non-responders corresponds with the results of other studies and is thought to depend on individual-specific variables, such as physiological status at the time of vaccination (Schuurs and Verheul 1990). In canids, an inadequate response to RABV vaccination may occur due to immunosuppression, which can be caused by underlying disease (Murray et al. 2009). It could also reflect the fact that, in some individuals, vaccination may stimulate a larger proportion of naïve B cells to differentiate into memory B cells, which will not immediately be active, as opposed to antibody-secreting B cells (Kennedy et al. 2007). As stated above, serum neutralization tests can only provide information on the humoral response to vaccination. Additional immunoassays, such as lymphocyte proliferation assays specifically aimed at evaluating the cellular response to rabies vaccination, would be needed to get a more-detailed picture of the immune response to vaccination (Overduin et al. 2019). However, the

311 elevated antibody titers after booster vaccination seen in the current study indicate that African
312 wild dogs generally do generate memory B cells during primary vaccination, which re-activate
313 following a booster vaccination (Spiegelberg 1978; Siegrist et al. 2018). Future studies could
314 include methods differentiating serum IgG and IgM antibodies to provide further information on
315 this.

316 The animal's age at vaccination, vaccine brand, and the time between first and second
317 (booster) vaccination were ultimately not found to be significant predictors of rabies antibody
318 titers. However, due to this study's retrospective nature, its small sample size and uneven
319 distribution of groups associated with different predictor variables (e.g. only six out of 35
320 vaccinated individuals received Canigen® rabies) the power of our analyses was limited. Studies
321 in several canid species have demonstrated that the timing of RABV vaccination and the age at
322 the initial inoculation significantly influence the generation of an immune response to a vaccine.
323 Notably, animals younger than one year of age have an increased probability of exhibiting an
324 inadequate response to rabies vaccination (Murray et al. 2009, Kennedy et al. 2007). To
325 accurately assess these parameters and further optimize rabies vaccination strategies in African
326 wild dogs, future research should follow a predetermined study design and, if possible, ensure
327 that group sizes are large enough to allow significance to be robustly ascertained.
328 In studies involving domestic dogs, it has been observed that the immune system responds more
329 rapidly after a second vaccine dose, and that elevated antibody titers (above 0.5 IU/ml) persist
330 for an extended period (Mansfield et al. 2004; Kennedy et al. 2007). We did observe that a
331 longer interval between vaccination and sampling was significantly associated with lower
332 antibody titers (Figure 2), which concurs with the results of previous studies in other canid
333 species (Mansfield et al. 2004; Kennedy et al. 2007). However, as samples examined in the

current study were taken at varying times after vaccination, it is not possible to assess when individuals first showed an antibody response post-vaccination, what their maximum titer was in between vaccination and sampling, or how long antibodies persisted in an individual animal. Both Canigen® rabies and Rabisin® have been reported to convey protection for about three years in domestic dogs (Boehringer Ingelheim Animal Health UK Limited 2020; MSD Animal Health UK Limited 2021), following a course of initial vaccination. For Canigen® rabies, the manufacturer recommends a single vaccination, whereas the manufacturer's instructions for Rabisin® suggest an additional booster vaccination one year after the initial inoculation (Boehringer Ingelheim Animal Health UK Limited 2020). The results of our study support a schedule similar to the one proposed for Rabisin® in African wild dogs, with three out of four boostered individuals sampled more than a year after booster vaccination showing titers above 0.5 IU/ml (Figure 2). It might be that elevated antibody titers persist for an even longer period of time, but the number of samples included in this study limited the conclusions we were able to draw regarding longevity of circulating antibodies after booster vaccination. Implementing an annual vaccination regimen for African wild dogs would likely be the most feasible strategy for conservation management. The species reproduces approximately annually, and packs mainly consist of yearlings and new pups (Fuller et al. 1992). Annual rabies vaccination of African wild dog packs would, therefore, offer a practical management option, serving the dual purpose of providing a booster vaccination to individuals which have already received a first dose and the vaccination of pups.

Based on the results of this study, a clear recommendation for rabies booster vaccination in African wild dogs can be given. This work illustrates the positive association between a booster vaccination in this species and elevated neutralizing serum antibody titers. Our findings

suggest that implementing an annual rabies vaccination regime could represent a viable strategy for protecting African wild dog packs against RABV infection.

While this study demonstrated no significant effect of vaccine brand, age at or timing of vaccination on antibody titers, these variables are known to affect immune responses in other canid species, and further immunogenicity studies would be warranted to optimize rabies vaccination strategies in African wild dogs.

This study serves to expand the current evidence base of African wild dog immunology with respect to rabies vaccination and contributes to the development of more effective RABV management strategies for this endangered species.

ACKNOWLEDGMENTS

We would like to thank Dr. Guanghui Wu, who made conducting the FAVN analyses possible. The authors would further like to express deep gratitude to Sam Webb, Tomasz Zborowski, Dr. Ad Vos and Dr. Thomas Müller for their advice during the preparation of this manuscript.

This research was partly funded by the Royal Veterinary College and Research England. This work was also supported by the Experiment Foundation as well as several backers via the Experiment.com platform. Additional funding was generously provided by the Zebra Foundation.

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Table 1. Overview of vaccination status and serum sampling schedule for African wild dogs (*Lycaon pictus*) kept at Port Lympne, UK from which blood samples were collected opportunistically between the years 2001 and 2019 before and after rabies vaccination. Numbers in brackets indicate the number of individual animals represented. NA = Not Applicable.

	Number of samples originating from animals that were vaccinated once (number of individuals)	**Number of samples originating from animals that were vaccinated twice** (number of individuals)	**Total** (number of individuals)
Sample taken before first vaccination	9 (8)	3 (3)	12 (11)
Sample taken after first vaccination; if applicable	17 (15)	7 (5)	24 (20)
Sample taken after second vaccination	NA	11 (7)	11 (7)
Total	26 (15)	21 (7)	47 (22)

Table 2. Explanatory variables included in statistical models analyzing their association with measured rabies antibody titers in blood samples of captive African wild dogs (*Lycaon pictus*) opportunistically collected at Port Lympne Reserve, UK, between the years 2001 and 2019. "Vaccination" denotes "rabies vaccination".

Variable	**Range/ Categories**	**Further explanation**
Vaccination status	0	Sample taken prior to vaccination
	1	Sample taken after first rabies vaccination
	2	Sample taken after rabies booster vaccination
Age	0.23 – 10.82	In years, at time of most recent rabies vaccination, if applicable
Sex	m	Male
	f	Female
Time since vaccination	0.20 – 110.99	In months; time between most recent vaccination and sample collection, if applicable
Booster interval	6.05 – 48.97	In months; time between first and booster vaccination, if applicable
Vaccine brand received	CG	Canigen® Rabies
	RS	Rabisin®

546 **Table 3.** Results of the multivariable models analyzing the association between rabies antibody
547 levels in blood samples from African wild dogs (*Lycaon pictus*) that were opportunistically
548 collected from captive individuals at Port Lympne Reserve, UK between 2001 and 2019
549 following rabies vaccination, and the relevant predictor factors. Only statistically significant
550 associations are shown.

			Estimate	**95 % CI**	**p – value**
Model 1	Vaccination status	2[1]	2.57	1.46 – 3.68	0.02
Log (TI) after vaccination	Serum interval		-0.02	-0.03 – -0.01	0.05
n = 35					
Model 2	Serum interval		-0.05	-0.06 – -0.04	1.1e-08
Log (TI)					
after booster vaccination					
n = 11					

[1] Reference: Vaccination status = 1
[2] Reference: Canigen® Rabies

551

Figure 1. Rabies antibody titers (IU/ml) according to rabies vaccination status (0 = no vaccinations received, 1 = one vaccination received, 2 = two vaccinations received) in blood samples of captive African wild dogs (*Lycaon pictus*) kept at Port Lympne Reserve, UK. Samples were collected opportunistically between 2001 and 2019. Horizontal jitter was imposed on individual data points to aid visualization, but categories of vaccination status are discrete. The dashed line marks the 0.5 IU/ml titer threshold generally accepted as denoting seroconversion in an individual vaccinated against RABV.

559 **Figure 2.** Rabies antibody titers (IU/ml) measured in the blood of RABV-vaccinated African
560 wild dogs (*Lycaon pictus*) according to time between rabies vaccination and serum collection in
561 months. Blood was collected opportunistically from captive individuals kept at Port Lympne
562 Reserve, UK, between the years 2001 and 2019. Vaccination status (VS) of individuals at time of
563 sample collection is represented by shape (1/ dots = one vaccination received, 2/ triangles = two
564 vaccinations received). The dashed line marks the 0.5 IU/ml titer threshold set by the World
565 Health Organization to mark seroconversion.

Figure 1

Click here to access/download;Figure;Figure 1_bw.tiff

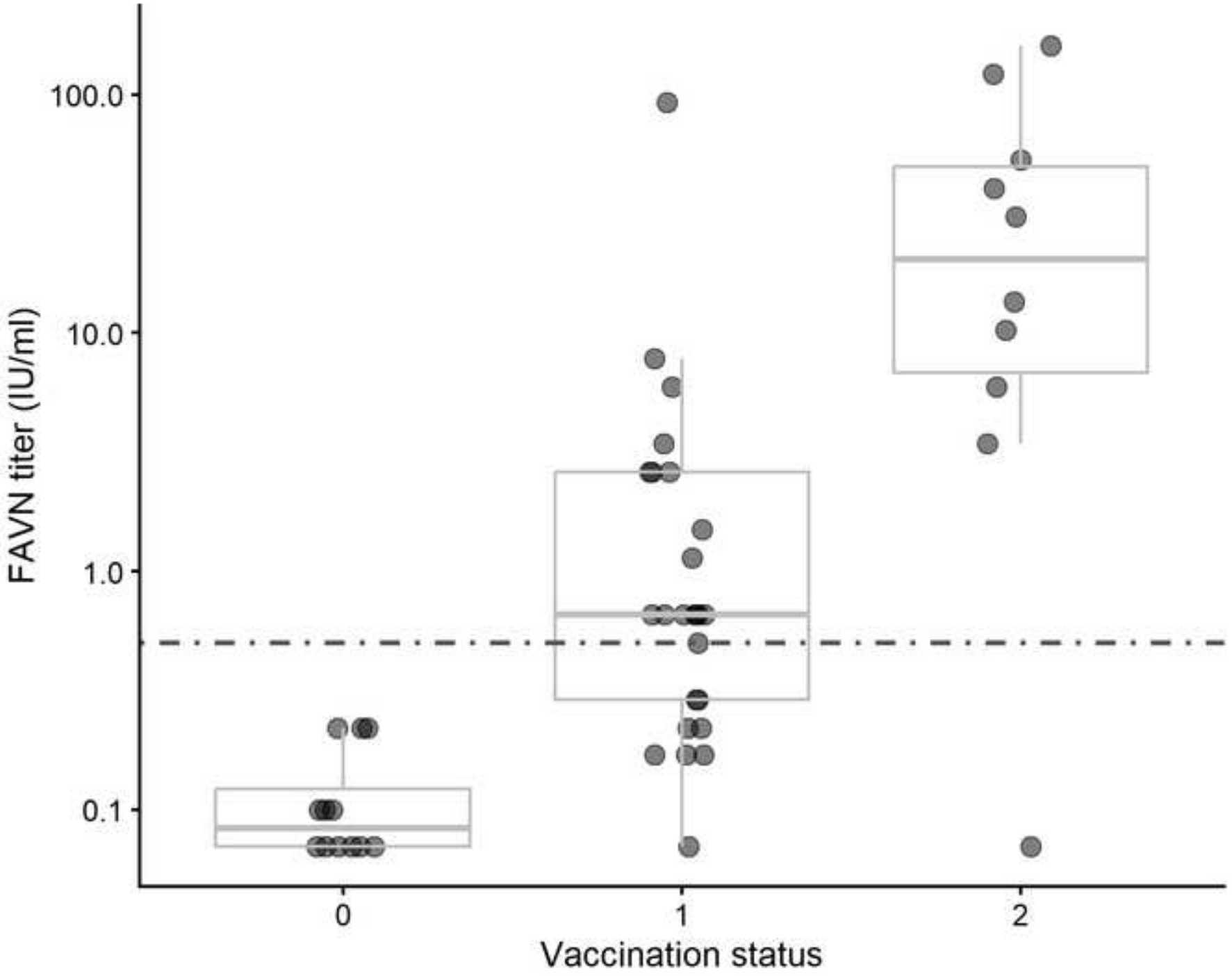


Figure 2

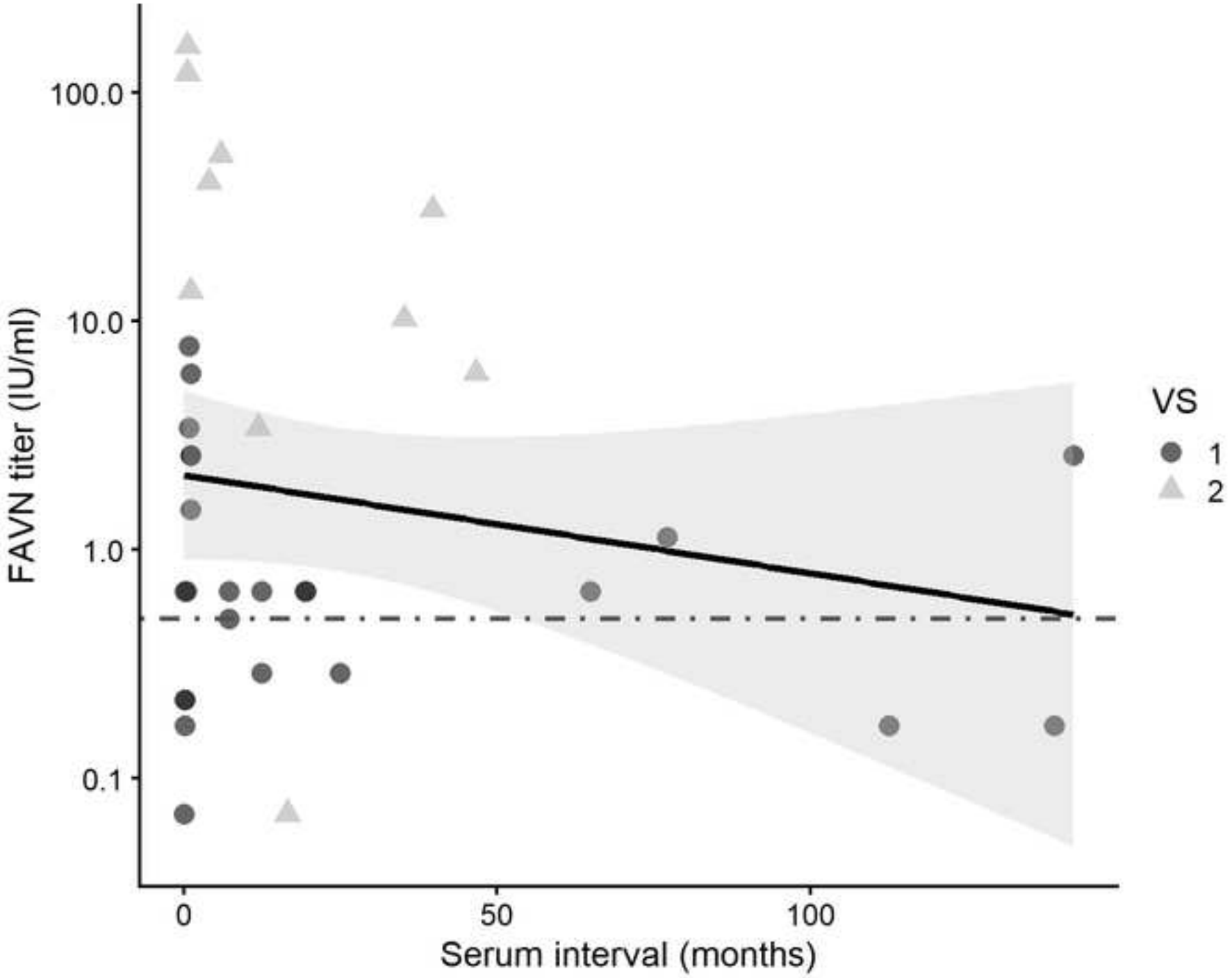
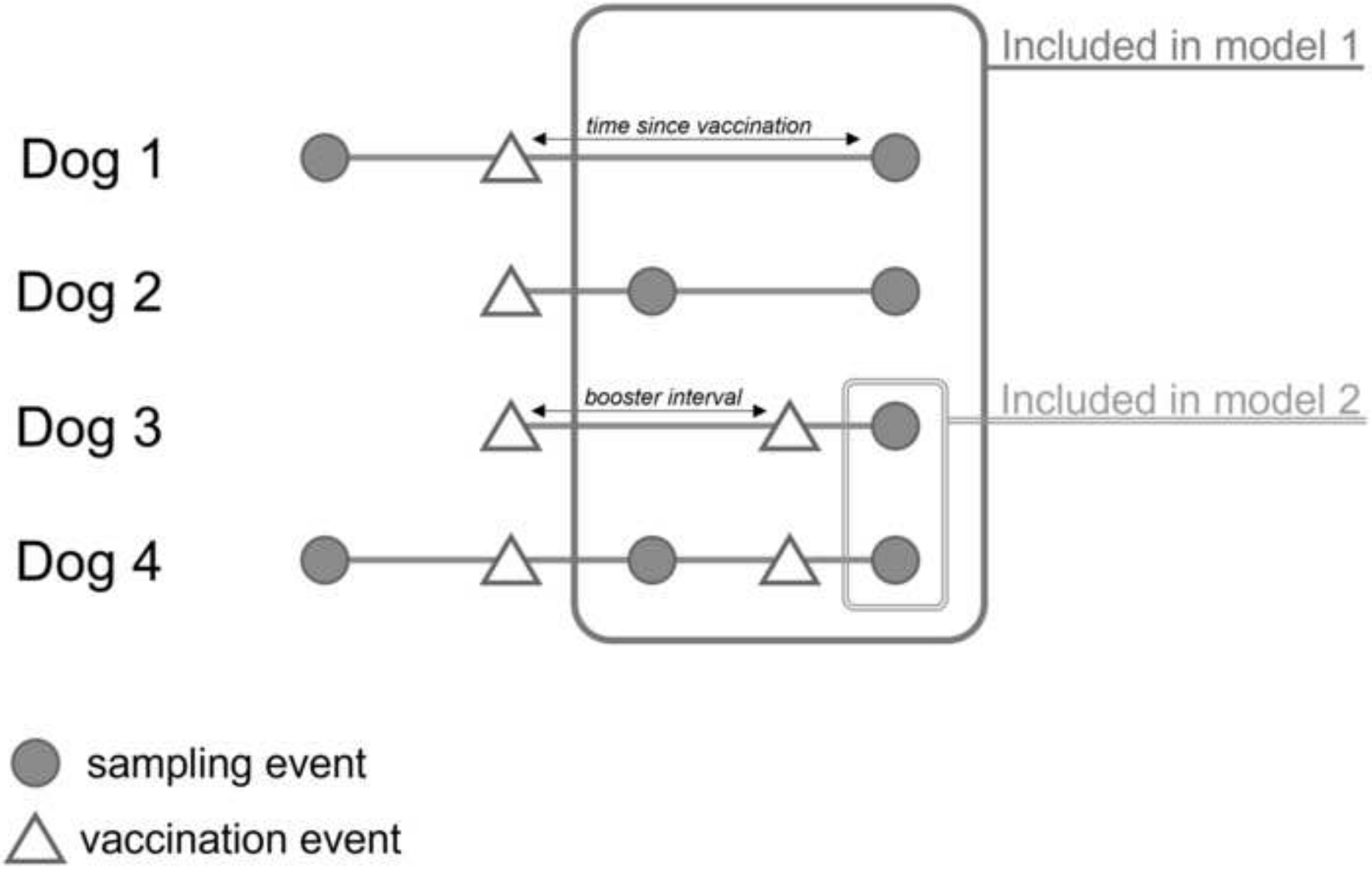


Figure S1



SUPPLEMENTAL MATERIAL

Table S1. Overview of individual data, vaccination information and serum sampling schedule for African wild dogs (*Lycaon pictus*) included in a study investigating the effect of booster vaccination on rabies antibody titers. Blood samples were collected opportunistically from captive individuals at Port Lympne Reserve, UK, both prior to and following rabies vaccination, spanning the years 2001 to 2019. # = sample number; ID = individual identifier (AWD = African wild dog); Age (y) = Age in years at time of most recent vaccination; VS = Vaccination status at time of sample collection (0 = no vaccinations received; 1 = one vaccination received; 2 = two vaccinations received); SI (mo) = Serum interval in months, time between most recent vaccination and sample collection, if applicable; BI (mo) = Booster interval in months, time between penultimate and most recent vaccination in months, if applicable; Vaccine = Type of vaccine received (CG = Canigen® Rabies; RS = Rabisin®).

#	ID	Age (y)	Sex	VS	SI (mo)	BI (mo)	Vaccine
1	AWD 1	4.99	m	1	1.05	-	CG
2	AWD 2	4.99	m	1	1.05	-	CG
3	AWD 3	-	m	0	-	-	-
4	AWD 3	6.99	m	2	1.05	6.05	CG
5	AWD 4	4.99	f	1	1.05	-	CG
6	AWD 5	10.82	m	2	11.67	22.03	RS
7	AWD 6	-	f	0	-	-	-
8	AWD 6	-	f	0	-	-	-
9	AWD 6	4.48	f	1	7.10	-	CG
10	AWD 7	-	m	0	-	-	-

#	ID	Age (y)	Sex	VS	SI (mo)	BI (mo)	Vaccine
11	AWD 7	4.12	m	1	86.07	-	RS
12	AWD 8	-	f	0	-	-	-
13	AWD 8	2.20	f	1	0.03	-	RS
14	AWD 8	2.20	f	1	0.83	-	RS
15	AWD 9	-	m	0	-	-	-
16	AWD 9	3.79	m	1	12.20	-	RS
17	AWD 9	4.80	m	2	0.50	12.20	RS
18	AWD 9	4.80	m	2	3.91	12.20	RS
19	AWD 10	-	f	0	-	-	-
20	AWD 10	3.29	f	1	0.82	-	RS
21	AWD 11	5.41	f	1	11.2	-	RS
22	AWD 11	9.49	f	2	39.33	48.97	RS
23	AWD 12	0.23	f	1	0.20	-	RS
24	AWD 13	0.70	f	1	7.16	-	RS
25	AWD 13	0.70	f	1	19.04	-	RS
26	AWD 13	0.70	f	1	19.20	-	RS
27	AWD 13	2.51	f	2	14.30	21.86	RS
28	AWD 14	-	m	0	-	-	-
29	AWD 14	6.42	m	1	69.37	-	RS
30	AWD 15	2.23	m	1	110.99	-	RS
31	AWD 16	-	m	0	-	-	-
32	AWD 16	3.79	m	1	12.20	-	RS
33	AWD 16	4.81	m	2	0.50	12.20	RS
34	AWD 16	4.81	m	2	3.91	12.20	RS

#	ID	Age (y)	Sex	VS	SI (mo)	BI (mo)	Vaccine
35	AWD 17	-	m	0	-	-	-
36	AWD 17	5.00	m	1	1.05	-	CG
37	AWD 18	0.23	m	1	0.20	-	RS
38	AWD 18	0.23	m	1	0.40	-	RS
39	AWD 19	3.77	f	1	24.53	-	RS
40	AWD 19	5.82	f	2	5.82	24.60	RS
41	AWD 19	5.82	f	2	34.65	24.60	RS
42	AWD 19	5.82	f	2	46.03	24.60	RS
43	AWD 20	-	f	0	-	-	-
44	AWD 20	0.7	f	1	7.17	-	RS
45	AWD 21	-	f	0	-	-	-
46	AWD 21	0.23	f	1	0.20	-	RS
47	AWD 22	0.23	f	1	0.20	-	RS

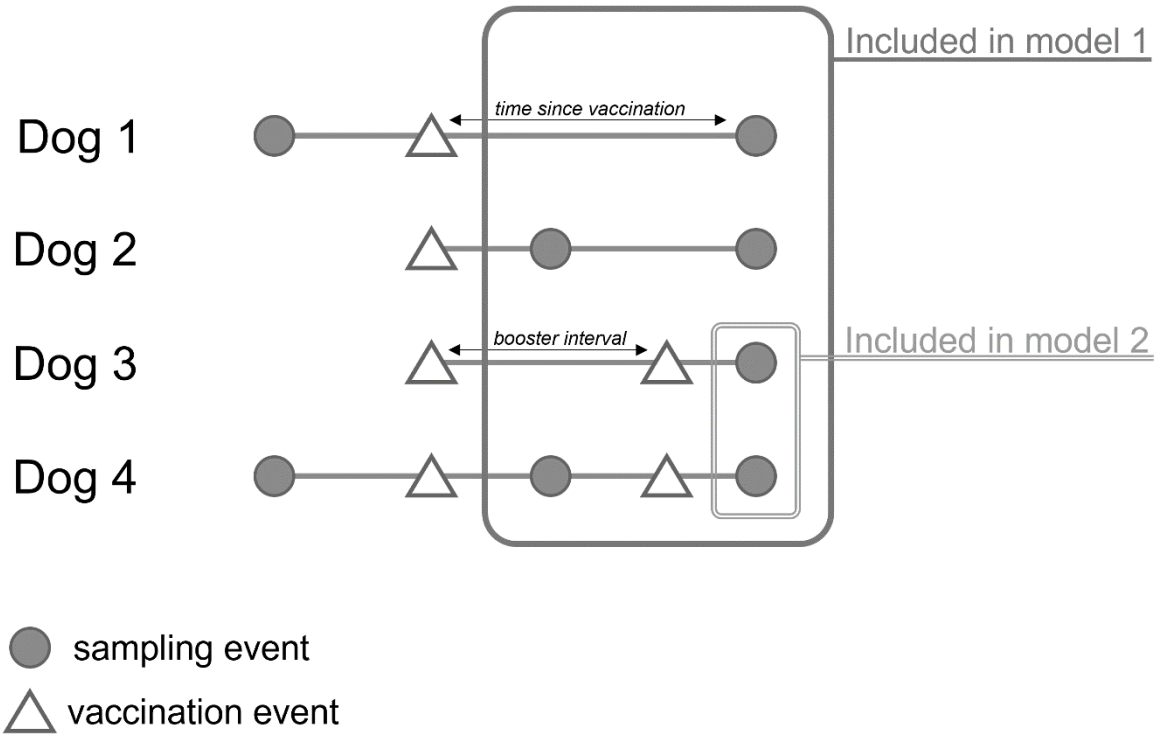


Figure S1. Schematic representation of samples included in multivariable models 1 and 2, analyzing the association between rabies antibody levels in blood samples of African wild dogs (*Lycaon pictus*) that were opportunistically collected from captive individuals at Port Lympne Reserve, UK between 2001 and 2019 following rabies vaccination, and the relevant predictor factors. While multivariable model 1 included all samples taken after vaccination, multivariable model 2 included only a subset of these samples, specifically those taken after booster vaccination. Note that the labels "Dog 1" through "Dog 4" denote example individuals, distinct from the identifiers "AWD 1" through "AWD 4" presented in table S1.

Univariable analysis

Assuming a significance threshold of $p < 0.2$ for univariable analyses, all variables except for sex were significantly associated with rabies antibody titers (table S2). FAVN values were found to be positively associated with age (coefficient (SE) = 0.40 (0.11), $p < 0.001$). The interval between vaccination and sample collection was negatively associated with titers (coefficient (SE) = -0.02 (0.01), $p = 0.01$), as was time between a first and second vaccination (coefficient (SE) = -0.08 (0.03), $p = 0.01$). Individuals vaccinated with Rabisin® were found to have lower antibody titers than those receiving Canigen® rabies (Rabisin®: coefficient (SE) = -1.66 (0.70), $p = 0.02$).

Association of vaccination status and rabies antibody titers

Both a single and a booster vaccination were significantly associated with measured rabies antibody titers ($p < 0.001$ for both analyses, table S2). The results of Tukey's multiple comparison of means test are shown in table S3. Significantly higher log-titers were observed in samples obtained from wild dogs that received a single vaccination compared to samples collected from unvaccinated individuals (coefficient (SE) = 1.56 (038), $p < 0.001$). Additionally, log-titers measured in samples collected after booster vaccination were significantly higher than those collected after a single vaccination (coefficient (SE) = 2.91 (0.59), $p < 0.001$). In comparison to rabies antibodies titers measured in unvaccinated wild dogs, booster vaccination was associated with log-titers more than twice as high as those observed after a single vaccination (coefficient (SE) = 5.00 (0.63), $p < 0.001$).

Table S2. Results of the univariable analysis investigating the association between rabies antibody titers measured in African wild dog (*Lycaon pictus*) blood samples and relevant predictor variables. Samples were collected opportunistically from captive individuals at Port Lympne Reserve, UK, both prior to and after rabies vaccination, during the period spanning from 2001 to 2019. Log (TI) = FAVN titer results in IU/ml, natural log transformed; Vaccination status = Vaccination status at time of sample collection (0 = no vaccinations received; 1 = one vaccination received; 2 = two vaccinations received); Age = Age in years at time of most recent vaccination; Serum interval = Time between most recent vaccination and sample collection, in months; if applicable; Booster interval = Time between penultimate and most recent vaccination, in months; if applicable; Vaccine type = Type of Vaccine received (Canigen® Rabies; Rabisin®); significance code < 0.2 = *; 95 % CI = 95% Confidence interval: lower – upper.

			Estimate	**95 % CI**	**p – value**
Log	Vaccination status	1[1]	1.57	1.20 – 1.96	2.7e-05*
(TI)		2[1]	4.50	3.85 – 5.12	9.8e-13*
	Age		0.40	0.29 – 0.52	4.3e-04*
	Sex	Male[2]	0.71	0.08 – 1.34	0.26
	Serum interval		-0.02	-0.03 - -0.01	0.01*
	Booster interval		-0.08	-0.11 - -0.05	0.01*
	Vaccine type	Rabisin®[3]	-1.66	-2.36 - -0.96	0.02*

[1] Reference: Vaccination status = 0
[2] Reference: Female
[3] Reference: Canigen® Rabies

Table S3. Results of Tukey's multiple comparison of means test, run on the univariable model analyzing the association between rabies antibody titers in the blood of African wild dogs (*Lycaon pictus*) and rabies vaccination status (0 = no vaccination received, 1 = one vaccination received, 2 = two vaccinations received). Blood was collected opportunistically between 2001 and 2019 from captive individuals kept at Port Lympne Reserve, UK.

Vaccination status	**Estimate**	**Standard error**	**z value**	**p – value**
1 vs. 0	1.56	0.38	4.20	2.7e-05
2 vs. 0	5.00	0.63	7.13	9.8e-13
2 vs. 1	2.91	0.59	4.96	6.9e-07

Table S4. Complete results of the multivariable model analyzing the association between rabies antibody titers in African wild dog (*Lycaon pictus*) blood samples taken after rabies vaccination and relevant predictor variables. Samples were collected opportunistically from captive individuals at Port Lympne Reserve, UK, between the years 2001 and 2019. As booster interval was only relevant to samples taken after a second vaccination, the influence of this variable on antibody titers had to be evaluated separately from vaccination status. Log (TI) = FAVN titer results in IU/ml, natural log transformed; Vaccination status = Vaccination status at time of sample collection (1 = one vaccination received; 2 = two vaccinations received); Age = Age in years at time of most recent vaccination; Serum interval = Time between most recent vaccination and sample collection, in months; if applicable; Booster interval = Time between penultimate and most recent vaccination, in months; if applicable; Vaccine type = Type of Vaccine received (Canigen® Rabies; Rabisin®); significance code < 0.05 = *; 95 % CI = 95% Confidence interval: lower – upper.

			Estimate	**95 % CI**	**p – value**
Model 1	Vaccination status	2[1]	2.57	1.46 – 3.68	0.02*
Log (TI) after	Age		0.16	-0.03 – 0.36	0.41
vaccination	Serum interval		-0.02	-0.03 – -0.01	0.05*
n = 35	Vaccine type	Rabisin®[2]	-1.40	-2.30 – 0.50	0.13
Model 2	Age		0.31	-0.20 – 0.81	0.54
Log (TI) after	Serum interval		-0.05	-0.06 – -0.04	1.1e-08*
booster	Booster interval		-0.07	-0.14 – 1.91e-03	0.33
vaccination	Vaccine type	Rabisin®[2]	1.56	0.40 - 2.72	0.33
n = 11					

[1] Reference: Vaccination status = 1
[2] Reference: Canigen® Rabies