Developmental and Comparative Immunology 114 (2021) 103841

Contents lists available at ScienceDirect

Developmental and Comparative Immunology

journal homepage: www.elsevier.com/locate/devcompimm

Perspective

# Retrospective anti-tetanus antibody responses of zoo-based Asian elephants *(Elephas maximus)* and rhinoceros (*Rhinocerotidae*)

Yasmine Sophia Sierra Muir [a], Benn Bryant [b], Michelle Campbell-Ward [b], Damien P. Higgins [a,*]

[a] Sydney School of Veterinary Science, The University of Sydney, Camperdown, 2006, NSW, Australia
[b] Taronga Western Plains Zoo, Obley Rd, Dubbo, 2830, NSW, Australia

ARTICLE INFO

*Keywords:*
Antibody
Elephant
ELISA
Rhinoceros
Tetanus toxoid

ABSTRACT

Tetanus toxoids (TT) commercially available for use in horses and livestock are commonly used to vaccinate elephants and rhinoceros that are in human care. Although recommendations for booster intervals have changed in human and horse protocols to reduce the risks associated with hyper-immunity (i.e. B-cell anergy and hypersensitivity reactions) these have generally not been adopted in zoo protocols. Additionally, there is no evidence to demonstrate commercial TT immunogenicity in rhinoceros. In this study, a preliminary analysis of rhinoceros antibody responses to TT was conducted, in addition to an exploration of the impact of various booster frequencies on antibody responses in elephant. Retrospective analysis of archived serum samples was conducted for 9 Asian elephants *(Elephas maximus)*, 7 southern black *(Diceros bicornis minor)*, one southern white *(Ceratotherium simum simum)*, and two greater one-horned *(Rhinoceros unicornis)* rhinoceros. Pre-vaccination (baseline) samples and those following priming vaccination (rhinoceros only), annual and non-annual boosters were targeted. A commercially available competitive ELISA kit was used to quantify serum anti-TT antibodies. Average baseline and post-vaccination anti-tetanus antibody concentrations were greater in elephant (92 mg/L ± 42, n = 3, N = 3; 125 ± 76, n = 82, N = 9) than in rhinoceros (47 mg/L ± 39, n = 8, N = 8; 44 mg/L ± 37, n = 16, N = 7). Rhinoceros antibody concentrations did not differ markedly following vaccinations from their naturally acquired high pre-vaccination concentrations. Eight elephants demonstrated antibody maintenance for 3–5 years without a tetanus booster. Additionally, although five out of nine elephants developed local reactions consistent with delayed type IV hypersensitivity following some boosters, there was no association between high antibody concentrations and increased incidence of adverse reactions. In addition, no decrease in antibody concentrations was detected as a result of annual vaccination in elephants, though this does not entirely rule out potential for B-cell anergy.

## 1. Introduction

Elephants and rhinoceros suffer from tetanus with varying degrees of susceptibility (Steel, 1885; Goss, 1942; Gupta, 1945; McGaughey, 1962; Burke, 1975; Rookmaaker et al., 1998; Hoar, 2017). For this reason, it is common practice in zoos to use tetanus toxoid (TT) vaccinations to protect Asian elephants *(Elephas maximus)* and rhinoceros species against the disease (Schmidt, 1986; Pye, 2005; Stevenson and Walter, 2006; Lindsay et al., 2010). It is important that current vaccination protocols are investigated to observe the effect of different intervals between vaccination on antibody responses to optimise vaccination protocols. A lethal case of suspected tetanus in a young greater-one-horned rhinoceros *(Rhinoceros unicornis)* in a regional zoo in Australia supports the presence of *Clostridium tetani* in the environment and the need for vaccination. While some published data exists on TT vaccination responses in elephants (Lindsay et al., 2010; Natalia et al., 2011), there remain questions around safety and efficacy; a review of clinical histories in one urban Australian zoo revealed recurring vaccination site reactions in Asian elephants (*unpublished data*). Increased hypersensitive reactions and a risk of B cell anergy have been associated with frequent antigen stimulation using both monovalent and polyvalent TT vaccines in humans (Peebles et al., 1969; Nanan et al., 2001; Cambier et al., 2007; Wassilak et al., 2008; Andrews and Wilson, 2010; Gowin et al., 2016; Hammarlund et al., 2016). Tetanus vaccination regimes used in elephant and rhinoceros are based on recommendations for horses and other hoof stock (Lindsay et al., 2010). Recently, changes

* Corresponding author.
*E-mail address:* damien.higgins@sydney.edu.au (D.P. Higgins).

https://doi.org/10.1016/j.dci.2020.103841
Received 23 June 2020; Received in revised form 19 August 2020; Accepted 19 August 2020
Available online 27 August 2020

have been made to both human and equine TT vaccination regime recommendations, to decrease the frequency of boosters and reduce risks associated with hyper-immunity (Gowin et al., 2016; Hammarlund et al., 2016; Kendall et al., 2016).

The induction of hyper-immunity through over-vaccination has multiple potential consequences. Over-vaccination is known to cause hypersensitive reactions in humans (Kuhns, 1962; Edsall et al., 1967; Facktor et al., 1973; Mayorga et al., 2003). Adverse reactions to TT vaccination in Asian elephants could increase the risk of stress-induced shedding of elephant endotheliotropic herpesvirus by asymptomatic animals (Bennett et al., 2015), exposing susceptible herdmates to potentially fatal disease. Another potential impact of over-vaccination is the induction of anergy by over-stimulation of the immune response. Anamnestic responses to TT vaccination have been demonstrated in Asian elephant however, further investigation of non-annual vaccinations is warranted (Lindsay et al., 2010; Natalia et al., 2011).

Alternatively, over-vaccination could reduce the overall quality of immune responses to TT boosters by impeding memory B cells, leaving animals susceptible to disease, with or without high concentrations of circulating antibodies. Effective immunological responses to tetanus antigens involve both B-lymphocyte activity and peripheral plasma cell antibody populations (Nanan et al., 2001). Decreased activity of B cell populations has been observed with increased booster administration in humans and mice (Nanan et al., 2001; Cambier et al., 2007; Andrews and Wilson, 2010). Independent regulation of antibody producing plasma cells and memory B cells has yet to be observed in Asian elephants. An initial analysis of antibody concentration following frequent vaccination may provide insight into elephant adaptive immunological pathways.

In this retrospective pilot study, archived serum samples from Asian elephant and three species of rhinoceros were assayed to investigate antibody responses to tetanus vaccination. Using a commercial anti-tetanus-toxoid competitive ELISA kit, species-independent examination of sera is possible as species-specific secondary antibodies are not required (Fischer-Tenhagen et al., 2000). The protective capacity of resultant antibody concentrations cannot be determined without toxin neutralisation tests or other challenge tests, which were beyond the scope of this study. Nonetheless, this investigation will be the first to explore the antibody responses of southern black *(Diceros bicornis minor)*, southern white *(Ceratotherium simum simum)*, and greater-one-horned *(Rhinoceros unicornis)* rhinoceros to tetanus vaccinations. This will determine whether vaccinations in rhinoceros will produce similar increases in antibody concentrations to those observed in elephants. Antibody concentrations will also be compared between elephants with annual boosters and elephants with longer intervals (2–6 years) between boosters to identify the optimal frequency of booster vaccination. Antibody concentrations from elephants with and without a history of adverse reaction will also be compared to determine if high antibody concentrations are associated with an increased risk of hypersensitive reactions as observed in humans (Edsall et al., 1967; Peebles et al., 1969; Gowin et al., 2016; Hammarlund et al., 2016).

## 2. Materials and methods

### 2.1. Selection of archived samples

Six elephants and 10 rhinoceros housed in a regional Australian open range zoo and three elephants housed in an urban Australian zoo were included. The vaccination histories of these animals were retrieved from records kept in the ZIMS. Species 360© database and reviewed. Vaccination timelines generated from this data are provided in Figs. 2 and 3. Histories were used to identify the class of each vaccination administered, e.g. two-dose priming, first, second or third individual booster. Using doses recommended for horses and cattle, rhinoceros and elephants were mostly administered monovalent TT vaccines (1 ml dose, Zoetis, Rhodes, New South Wales, Australia), with some use of a polyvalent '8 in 1' clostridial vaccine in rhinoceros (5 ml dose, Coopers Animal Health, Bendigo East, Victoria, Australia). Serum samples corresponding to the following time points were selected from archives, when available: baseline samples (pre-vaccination) were targeted for elephants (n = 3, N = 2) and rhinoceros (n = 8, N = 8); samples following priming courses in rhinoceros (n = 16, N = 7); and samples following annual boosters and non-annual boosters in elephants (n = 82, N = 9). Where 'n' refers to the number of individual samples from the number of sampled individuals 'N'. Demographics of the elephants and rhinoceros used in this study, as well as the number of samples targeted in comparison to the number of samples obtained are summarised in Table S1. To avoid sample bias, cases of adverse reactions following toxoid administration were not identified in the elephant study population until analysis was completed (Borrow et al., 2006). Separated serum samples had been stored at −80 °C and, following extraction from the archive, were transported without thawing to −80 °C freezers to await analysis. Duration in archived storage ranged from 1 month to 14 years.

### 2.2. Competitive ELISA (cELISA)

Concentrations of serum antibody against TT were determined using a commercial anti-tetanus antibody (TTAb) competitive enzyme-linked immunosorbent assay kit (cELISA) (MBS726075, MyBiosource, San Diego, California, USA) according to the manufacturer's directions. Briefly, three 96 strip-well microtitre plates came pre-coated with TT to which 100 μL of diluted samples (1:4) were applied. The dilution chosen (1:4) was selected to allow for accurate quantification across the greatest possible range of antibody concentrations in samples (Appendix, Fig. S1) and was determined using neat, 1:2, and 1:4 dilutions of predicted high, medium, and low antibody concentrations from a single elephant, and four individual rhinoceros, as well as four positive standard controls of tetanus antibody purified from human sera (0, 25, 50 & 100 mg/L).

Standard tetanus antitoxin controls were applied to each test plate at 0, 5, 10, 25, 50 & 100 mg/L to generate a standard curve for the interpolation of sample antibody concentrations. Tetanus antibody conjugated to horse-radish-peroxidase (HRP) was used as a reference for competitive binding. Blank samples, consisting of 100 μL PBS (pH 7.0–7.2), were included to detect background signals. Hydrogen peroxidase ($H_2O_2$) substrate for HRP was used with 3,3′,5,5' Tetramethylbenzidine (TMB) to generate a colorimetric reaction, which was stopped with 50 μL sulfuric acid. Optical densities ($A_{450}$) were measured using a microplate reader (Dynamica HALO LED 96). Selected samples with unexpected antibody concentrations, such as non-vaccine associated rises in antibody concentrations, or those above the limit of quantification were repeated at a higher dilution (1:6) for more accurate interpretation. Serum from a dog and cat of unknown origins or medical history (but expected to not have been vaccinated against tetanus) were also included to compare with rhinoceros and elephant average baseline concentrations. Assay sensitivity was 1.0 mg/L and the detection range included antibody concentrations within 5–100 mg/L. The intra-assay coefficient of variation (CV) for each cELISA completed, as calculated by obtaining the mean CV% of all measured samples (SD of replicates/mean of replicates), did not exceed 8%.

## 3. Results

### 3.1. Rhinoceros antibody responses to TT

Antibody concentrations in all rhinoceros samples, including pre-vaccination (baseline) samples, exhibited concentrations above the human minimum protective titre 0.17 mg/L (0.01 IU/ml) (Borrow et al., 2006). Mean rhinoceros baseline antibody concentrations (47 mg/L ± 39; n = 8, N = 8) were smaller than those observed in the elephant samples in this study (92 mg/L ± 42; n = 3, N = 2) (Fig. 1) but were similar to average post-vaccination antibody concentrations (44 mg/L

± 37; n = 16, N = 7) (Fig. 1). Within individual rhinoceros, the post-vaccination deviation of antibody concentrations from baseline values ranged from 0.4 to 1.8-fold (Figs. 1 and 2). This was in contrast to the one elephant for which a baseline and post-two-dose priming course sample were available (4-fold increase, elephant 9, Fig. 3I). No effect of age, sub-species, gender or vaccination type on antibody concentrations could be observed (Appendix, Figs. S2, S3, S4, S5).

The available sample set was fragmented by irregularities in vaccine administration and serum sampling, dividing animals into those that received a single vaccination (Fig. 2B), an initial two-dose priming course (Fig. 2A, D), two individual boosters (Fig. 2B, E, F, G), a two-dose priming course with a booster (Fig. 2C), three individual boosters (Fig. 2D and E), and four individual boosters (Fig. 2E, G). Antibody responses varied within these groups. Rhinoceros 5 and 6 exhibited maintenance of antibody concentrations for multiple years between vaccinations (Fig. 2E and F). Rhinoceros 4 (Fig. 2D) demonstrated markedly higher antibody concentrations before and after vaccination than all other study rhinoceros. Clinical histories for rhinoceros 4 detailed multiple cases of 'fissures over solar surfaces of all feet', face, horn, and ears, 'contaminated wounds' with 'superficial infections' and 'necrotic tissue'.

### 3.2. Comparison of elephant antibody responses to annual administration and non-annual administrations of TT

Little difference in antibody maintenance was observed between annually administered vaccinations and those administered at reduced frequencies. Of the nine elephants, six had received annual boosters for 4–5 consecutive years. These elephants maintained antibody concentrations throughout this period with minor (elephants 1, 4 & 5; Fig. 3A, D and E) or marked fluctuations (elephants 6, 7 & 8; Fig. 3F, G, and H).

All elephants in this study experienced a period without vaccination ranging from 2 to 6 years (Fig. 5). There were no clear differences in antibody concentrations throughout this period, relative to those observed following annual vaccination. Elephants 1, 2, & 4–6 demonstrated steady maintenance of antibody concentrations for up to 3–5 years without any additional boosters (Fig. 5). Greater fluctuations in antibody concentrations were observed in elephants 7–9 in this period without boosters (Fig. 5). Although variable, these concentrations remained above those maintained in elephants 1, 2, & 4–6. Concentrations decreased around the 3-5-year mark depending on the animal.

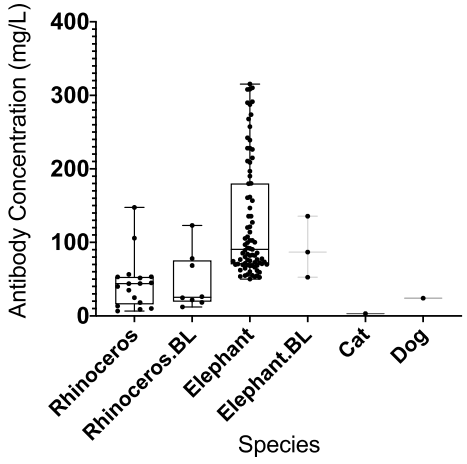


**Fig. 1.** Comparison of baseline (BL) and post-vaccination antibody concentrations (mg/L) from rhinoceros (n = 17, N = 8) and elephant (n = 82, N = 3) with additional cat (n = 1) and dog samples (n = 1) for reference. Raw data points are shown against box plots exhibiting median and 95% interquartiles, with minimum and maximum values where applicable.

### 3.3. Comparison of elephant antibody responses from individuals with histories of adverse reaction and those without

There was no evidence of association between high anti-tetanus antibody concentrations and increased incidence of adverse reaction. Of the 9 elephants, 5 had at least one case of adverse reaction following a TT vaccination (Fig. 3D, E, F, G, H). Clinical records of adverse reactions following vaccination were the same for elephants 4, 5 & 6, detailing an 18 cm "rockmelon sized" swelling at the site of vaccination, which increased to nearly double the size ("soccer ball") three days later. These three elephants reacted to the same toxoid administration which represented their 5th or 6th vaccinations. Elephant 7 had the most extensive history of adverse reaction to TT's. This animal demonstrated difficulty laying down, varying degrees of stiffness and lameness, and swelling of varying diameters lasting up to a month following vaccination. Elephant 7 adversely reacted to all tetanus vaccinations apart from the last, and elephant 8 reacted to two of the same vaccinations given to elephant 7 and matched clinical signs. Only elephants 7 & 8 demonstrated high antibody concentrations along with hypersensitive-like symptoms (2/5) (Fig. 4). Furthermore, elephant 9 had elevated antibody concentrations but had no history of adverse reactions to the toxoid (Fig. 4). Therefore, there was no clear evidence to illustrate that high antibody concentrations were associated with adverse vaccination reactions.

## 4. Discussion

This study demonstrates variability in antibody concentrations and responses to tetanus vaccinations in the elephants and rhinoceros studied. This first investigation of rhinoceros anti-tetanus antibody concentrations provides no evidence to show that either monovalent or polyvalent vaccinations stimulate expected post-vaccination rises in antibody concentrations beyond already elevated baseline concentrations. In addition, this study provides no evidence to suggest any reduction in antibody concentrations following subsequent annual vaccinations in elephants. Maintenance of antibody concentrations in study elephants with greater than annual vaccination intervals are supportive of previous work that found successful maintenance of antibody concentrations following a 4-year interval (Lindsay et al., 2010). Although the majority of elephants used in this study had experienced possible hypersensitive adverse reactions following vaccination, association with high antibody concentration was not evident.

This study provides evidence for natural development of anti-tetanus antibodies in rhinoceros, to the extent that they appear to be inhibiting vaccination responses. This same phenomenon is observed when maternal antibodies are still present at the time of vaccination (Maselle et al., 1991; Borrow et al., 2006). In humans, a 4-fold increase in antibody concentrations following vaccination is considered to represent successful stimulation by TT (Zaccaro et al., 2013). However, this is mostly used to define responses from a naïve population. As the rhinoceros antibody concentrations suggests a degree of prior exposure to tetanus antigens and no four-fold increase in antibody concentrations, it is difficult to determine the quality of vaccination responses. There is strong evidence to support the presence of *C. tetani* within the environment in which these rhinoceros reside. A suspected case of lethal tetanus in a greater one-horned rhinoceros enclosure-mate of study animals suggests the environmental presence of the organism. Evidence to support the presence of environmental challenge is observed in the antibody concentrations of rhinoceros 5, which suddenly increased without vaccination (Fig. 3E) in one instance. Furthermore, the high antibody concentrations from rhinoceros 4 were associated with chronic foot disease and injury. Such injuries support optimal anaerobic environment for this bacterium and could generate small antigenic challenges over the animal's lifetime (Cook et al., 2001; Ribeiro et al., 2018). As hypothesised in other investigations, naturally acquired anti-TT antibodies could potentially develop through exposure within gastrointestinal (GI) tracts (Matzkin and Regev, 1985; Veronesi et al., 1975;

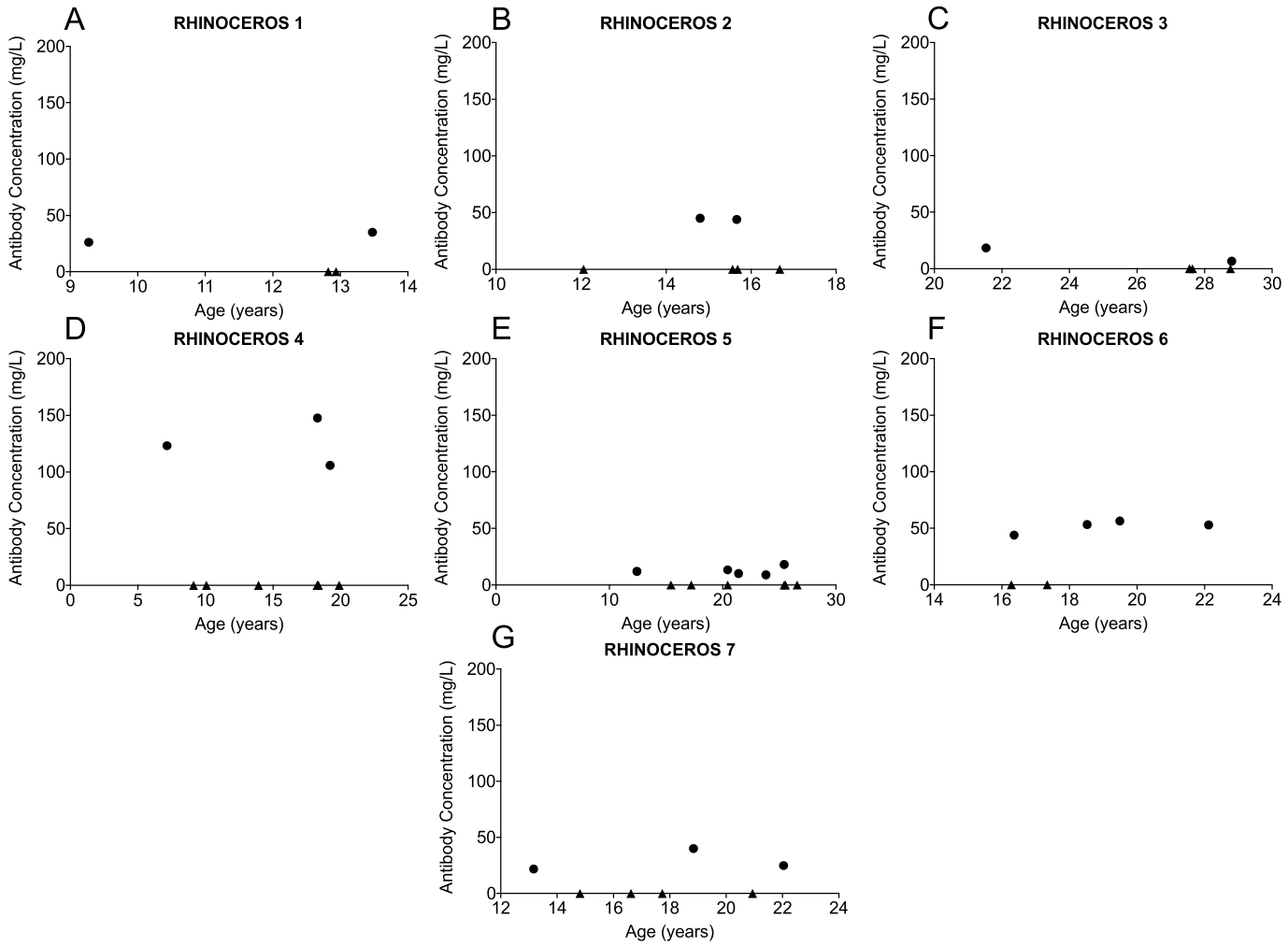


**Fig. 2.** Individual rhinoceros vaccination timelines (years) with associated serum sample anti-tetanus antibody concentrations (mg/L). Points (●) represent serum sample antibody concentrations (mg/L) and (▲) represent vaccination events. Graphs A-G represent rhinoceros 1–7 respectively.

Pichichero, 2008). Environmental loads of *C. tetani* may prime the immune system at a young age and climatic changes, which alter the abundance of this bacteria, may be responsible for the observed fluctuations in antibody concentrations without vaccination (Matzkin and Regev, 1985). However, the potential impact of *C. tetani* within the GI tract on anti-TT antibody production has yet to be explored. These high antibody concentrations in baseline samples are less likely to result from inaccurate measurement. Non-specific binding detected in both blank control wells and TTAb negative cat serum were below quantifiable limits, eliminating effects of methodological artefacts. Dog serum did have quantifiable concentrations of anti-TT antibodies, however, without any medical histories it is possible that this animal may have been exposed to tetanus antigens within the environment or its diet. If baseline antibody concentrations from non-related-species are required for future research, chicken serum is suggested as a better alternative as TT is not routinely provided due to greater resistance in avian species (Muir et al., 2002).

To increase the sample size available for further research, zoological facilities should be encouraged to collect serum samples before a primary TT vaccination and before each subsequent booster. With this, stronger evidence may be provided for differing immune responses to tetanus vaccinations between different species and age cohorts of rhinoceros. Although this study lacked in sample size, the preliminary data revealed lower black rhinoceros (n = 5) average antibody concentrations in comparison to those seen in greater-one-horned (n = 2) rhinoceros; suggestive enough to warrant further examination. No meaningful comparison could be made between these species and white rhinoceros as only one white rhinoceros was included in this study. Of the limited available information on rhinoceros immunology, literature suggests that variations in immune capabilities and function exist, particularly between captive black and white rhinoceros, and their free-ranging conspecifics (Van Heerden et al., 1985; Kock et al., 1990). These studies demonstrated higher average serum glucose, cortisol and lactate dehydrogenase concentrations in the captive black rhinoceros in comparison to the white (Van Heerden et al., 1985; Kock et al., 1990). These chemical parameters are associated with acute and chronic stress, and therefore have the potential impact to reflect immune function (Kock et al., 1990). Further studies to determine if the black rhinoceros from this study are experiencing chronic stress and immunosuppression could be undertaken by monitoring these chemical parameters.

Attenuated immune responses are also evident in elephants with pre-existing high antibody concentrations from natural toxin exposure or frequent vaccination. Two pre-vaccination samples taken one year apart were obtained in elephant 7. A 1.5-fold increase suggestive of natural exposure was observed within that period. No marked increase was seen following the animals first vaccination at 15-years-old in the presence of this pre-existing rise in anti-tetanus antibody concentration. In comparison, elephant 9 was 8-months-old at first vaccination, and was the only elephant observed to have produced a 4-fold increase in antibody concentrations. As the sample was taken 15 days post vaccination, it is possible that the level of this initial response could have increased further, as peak responses have been identified one month following

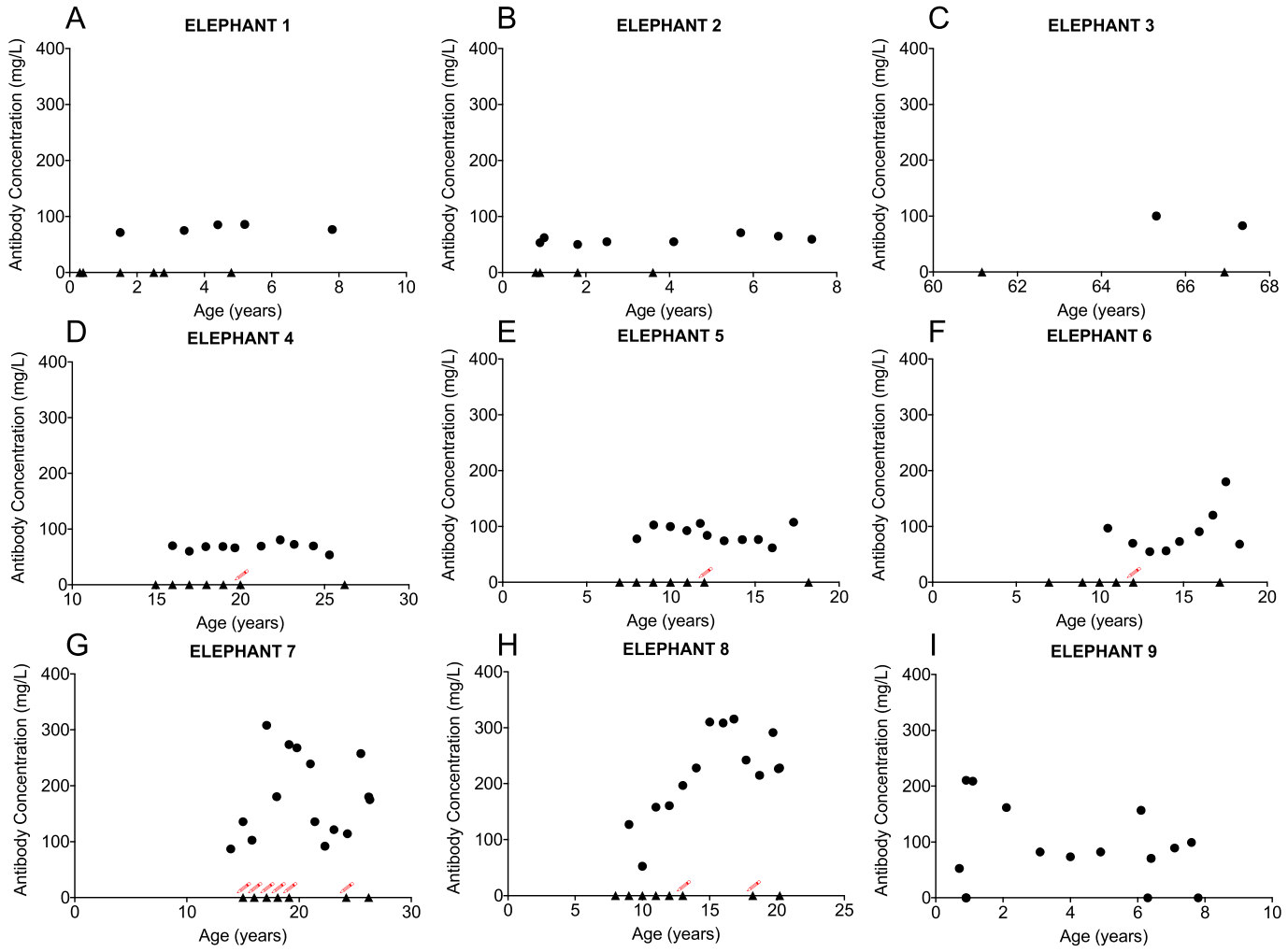


**Fig. 3.** Individual elephant vaccination timelines (years) with associated serum sample anti-tetanus antibody concentrations (mg/L). Points (●) represent serum sample antibody concentrations (mg/L) and (▲) represent vaccination events. (★) represents adverse reactions to vaccine administration. Graphs A-I represent elephants 1–9 respectively.

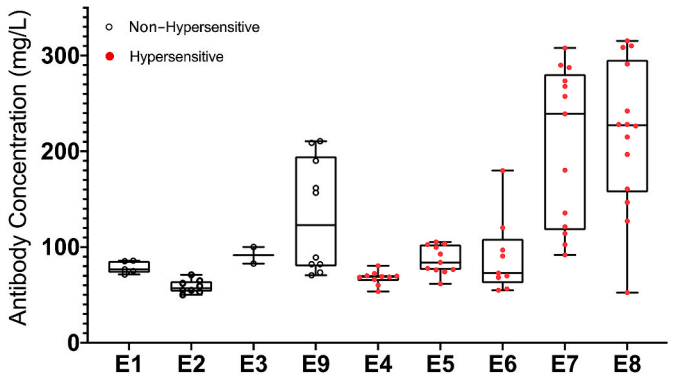


**Fig. 4.** Comparison of post-vaccination anti-tetanus antibody concentrations (mg/L) from elephants with record of hypersensitive reaction(s) following TT administration (●) (N = 5) and elephants with no history of hypersensitivity (○) (N = 4). Each box and whisker plot demonstrates the smallest and largest values, lower and upper quartiles, and median value.

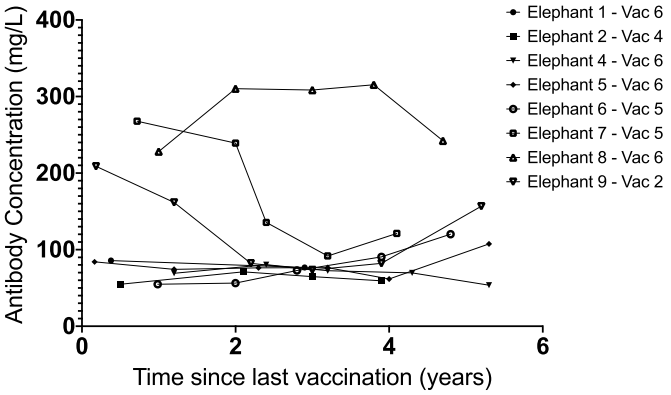


**Fig. 5.** Antibody concentrations (mg/L) within serum samples of elephants taken over a period of 2–6 years after their last vaccination. Elephant 3 was excluded for lack of appropriate samples. Adjacent to each elephant in the key details after which vaccination (Vac) this period occurred.

vaccination (Lindsay et al., 2010; Natalia et al., 2011). Attenuated antibody responses to tetanus vaccination in humans with high initial concentrations have been documented (Peebles et al., 1969; Aggerbeck et al., 1997; Olander et al., 2001; Danilova et al., 2005). Furthermore, studies indicate that increasing the number of doses of TT does not continuously increase concentration of antibodies (Peebles et al., 1969; Danilova et al., 2005). Essentially, when the maximum response level is reached, additional doses will not induce any further increase in

antibodies (Natalia et al., 2011). It is important that further studies attempt to determine what is considered to be a protective antibody concentration in elephants so that unnecessary vaccinations can be avoided, stronger responses stimulated and the risk of hypersensitivity reactions reduced.

This study supports somewhat the reduction of tetanus vaccination frequencies in elephants. Lindsay et al. (2010) used a controlled experimental design and a larger sample size to demonstrate antibody maintenance for up to 4 years in 90% (20/22) of elephants investigated. All eight elephants demonstrated this in the current study. It is possible that the marked fluctuation in the three elephants (7, 8, & 9) with extremely high antibody concentrations was an artefact of concentrations lying close to the upper limit of quantification of the assay. Limited resources precluded further re-analysis of these sera following titration, which may have allowed optical densities to be more accurately interpolated.

Further studies into allergic responses in elephants, via intradermal skin tests, lymphocyte proliferation responses, and characterization of immunoglobulin subclasses in these species, would help define the cause of the observed hypersensitive responses. Our investigation of adverse reactions in elephants indicated that these were not associated with the number of prior vaccinations or the intervals between each dose. Temporal clustering of adverse reactions occurred that suggested a non-hypersensitivity variable was contributory (eg. injection technique). Alternatively, it is also possible that some adverse reactions or additional vaccination events may not have been observed or noted in medical records. In a comparative study of potential causative agents of immediate adverse reactions to TT, TT antigens were the only component able to fix both IgG and IgE antibodies (Mayorga et al., 2003). Furthermore, both alum adjuvants and thimerosal (mercury) skin tests performed on children with adverse reactions were negative (Mayorga et al., 2003). These results imply that tetanus neurotoxin antigens could be the main immunogenic protein in TT's. Due to the delayed nature of elephant reactions following vaccination it is unlikely that alum adjuvant (aluminium phosphate) used in TT formulations is the causative agent. Nonetheless, cutaneous prick testing should be performed to confirm this (Jacobs et al., 1982).

Clinical reports of adverse reactions to tetanus vaccination in elephants are most supportive of cell mediated (type IV) delayed hypersensitivities. Although varying in severity, the reported symptoms were not systemically presented and did not resolve quickly as might be expected for type I or II hypersensitivities (Facktor et al., 1973; Stratton et al., 1994). The inconsistency of antibody concentrations and occurrence of adverse reactions was also unlike those reported from localised type III hypersensitivities (Arthus type) (Peng et al., 2019; Stratton et al., 1994). Type IV reactions are classified by peak clinical signs approximately 3 weeks following the first exposure, or from 24 to 48 h after re-exposure (Facktor et al., 1973; Stratton et al., 1994; Chung, 2014). As type IV hypersensitivities are mediated mostly by sensitised T cells and do not involve antibodies, further exploration of vaccination responses in hypersensitive animals should include monitoring of cellular TH1 components such as cytotoxic $CD8^+$ T cells, cytokines (IFN-γ, TNF-α & TNF-β) and macrophages (Salmon, 2012; Actor, 2019). Inclusion of assays for complement factors, such as chemotactic FcRIII, could help distinguish between type III and type IV reactions (Peng et al., 2019).

Persistence of antibodies despite frequent vaccination presents no evidence for, but cannot rule out, presence of B cell anergy, which could compromise protection. As detailed in previous studies, increased antigenic challenge may diminish the quality and overall activity of B-cell lymphocyte responses, which is equally influential in maintaining immune defence (Nanan et al., 2001; Cambier et al., 2007). The method used in this study would not have allowed for the observation of this effect as B-cell activity and circulating antibody concentrations are independently regulated (Amanna et al., 2007; Yoshida et al., 2010; Slifka and Amanna, 2014). It has been considered that antibody concentrations may not adequately reflect the true susceptibility of humans, and potentially other animals, to tetanus (Crone and Reder, 1992). Therefore, continued exploration into the different antibody and lymphocyte classes that are stimulated against tetanus antigens may offer a better perspective on what is considered an appropriate immune response to tetanus vaccinations. Additionally, overall avidity of antibody sub-classes, measured using affinity, valency, and structural parameters, would also provide important information on the quality of responses generated by TT in elephant and rhinoceros (Feng et al., 2009). The use of a larger sample population, particularly for the rhinoceros, would help demonstrate clearer relationships that could be tested for statistical significance in future research.

## 5. Conclusion

In summary, this study demonstrated that existing high concentrations of antibodies, either naturally acquired or from previous vaccination, may be inhibiting appropriate vaccination responses in rhinoceros and elephants. Additionally, these high antibody concentrations can be maintained for a minimum of 3 years in elephants. Whilst adverse reactions typical of hypersensitivity following vaccination is evident, further study into the origins and risk factors associated with these reactions in elephants is required. This investigation provides a good foundation to further explore the many unexpected peculiarities of elephant and rhinoceros antibody responses. Acquiring a larger sample population and using a controlled experimental design with equal sampling for more accurate observation of responses to vaccination is suggested. Furthermore, examining the immune response as an integrated system by targeting specific cellular and humoral components could give a more holistic indication of immune responses.

## 6. Ethics statement

All samples used were archived surplus material initially collected for clinical management of animals independent of this study. Approval for use was granted by the Taronga Western Plains Zoo Animal Ethics Committee (Wildlife 5.4.1 Specimen Licence Agreement – #R19B283) and the Animal Ethics Committee of the University of Sydney was notified.

## Declaration of interest

There are no conflicts of interest to declare.

## Acknowledgements

The staff at the Wildlife Hospital at Taronga Western Plains Zoo, Dubbo and Paul Thompson at Taronga Zoo, Sydney helped provide samples for this study. Literary guidance was provided by Dr Wendy Muir and Dr Kumudika de Silva from the University of Sydney.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.dci.2020.103841.

## References

Actor, J.K., 2019. Immune hypersensitivities. In: Versteeg-Buschman, L. (Ed.), Introductory Immunology: Basic Concepts for Interdisciplinary Applications. Elsevier Academic Press, London, pp. 103–110.

Aggerbeck, H., Gizurarson, S., Wantzin, J., Heron, I., 1997. Intranasal booster vaccination against diphtheria and tetanus in man. Vaccine 15, 307–316. https://doi.org/10.1016/s0264-410x(96)00175-2.

Amanna, I.J., Carlson, N.E., Slifka, M.K., 2007. Duration of humoral immunity to common viral and vaccine antigens. N. Engl. J. Med. 357, 1903–1915. https://doi.org/10.1056/NEJMoa066092.

Andrews, S.F., Wilson, P.C., 2010. The anergic B cell. Blood 115, 4976–4978. https://doi.org/10.1182/blood-2010-03-276352.

Bennett, L., Dunham, S., Yon, L., Chapman, S., Kenaghan, M., Purdie, L., Tarlinton, R., 2015. Longitudinal study of Asian elephants, Elephas maximus, indicates intermittent shedding of elephant endotheliotropic herpesvirus 1 during pregnancy. Vet. Rec. Open. 2, e000088 https://doi.org/10.1136/vetreco-2014-000088.

Borrow, R., Balmer, P., Roper, M.H., 2006. Module 3: tetanus update 2006. In: The Immunological Basis for Immunization Series. World Health Organization.

Burke, T.J., 1975. Probable tetanus in an Asian elephant. J. Zoo Anim. Med. 6, 22–24. https://doi.org/10.2307/20094283.

Cambier, J.C., Gauld, S.B., Merrell, K.T., Vilen, B.J., 2007. B-cell anergy: from transgenic models to naturally occurring anergic B cells? Nat. Rev. Immunol. 7, 633. https://doi.org/10.1038/nri2133.

Chung, E.H., 2014. Vaccine allergies. Clin. Exp. Vaccine. Res. 3, 50–57. https://doi.org/10.7774/cevr.2014.3.1.50.

Cook, T.M., Protheroe, R.T., Handel, J.M., 2001. Tetanus: a review of the literature. Br. J. Anaesth. 87, 477–487. https://doi.org/10.1093/bja/87.3.477.

Crone, N.E., Reder, A.T., 1992. Severe tetanus in immunized patients with high anti-tetanus titers. Neurology 49, 761–764. https://doi.org/10.1212/wnl.42.4.761.

Danilova, E., Shiryayev, A., Kristoffersen, E.K., Sjursen, H., 2005. Attenuated immune response to tetanus toxoid in young healthy men protected against tetanus. Vaccine 23, 4980–4983. https://doi.org/10.1016/j.vaccine.2005.05.028.

Edsall, G., Elliott, M.W., Peebles, T.C., Levine, L., Eldred, M.C., 1967. Excessive use of tetanus toxoid boosters. J. Am. Med. Assoc. 202, 17–19. https://doi.org/10.1001/jama.202.1.111.

Facktor, M.A., Bernstein, R.A., Fireman, P., 1973. Hypersensitivity to tetanus toxoid. J. Allergy Clin. Immunol. 52, 1–12. https://doi.org/10.1016/0091-6749(73)90115-2.

Feng, J., Gulati, U., Zhang, X., Keitel, W.A., Thompson, D.M., James, J.A., Thompson, L. F., Air, G.M., 2009. Antibody quantity versus quality after influenza vaccination. Vaccine 27, 6358–6362. https://doi.org/10.1016/j.vaccine.2009.06.090.

Fischer-Tenhagen, C., Hamblin, C., Quandt, S., Frolich, K., 2000. Serosurvey for selected infectious disease agents in free-ranging black and white rhinoceros in Africa. J. Wildl. Dis. 36, 316–323. https://doi.org/10.7589/0090-3558-36.2.316.

Goss, L.J., 1942. Tetanus in an elephant, Elephas maximus. Zoologica 27, 5–6. https://www.biodiversitylibrary.org/page/51008374.

Gowin, E., Wysocki, J., Kałużna, E., Świątek-Kościelna, B., Wysocka-Leszczyńska, J., Michalak, M., Januszkiewicz-Lewandowska, D., 2016. Does vaccination ensure protection? Assessing diphtheria and tetanus antibody concentrations in a population of healthy children: a cross-sectional study. Medicine 95, e5571. https://doi.org/10.1097/MD.0000000000005571.

Gupta, G.K., 1945. Tetanus in an elephant. J. Am. Vet. Med. Assoc. 107, 241.

Hammarlund, E., Thomas, A., Poore, E.A., Amanna, I.J., Rynko, A.E., Mori, M., Chen, Z., Slifka, M.K., 2016. Durability of vaccine-induced immunity against tetanus and diphtheria toxins: a cross-sectional analysis. Clin. Infect. Dis. 62, 1111–1118. https://doi.org/10.1093/cid/ciw066.

Hoar, J., 2017. Dubbo Remembers Taronga Western Plains Zoo's Little Prince Rajah. The Courier. https://www.thecourier.com.au/story/.

Jacobs, R.L., Lowe, R.S., Lanier, B.Q., 1982. Adverse reactions to tetanus toxoid. J. Am. Med. Assoc. 247, 40–42.

Kendall, A., Anagrius, K., Gånheim, A., Rosanowski, S.M., Bergström, K., 2016. Duration of tetanus immunoglobulin G concentrations following basic immunisation of horses. Equine Vet. J. 48, 710–713. https://doi.org/10.1111/evj.12502.

Kock, M.D., du Toit, R., Morton, D., Kock, N., Paul, B., 1990. Baseline biological data collected from chemically immobilized free-ranging black rhinoceroses (Diceros bicornis) in Zimbabwe. J. Zoo Wildl. Med. 283–291. https://www.jstor.org/stable/20095065.

Kuhns, W.J., 1962. The relationship of immediate wheal reactions to the repeated administration of diphtheria and tetanus toxoid. J. Immunol. 89, 652–659.

Lindsay, W.A., Wiedner, E., Isaza, R., Townsend, H.G., Boleslawski, M., Lunn, D.P., 2010. Immune responses of Asian elephants (Elephas maximus) to commercial tetanus toxoid vaccine. Vet. Immunol. Immunopathol. 133, 287–289. https://doi.org/10.1016/j.vetimm.2009.08.005.

Maselle, S.Y., Matre, R., Mbise, R., Hofstad, T., 1991. Neonatal tetanus despite protective serum antitoxin concentration. FEMS Microbiol. Lett. 76, 171–176. https://doi.org/10.1111/j.1574-6968.1991.tb04211.x.

Matzkin, H., Regev, S., 1985. Naturally acquired immunity to tetanus toxin in an isolated community. Infect. Immun. 48, 267–268. https://doi.org/10.1128/IAI.48.1.267-268.1985.

Mayorga, C., Torres, M.J., Corzo, J.L., Alvarez, J., CornejoGarcía, J.A., Rodríguez, C.A., Blanca, M., Jurado, A., 2003. Immediate allergy to tetanus toxoid vaccine: determination of immunoglobulin E and immunoglobulin G antibodies to allergenic proteins. Ann. Allergy Asthma Immunol. 90, 238–243. https://doi.org/10.1016/s1081-1206(10)62148-5.

McGaughey, C.A., 1962. Diseases of elephants. Part IV. Ceylon Vet. J. 10, 3–9.

Muir, W.I., Husband, A.J., Bryden, W.L., 2002. Dietary supplementation with vitamin E modulates avian intestinal immunity. Br. J. Nutr. 87, 579–585. https://doi.org/10.1079/BJNBJN2002562.

Nanan, R., Heinrich, D., Frosch, M., Kreth, H.W., 2001. Acute and long-term effects of booster immunisation on frequencies of antigen-specific memory B-lymphocytes. Vaccine 20, 498–504. https://doi.org/10.1016/s0264-410x(01)00328-0.

Natalia, L., Priadi, A., Wahyu, M., Lubis, A., Stremme, C., 2011. ELISA for detecting anti-tetanus antibodies in Sumatran elephants. Gajah 34, 49–55.

Olander, R.M., Wuorimaa, T., Kayhty, H., Leroy, O., Dagan, R., Eskola, J., 2001. Booster response to the tetanus and diphtheria toxoid carriers of 11-valent pneumococcal conjugate vaccine in adults and toddlers. Vaccine 20, 336–341. https://doi.org/10.1016/s0264-410x(01)00375-9.

Peebles, T.C., Levine, L., Eldred, M.C., Edsall, G., 1969. Tetanus-toxoid emergency boosters: a reappraisal. N. Engl. J. Med. 280, 575–581.

Peng, B., Wei, M., Zhu, F.C., Li, J.X., 2019. The vaccines-associated Arthus reaction. Hum. Vaccines Immunother. 15, 1–9. https://doi.org/10.1080/21645515.2019.1602435.

Pichichero, M.E., 2008. Vaccine-induced immunologic memory and pace of pathogenesis: predicting the need for boosters. Expert Rev. Vaccines 7, 1299–1303. https://doi.org/10.1586/14760584.7.9.1299.

Pye, G., 2005. Species Survival Plan Guidelines for Comprehensive Elephant Health Monitoring Program. American Zoo Association.

Ribeiro, M.G., de Nardi Júnior, G., Megid, J., Franco, M.M.J., Guerra, S.T., Portilho, F.V. R., Rodrigues, S.A., Paes, A.C., 2018. Tetanus in horses: an overview of 70 cases. Pesqui. Vet. Bras. 38, 285–293. https://doi.org/10.1590/1678-5150-pvb-5441.

Rookmaaker, L.C., Jones, M.L., Kloes, H.G., Reynolds, R.J., 1998. The Sumatran rhinoceros (Dicerorhinus sumatrensis) in captivity to 1994. In: The Rhinoceros in Captivity: a List of 2439 Rhinoceroses Kept from Roman Times to 1994. SPB Academic Publishing, The Hague, pp. 129–153.

Salmon, J.E., 2012. Mechanisms of immune-mediated tissue injury. In: Goldman, L., Schafer, A. (Eds.), Cecil Textbook of Medicine. Elsevier Saunders, Philadelphia, pp. 226–230.

Schmidt, M., 1986. Elephants (Proboscidea). In: Miller, R.E., Fowler, M.E., Saunders, W. B. (Eds.), Fowler's Zoo and Wild Animal Medicine. Elsevier, Philidelphia, pp. 883–923.

Slifka, M.K., Amanna, I., 2014. How advances in immunology provide insight into improving vaccine efficacy. Vaccine 32, 2948–2957. https://doi.org/10.1016/j.vaccine.2014.03.078.

Steel, J.H., 1885. A Manual of the Diseases of the Elephant and of His Management and Uses. Lawrence Asylum Press, Madras, p. 50.

Stevenson, M.F., Walter, O., 2006. Elephants: Loxodonta africana and Elephas maximus. In: Management Guidelines for the Welfare of Zoo Animals. British and Irish Association of Zoos and Aquariums, London, pp. 116–117.

Stratton, K.R., Howe, C.J., Johnston, R.B., 1994. Adverse Events Associated with Childhood Vaccines: Evidence Bearing on Causality. National Academies Press, Washington (DC), pp. 59–62.

Van Heerden, J., Keffen, R.H., Dauth, J., Dreyer, M.J., 1985. Blood chemical parameters in free-living white rhinoceros Ceratotherium simum. J. S. Afr. Vet. Med. Assoc. 56, 187–189.

Veronesi, R., Cecin, H., Correa, A., Tavares, J., Moraes, C., Bertoldo, O.J., 1975. New concepts on tetanus immunization: naturally acquired immunity. J. Hyg. Epidemiol. Microbiol. Immunol. (Prague) 19, 126–134.

Wassilak, S.G., Roper, M.H., Kretsinger, K., Orenstein, W.A., 2008. Tetanus toxoid. In: Plotkin, S.A., Orenstein, W.A., Offit, P.A. (Eds.), Vaccines. Saunders, Elsevier, Philadelphia, pp. 805–839.

Yoshida, T., Mei, H., Dörner, T., Hiepe, F., Radbruch, A., Fillatreau, S., Hoyer, B.F., 2010. Memory B and memory plasma cells. Immunol. Rev. 237, 117–139. https://doi.org/10.1111/j.1600-065X.2010.00938.x.

Zaccaro, D.J., Wagener, D.K., Whisnant, C.C., Staats, H.F., 2013. Evaluation of vaccine-induced antibody responses: impact of new technologies. Vaccine 31, 2756–2761. https://doi.org/10.1016/j.vaccine.2013.03.065.