Sex-differential non-specific effects of rabies vaccine in dogs: An extended analysis of a randomized controlled trial in a high-mortality population

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Highlights

- Rabies vaccine in dogs has nonspecific effects on survival that are modified by sex.
- 1st rabies vaccination at 6 wks increased mortality in females, compared to placebo.
- 2nd at 13 wks decreased mortality in males, compared to 1st dose at that age.
- Conditioning on body weight as a predictor of loss to follow up didn't change results.

Abstract

Non-live rabies vaccines have been associated with both beneficial and detrimental effects on host population morbidity and mortality rates to unrelated infections in people and animals, and these non-specific effects may differ by sex. Previous animal studies may have been affected by bias, including selection bias due to loss to follow up in randomized controlled trials (RCTs). We previously reported results of an RCT in dogs on the effect of primary rabies vaccine administered at 6 weeks of age on all-cause mortality over a 7-week follow-up period, in a high-mortality population of owned dogs. Here, we report the results from the same trial of a second vaccination at 13 weeks of age, compared to a primary vaccination. Because a relatively high proportion of study subjects (30%) were lost to follow-up in the RCT, we also conducted an analysis to control for possible selection bias over both periods (6 to 13 weeks and 13 to 20 weeks of age). We found that primary rabies vaccination at 6 weeks of age substantially increased the hazard of death from all causes over the next 7 weeks among females (hazard ratio [HR] 2.69, 95% confidence intervals [CI] 1.27–5.69), but not among males (HR 0.91, 95% CI 0.32–2.59). Among survivors, administration of a second dose of rabies vaccine at 13 weeks of age was associated with a decreased hazard of death among males (HR 0.33, 95% CI 0.10–1.02) but not females (HR 1.64, 95% CI 0.59–4.58), when compared to the group receiving their first dose at this age. Based on our causal assumptions, we show that these results were not affected by selection bias. In this high-mortality dog population, receipt of a non-live rabies vaccine substantially affected all-cause mortality rates, with this effect being strongly modified by sex.

Keywords: Non-specific effects; Vaccine; Sex; Mortality; Rabies; Dogs

1 Introduction

Vaccines work by stimulating the body to produce a rapid and specific protective immune response in recipients upon subsequent exposure to infection by the particular pathogen targeted by the vaccine. It has been proposed that vaccines have additional, unanticipated effects that manifest as a general increase or decrease in morbidity or mortality of vaccinated populations that cannot be explained by this specific protective effect, nor conversely by typical expected adverse reactions to the vaccine [1,2]. These effects of vaccines have been termed "non-specific effects" (NSEs). It is presumed that NSEs are mediated through the host immune system by altering the response to subsequent infections by unrelated pathogens, although the specific biological mechanism(s) remains unclear [3,4].

As general principles, it has been proposed that live vaccines enhance resistance towards unrelated infections, while non-live vaccines enhance the susceptibility of females to unrelated infections [5]. These principles are supported by a systematic review of the evidence for NSEs of three common childhood vaccines–Bacillus Calmette-Guérin (BCG), measles-containing vaccines (MCV) and diphtheria-tetanus-pertussis (DTP)–on all-cause mortality in children up to 5 years of age, which concluded that receipt of BCG and MCV (both live vaccines) was associated with lower mortality and receipt of DTP (a non-live vaccine) was associated with higher mortality, with the effect tending to be stronger in girls [6]. Besides DTP, receipt of other non-live vaccines has been associated with higher all-cause mortality in girls than in boys, including inactivated polio vaccine [7], hepatitis B vaccine [8], RTS,S malaria vaccine [9] and pentavalent vaccine [10.11].

The current generation of rabies vaccines widely used in humans and animals are non-live vaccines. In contrast to the reported pattern of detrimental NSEs for non-live vaccines, receipt of non-live rabies vaccine in people has been associated with lower rates of meningitis and cerebral malaria in children aged 5 to 17 months compared to receipt of RTS,S/AS01 malaria vaccine [12], and a possible protective effect against diarrheal disease in young adult males compared to receipt of a placebo injection [13]. In an observational animal study, a beneficial association between owner-reported rabies vaccination and overall survival in owned dogs was also reported [14], but we consider that study to be at high risk of bias (residual confounding as well as measurement bias due to the retrospective updating of vaccination status [15]). In a randomized controlled trial of the NSEs of a dog rabies vaccine in the same population, we found that survival decreased substantially in females in the 7 weeks following vaccination, but not in males [16]. Thus there is inconsistent evidence for NSEs of rabies vaccine, in part due to potential biases in previous studies. Estimation of the unbiased causal effect is important in view of the global effort to increase use of human and animal rabies vaccines in a bid to eliminate human deaths from rabies by 2030 [17], and may assist in identifying the mechanism of action of any NSEs of non-live vaccines.

The risk of bias is a common criticism in general of studies showing that some non-live vaccines may have overall deleterious effects on the health of females in populations with a high burden of unrelated infections, contributing to a lack of scientific consensus on the topic [6,18,19]. Potential sources of bias include failure to appropriately control for common causes of vaccination and mortality, such as health status (confounding bias); bias resulting from processes by which individuals are selected into the analysis (selection bias); and bias resulting from processes by which data on vaccination, mortality or confounding variables are measured (measurement bias, also called information or misclassification bias) [20]. Notably, while well-performed randomized controlled trials (RCTs) are free of confounding bias, they are not

immune to the risk of either selection bias or measurement bias. In the analysis of survival data from such RCTs, follow-up is said to be censored when information about the time to death (or other event of interest) is incomplete. If subjects' censoring times are associated with their (unobserved) event times, then censoring is said to be informative and will lead to selection bias. Appropriate adjustment for selection bias is important for valid causal effect estimates in any studies of NSEs with high rates of censoring, including RCTs [21].

We previously reported results from our RCT in dogs on the effect of rabies vaccine compared to placebo administered at 6 weeks of age on all-cause mortality over a 7-week follow-up period [16], in a high-mortality population of owned dogs in a resource-poor community in South Africa. After the initial 7 weeks of follow-up, to comply with South African rabies control legislation all puppies remaining in the trial received rabies vaccine at 13 weeks of age and were followed up for a further 7 weeks. In this paper, we present the results of this extended follow up. Because a relatively high proportion of study subjects (30%) were lost to follow-up in the RCT, we also present the results of an analysis to control for possible selection bias due to informative censoring, using body weight as a measured mediator for the effect of underlying health status on censoring [22]. Fig. 1 shows the assumed causal structure for this analysis in the form of directed acyclic graphs (DAGS, or causal diagrams).

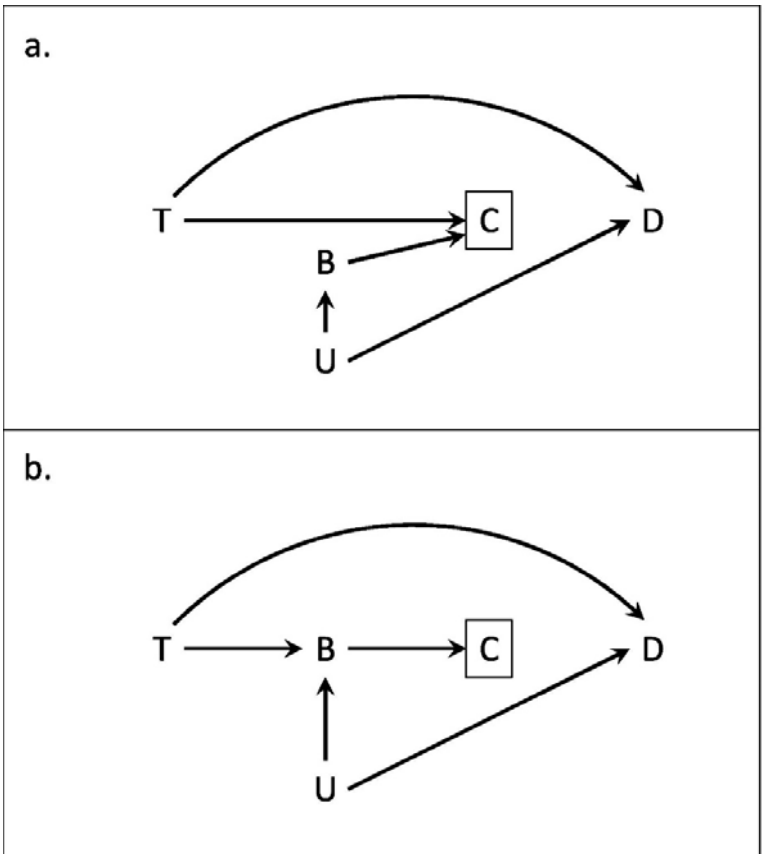

Fig. 1. Causal directed acyclic graphs (DAGs) showing casual assumptions in the analysis. The graphs show dichotomous treatment *T* (rabies vaccine or placebo), the outcome *D* (time to death) and censoring due to loss to follow-up *C* . *U* represents unmeasured underlying health status, which affects both the outcome and body weight, *B* . The association between *T* and *D* may be due to selection bias introduced by conditioning on *C* (represented by the box around *C*) [22]. This selection bias can be removed by conditioning on the probability of loss to follow up through inverse probability of censoring weighting, using *B* as a predictor of loss to follow up.

2 Materials and methods

2.1 Study design

The study design was a single-site, owner-blinded, randomized, placebo-controlled trial in puppies, restricted to owned puppies born to mothers vaccinated against rabies within the 12 months prior to the start of the study. Details of the study design and ethical approvals are provided in [16]. Briefly, puppies within litters were randomly assigned in a 1:1 ratio to receive a subcutaneous injection of either rabies vaccine (RV; Defensor® 3, Zoetis, South Africa) or sterile water (SW) at 6 weeks of age, and followed up for 7 weeks. At 13 weeks of age, remaining puppies received a subcutaneous injection of RV; thus, treatment groups at allocation were defined by planned treatment at 6 and 13 weeks of age: $RV_6 + RV_{13}$ and $SW_6 + RV_{13}$. Puppies were followed up for a further 7 weeks, until exit from the study at 20 weeks of age. Owners were blind to the allocation of their dogs until exit from the study. The primary outcome was death due to any cause. Body weight was measured at 6, 9, 13, 16 and 20 weeks of age.

2.2 Survival analysis

Details of sample size estimates are provided in [16]. For the analysis, we used an extended mixed-effects Cox proportional hazard model. We included litter as a random effect, to account for litter as a stratifying variable in the randomization. We included an interaction term between treatment group and sex. Unlike our previous analysis [16], we did not include body weight as a covariate in the unweighted model, but rather accounted for it as a predictor of censoring through the inverse probability weighting, described below. To account for the application of the treatment at two time points (RV or SW at 6 weeks of age; RV at 13 weeks of age), the Cox model was extended with a Heaviside function for treatment and for sex [23], which provides two hazard ratios for each covariate: one that is constant for the period 6 to 13 weeks, and one that is constant for the period 13 to 20 weeks. The proportional hazards assumption was tested using a goodness-of-fit test of the Schoenfeld residuals [24].

2.3 Bias analysis

Inverse probability of censoring weighting requires estimates of covariates at all event and censoring times. Missing values of body weight at 6, 9, 13, 16 and 20 weeks (which were fewer than 30% of measured values at each time point, with the exception of 20 weeks where 46% of values were missing) were imputed using multiple imputation based on litter, sex and previous measured weights, when available [25]. Given the linear growth rate between 6 and 20 weeks observed in dog breeds of similar adult size to our study population [26], we used linear interpolation to estimate body weights at relevant points between measurement times. Inverse probability of censoring weights were calculated using the 'ipw' package [27]. We then refit the extended model Cox model with the Heaviside function for treatment described above, using the calculated weights. All analyses were performed in R [28].

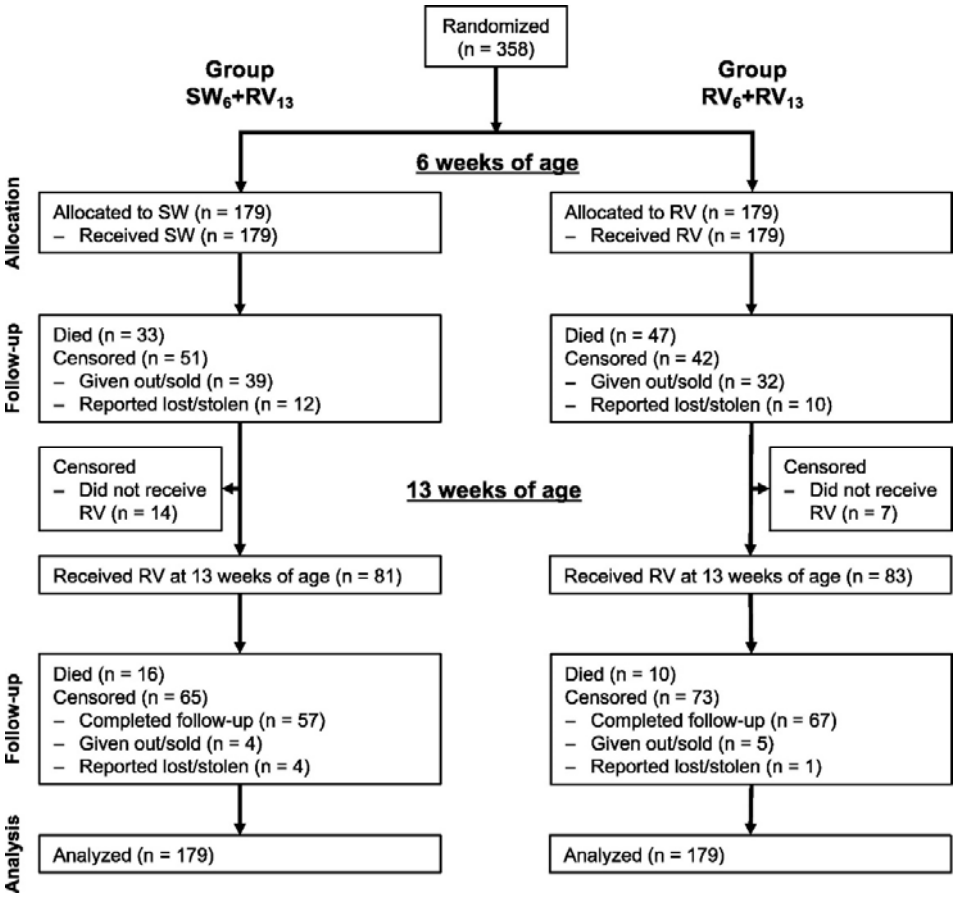

Fig. 2. Flow chart of study subjects. SW = sterile water; RV = rabies vaccine; subscript numbers show age (in weeks) at which interventions were administered.

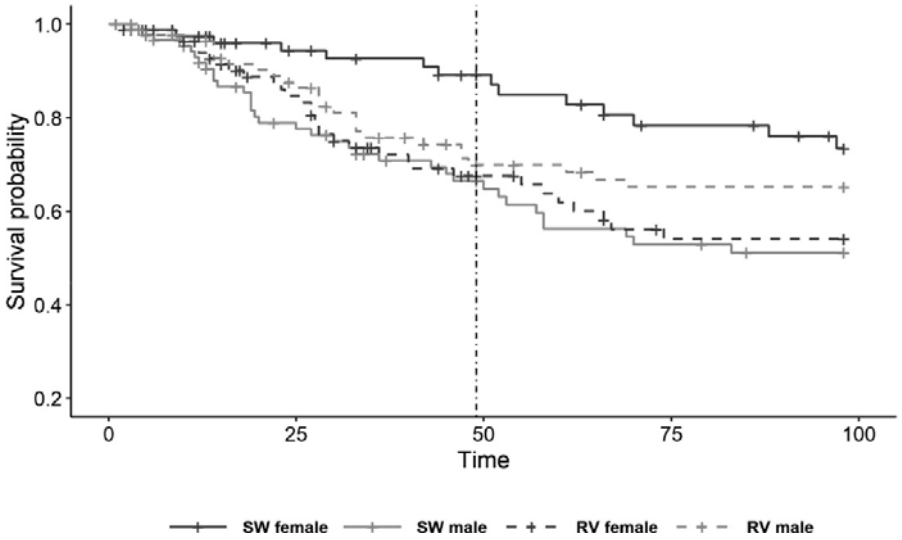

Fig. 3. Survival curve of study subjects (n = 358) by treatment group and sex. The x-axis shows time since first injection (in days). SW = sterile water at 6 weeks of age (time 0); RV = rabies vaccine at 6 weeks of age. Remaining puppies in both groups received RV at 13 weeks of age (time 49; vertical dashed line).

Table 1
Estimated hazard ratios (HRs) with 95% confidence intervals (CI) for allocation to the $RV_6 + RV_{13}$ group (n = 179) compared to the $SW_6 + RV_{13}$ group (n = 179) from an unweighted extended Cox proportional hazards model with a Heaviside function (for the periods pre- and post-second injection), and from the weighted model correcting for potential bias due to loss to follow up.

Period	Sex	Mortality rate[a] (deaths/dog-years[b])		HRs (95% CI) for $RV_6 + RV_{13}$ relative to $SW_6 + RV_{13}$ within strata of sex	
		$SW_6 + RV_{13}$	$RV_6 + RV_{13}$	Unweighted	Weighted
6 to 13 weeks	Females	853 (7/8.2)	2,854 (24/8.4)	2.66 (1.24–5.73)	2.69 (1.27–5.69)
	Males	3,065 (26/8.5)	2,546 (23/9.0)	0.93 (0.32–2.74)	0.91 (0.32–2.59)
HRs (95% CI) for males relative to females within strata of treatment[c]		2.90 (1.32–6.37)	0.80 (0.28–2.32)		
13 to 20 weeks	Females	1,467 (7/4.8)	1,780 (7/3.9)	1.64 (0.58–4.68)	1.64 (0.59–4.58)
	Males	2,125 (9/4.2)	528 (3/5.7)	0.34 (0.10–1.10)	0.33 (0.10–1.02)
HRs (95% CI) for males relative to females within strata of treatment[c]		2.87 (1.02–8.00)	0.21 (0.07–0.67)		

[a] Mortality rate per 1,000 dog-years.
[b] Dog-years and hence mortality rates for the period 6–13 weeks differ from those presented in Table 4 in Arega et al. (2020) due to differences in cut-off between the first and second period between the studies.
[c] Results from unweighted model only.

3 Results

The study was conducted from December 2016 through August 2018. We enrolled 358 puppies and randomly assigned them to treatment group at 6 weeks of age (179 in the $RV_6 + RV_{13}$ group and 179 in the $SW_6 + RV_{13}$ group). The randomization resulted in balanced groups with regard to baseline demographics, health and owner care (Table 1 in [16]). Fig. 2 shows the flow chart of study subjects from 6 to 20 weeks of age. Fig. 3 shows the survival curves by treatment group and sex.

From 6 to 13 weeks of age, there were 80 deaths (47 in the $RV_6 + RV_{13}$ group and 33 in the $SW_6 + RV_{13}$ group) and 93 puppies (26%) were lost to follow-up (42 in the $RV_6 + RV_{13}$ group and 51 in the $SW_6 + RV_{13}$ group). Mean follow-up time over this period was 35.6 days (median 49.0, interquartile range [IQR] 22.5–49.0) for the $RV_6 + RV_{13}$ group and 34.1 days (median 49.0, IQR 15.0–49.0) for the $SW_6 + RV_{13}$ group. Of the 185 subjects remaining in the study at 13 weeks of age, 21 did not receive the injection of RV (because of unavailability or refusal by owners) and were administratively censored at this time point. From 13 to 20 weeks of age, there were 26 deaths (10 in the $RV_6 + RV_{13}$ group and 16 in the $SW_6 + RV_{13}$ group) and 14 puppies (9%) were lost to follow-up (6 in the $RV_6 + RV_{13}$ group and 8 in the $SW_6 + RV_{13}$ group). Mean follow-up time over this period was 42.3 days (median 49.0, IQR 49.0–49.0) for the $RV_6 + RV_{13}$ group and 40.6 days (median 49.0, IQR 39.0–49.0) for the $SW_6 + RV_{13}$ group.

Table 1 shows the estimated hazard ratios (by time period) of allocation to the $RV_6 + RV_{13}$ group compared to the $SW_6 + RV_{13}$ group from the unweighted extended Cox proportional hazards model, and from the weighted model correcting for potential bias due to loss to follow up. The estimated hazard ratios comparing males to females within strata of treatment group are also shown.

4 Discussion

In this high-mortality dog population, receipt of a non-live rabies vaccine substantially affected all-cause mortality rates, with this effect being strongly modified by sex. Rabies vaccination at 6 weeks of age substantially increased the hazard of death from all causes over the next 7 weeks among females, but not among males. Administration of a second dose of rabies vaccine at 13 weeks of age was associated with a decreased hazard of death among males but not females, when compared to the group receiving their first dose at this age. Among this previously-unvaccinated control group, mortality rates increased in females but decreased in males in the 7 weeks after receipt of rabies vaccine at 13 weeks of age, compared to the preceding 7 weeks.

Despite the high rate of loss to follow up of subjects, our analysis shows that this did not introduce selection bias, based on our assumed causal structures. If anything, the inverse probability of censoring weighting analysis suggests that the original, unweighted effect estimates may have been slightly biased towards the null, particularly in the first time period (6–13 weeks of age). Among other possible explanations, this pattern would be consistent with owners retaining puppies of larger body weight while giving out smaller litter mates, with body weight being positively associated with survival. Further studies should however plan to collect data on additional time-varying predictors of loss to follow up to more rigorously control for selection bias.

Rabies vaccines are not licensed for use in dogs younger than 12–14 weeks of age, but guidelines from the World Health Organization [29] and the World Organization for Animal Health [30], as well as South African legislation [31], allow for the vaccination of dogs younger than this in the context of annual mass rabies vaccination campaigns. Based on our results, we advise that these recommendations be revisited and that caution in vaccination of female puppies in particular be exercised until such time as our results are confirmed or refuted. The pattern of mortality rates before and after first vaccination at 13 weeks of age is consistent with the sex-differential pattern found in younger puppies (increased female and decreased male mortality following vaccination), but the lack of a contemporaneous, age-matched control group prevents causal inference. There is an absence of data on age- and sex-specific mortality rates in free-roaming dog populations against which this finding could be assessed. This finding should be more rigorously assessed through a randomized controlled trial of vaccination at 13 weeks of age, with a sample size sufficient to detect modification of the effect of rabies vaccination on all-cause mortality by sex. Among the treatment group, administration of a second (booster) vaccine at 13 weeks of age appears to amplify the sex-differential effect, consistent with the pattern reported for DTP [32].

Our study has additional limitations. Although the estimation of period-specific hazard ratios through the Heaviside function accounts for changes in the hazard ratio over time, causal interpretation for the second period is complicated by the selection bias introduced through conditioning on a post-treatment variable; that is, being alive at the time of the second injection. As a result, the effect estimate for the second period (which is the estimate of the effect of a booster vs. a primary rabies vaccination) may be confounded by unmeasured factors that affect mortality in both periods. This could potentially explain the lack of an effect of the second dose among females. Our study was also restricted to puppies born to females who had been vaccinated against rabies within the preceding 18 months; thus if maternal immune status modifies the sex-differential effect of offspring vaccination on all-cause mortality (as may be the case [33]), our causal effect estimates may not be transportable to other populations with different distributions of maternal immune status. A further limitation of our study is the lack of information on causes of death in our study subjects, due to difficulties in obtaining carcasses soon enough after death to perform necropsies. Aside from 10 deaths reported due to accidents, information collected on deaths was consistent with infectious or parasitic aetiologies, but could not rule out other causes. Sensitivity analysis showed that censoring accidental deaths did not substantively affect the results for the first time period [16]. Rabies was tested for and ruled out only in a single case with a presumptive history; the study's conclusions would be strengthened had the disease been ruled out as a cause of death in all cases (although we note that this limitation should not affect our conclusion of a non-specific detrimental effect of the vaccine in females).

Our findings of a detrimental effect of rabies vaccine in young female puppies may be contrasted with reports of beneficial NSEs of rabies vaccine in people [12.13]. One possible reason for this is the difference in excipients between human rabies vaccines and most licensed animal rabies vaccines: while the antigenic component of both vaccine types comprise inactivated cell-cultured rabies virus, most animal rabies vaccines (including the one used in our study) contain alum adjuvant, whereas human rabies vaccines are unadjuvanted. It has been demonstrated that alum activates immunosuppressive mechanisms following vaccination, mediated through increased production of the immune regulatory cytokine IL-10 [34]. We speculate that this mechanism may underpin the detrimental effects attributed to non-live vaccines, by enhancing susceptibility to unrelated infections, and could also explain the heterogeneity of these effects between the sexes [35]. We hypothesize that the antigenic

component of rabies vaccine (common to human and animal vaccines) may have a non-specific protective effect, but that any such effect in adjuvanted vaccines is countered by a strong immune suppressive effect of the alum adjuvant that enhances susceptibility to unrelated infections. We speculate that the consequence of this immune suppressive effect on mortality is only noted in females and not in males, as the latter are already susceptible to these unrelated infections through sex-linked factors including gonadal sex hormones [36], consistent with our finding of high male mortality relative to female mortality in our control group. In populations with a high incidence of these infections, this manifests as an increase in female mortality after vaccination, whereas male mortality may decrease due to the specific and/or non-specific protective effects of the vaccine. This hypothesis should be tested in randomized trials in these populations comparing the effects of adjuvanted and non-adjuvanted rabies vaccine, and the modification of these effects by sex. Such trials should also examine underlying immunological mediators of any effects.

In conclusion, our study in this novel, high-mortality animal population is consistent with the emerging principle of increased female mortality following non-live vaccination [5]. Confirmation of this finding and identification of the underlying immunological mechanism are essential so that any causal effect can be mitigated. Fundamental to this endeavour will be integration of epidemiological and immunological studies, if we are to achieve consensus on the existence and importance of NSEs of vaccines.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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