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# Vaccination of koalas with a prototype chlamydial vaccine is safe, does not increase the incidence of lymphoma-related disease and maybe associated with increased lifespan in captive koalas

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ABSTRACT

*Objectives:* To assess the impact of *Chlamydia* vaccination on survival of captive koalas, and to compare the incidence of lymphomas and neoplasias between vaccinated and unvaccinated koalas.
*Methods:* Survival analysis using Cox and Weibull regressions on 54 vaccinated and 52 matched unvaccinated koalas, and chi-square contingency table for incidence of lymphomas/neoplasias.
*Results:* Vaccination was found to have a significant positive effect on koala lifespan ($P = 0.03$), with vaccinated koalas having a median lifespan of 12.25 years compared to 8.8 years for unvaccinated ones. The effect of sex on lifespan was not significant ($P = 0.31$). The risk ratio of unvaccinated over vaccinated koalas was 2.2 with both Cox and Weibull regressions. There was no association between the incidence of lymphoma/neoplasias and vaccination status ($P = 0.33$).
*Conclusions:* Koalas vaccinated with a prototype *Chlamydia* vaccine may live longer than unvaccinated ones. There was no known *Chlamydia* infection among koalas, so our interpretation is that vaccination may have boosted the innate and adaptive immune systems to protect against a wide spectrum of bacteria, fungi and parasites. Vaccinated koalas did not show negative physiological effects of the vaccine, for example, the frequency of deaths due to lymphomas/neoplasias was the same in both vaccinated and unvaccinated animals.



## 1. Introduction

Many koala populations are under threat in Australia due to a combination of habitat destruction and fragmentation, motor vehicle injury, dog attacks and disease. The rate of decline is serious and likely to lead to localized extinction in many areas [1,2]. Reducing mortality due to disease is predicted to have the greatest impact on stabilizing populations [3]. *Chlamydia* are obligate intracellular bacteria associated with significant diseases in koalas. *Chlamydia pecorum* is the most widespread and pathogenic chlamydial species infecting koalas. Ocular infections cause keratoconjunctivitis and blindness, urinary tract infections cause cystitis and continual urinary soiling (wet bottom) and genital tract infections cause severe inflammation, fibrosis and scarring that can lead to infertility [4]. For these reasons, an effective vaccine that prevents the adverse health outcomes associated with chlamydial infection would be an important management tool to reverse the decline in koala numbers. We have recently developed and tested a vaccine designed to protect koalas against chlamydial infection [5–7]. The vaccine proved to be safe in captive koala populations, induced long-lasting neutralizing antibody in serum and strong lymphocyte proliferative responses, each lasting more than a year [5]. The vaccine was also shown to be safe in previously infected koalas that had been treated with antibiotics for chlamydial infections prior to vaccination [6]. Previously infected animals mounted similar antibody and cell-mediated immune responses to chlamydia-naïve animals and, importantly, no evidence of enhanced infection-associated inflammatory disease was observed in previously infected and vaccinated koalas. The vaccine is now being evaluated in a group of wild koalas in SE Queensland.

Canfield et al. [8] observed retroviral particles in koalas with leukemia and lymphoma, and, in 2000, Hanger et al. [9] isolated the first koala retrovirus (KoRV) and determined its nucleotide

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sequence. Initially it was reported that KoRV provirus was present in almost all northern koalas but absent in the genomes of some southern koala populations. Now, however, it is evident that koalas across the entire east coast of Australia carry the provirus, with 100% prevalence in Queensland and NSW, and a high prevalence even detected on Kangaroo Island, previously thought to be free of KoRV, where 24/162 animals sampled harbored provirus [10]. These data suggest that this unique retrovirus is currently in the process of endogenization. There have now been at least four KoRV genotypes identified (A, J, C and D) and in one study involving zoo populations [11] KoRV-B has been associated with the development of lymphoma [12,13].

Lymphoma and leukemia are common causes of death in both wild and captive koala populations, and it is possible that KoRV may play a role in the development of these neoplasias, although this has yet to be proven. If KoRV is in the process of endogenization in koalas, it is possible that activation of the immune system by vaccination could enhance viral replication, and thereby increase the incidence of lymphoma and/or leukemia. This has been demonstrated in HIV infected individuals following booster immunization with a tetanus vaccine [14]. To investigate this possibility, we retrospectively determined the lifespan and the incidence of lymphoma/neoplasia as a cause of death in vaccinated and unvaccinated koalas at the Lone Pine Koala Sanctuary in Brisbane, Australia.

## 2. Materials and methods

Koalas at the Lone Pine Koala Sanctuary (Brisbane, Australia) were included in two *Chlamydia* vaccination trials, one in 2007, the other in 2011 [5,7]. In both trials the vaccine consisted of the adjuvant Immune Stimulating Complex combined with recombinant chlamydial proteins, produced in *Escherichia coli*. Vaccinated koalas were born between 2002 and 2010. A cohort of all unvaccinated individuals born between those years was selected as the control group. Both vaccinated and control animals were housed at Lone Pine Koala Sanctuary under identical conditions and did not differ in median birth year (Mann–Whitney $p$-value = 0.62; supplementary material). Selecting this control group as opposed to, for example, all individuals alive at the beginning of vaccination, was crucial to prevent the "healthy worker effect" [15]. The null hypothesis was that lifespan (in months) was not affected by vaccination. The other explanatory variable was sex.

We used a Cox Proportional Hazards regression (Cox regression) to assess the impact of vaccination status and sex on the hazard (rate) ratio of dying at any month in the koala's life [16]. The actual time for each individual was its lifespan in months from birth to 1st November 2013 (the date of first analysis). Individuals still alive at the end of the study were censored. Cox regression models the change in hazard due to predictors but not the (baseline) hazard itself. The hazard, or the instantaneous probability of dying, can be obtained using Kaplan–Meier survival tables [17].

A parametric model assuming a Weibull distribution of survival times was also fitted to the data. This is appropriate when baseline hazard changes monotonically with time. A parametric model extracts more information from data than a semi-parametric one such as Cox regression, for example baseline hazard and the probability distribution of survival ages.

The package survival within the free software R was used [18–20].

## 3. Results

We studied a cohort of 106 animals across a total period of up to 12 years. During this period, there were 30 deaths and the median lifespan for the whole sample was 10.2 years (122 months), as estimated with the Kaplan–Meier table. Table 1 shows the distribution of individuals by vaccination status and sex.

**Table 1**
Distribution of koalas by vaccination status and sex.

| | Males | Females |
|---|---|---|
| Vaccinated | 20 | 34 |
| Unvaccinated | 40 | 12[a] |

[a] Baseline group.

**Table 2**
Results of Cox and Weibull regressions on survival of koalas with vaccination status as predictor (log scale estimates).

| Regression | Predictor | Effect | se (Effect) | 95% CI Effect | $P$ |
|---|---|---|---|---|---|
| Cox | $b_c$ | −0.82 | 0.38 | −1.57, −0.07 | 0.03 |
| Weibull | $a_w$ | 4.81 | 0.10 | 4.62, 5.01 | ∼0 |
| Weibull | $b_w$ | 0.32 | 0.16 | 0.03, 0.61 | 0.04 |
| Weibull | Log(scale) | −0.92 | 0.15 | −1.21, −0.63 | ∼0 |

Effect: estimate of parameter; se: standard error; CI: confidence interval; $P$: significance of testing Effect/se(Effect) = 0; $b_c$: Cox's log hazard ratio of vaccinated vs. unvaccinated koalas; $a_w$: log survival time for unvaccinated koalas (intercept in Weibull regression); $b_w$: log increase in survival time due to vaccination (Weibull regression).

### 3.1. The Cox regression

The initial model estimated the main effects of vaccination, sex and their interaction on the log hazard ratio. The interaction was not significant ($P = 0.66$). In a second model with vaccination and sex but without their interaction, neither sex ($P = 0.31$) nor vaccination was significant ($P = 0.12$). After dropping sex, vaccination was significantly associated with an increased lifespan ($P = 0.03$).

Table 2 shows the results of the Cox regression. The hazard (risk) ratio of vaccinated over unvaccinated koalas is 0.44 with a 95% CI ranging from 0.21 to 0.93. That is, unvaccinated koalas were 2.3 times more likely to die than vaccinated ones at any given month during their lifetime.

Cox regression assumes proportional hazards, implying that hazard ratios are constant over time. A test to reject the proportional hazard assumption was not significant ($P = 0.7$) [20].

### 3.2. The Weibull regression

The initial model contained vaccination status, sex and their interaction. The interaction was not significant ($P = 0.66$). In a model without interaction, neither vaccination ($P = 0.12$) nor sex ($P = 0.39$) was significant. The final model only contained the significant factor vaccination status ($P = 0.04$). Table 2 shows the parameter estimates from the final Weibull regression. As with Cox regression, a test to reject the proportional hazards assumption was not significant ($P = 0.2$).

Table 3 shows the survival, $S(t)$, and hazard, $h(t)$, functions built with the above parameters. The ratio $S_v(t)/S_u(t) = \exp(3.3 \times 10^{-6} t^{2.5})$ between vaccinated and unvaccinated koalas shows how much the surviving advantage of vaccinated koalas increases over time compared to unvaccinated ones. The risk of dying for unvaccinated koalas is $h_u(t)/h_v(t) = 2.2$

**Table 3**
Parametric survival, $S(t)$, and hazard, $h(t)$, functions given vaccination status under the Weibull regression.

| | $S(t)$ | $h(t)$ |
|---|---|---|
| Unvaccinated[a] | $\exp(-0.000006t^{2.5})$ | $0.000015t^{1.5}$ |
| Vaccinated | $\exp(-0.0000027t^{2.5})$ | $0.00000675t^{1.5}$ |

[a] Baseline group; $t$ is time in months.