Supplementary Materials for

Vaccination protects endangered albatross chicks against avian cholera

by

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Method details

Serological assay setup: antibody titration by slow agglutination (SEROPAST® method).

The aim of this method, developed at CEVA BIOVAC, is to titrate antibodies to *Pasteurella multocida* in bird plasma by slow agglutination on microplates.

Ninety-six well, U-bottomed microplates are used. Each plasma undergoes serial twofold dilutions in sterile physiological water on the first 11 wells of a row (the last well being used as a negative control containing only physiological water). A hen serum sample positive to the Heddleston 1 type is used as a control. An inactivated antigenic suspension of *P. multocida* strain D2C is then added to all wells, resulting in final serum dilutions ranging from 1:10 to 1:10240.

Plates are read following incubation at 37°C for 18 to 24 hours. Sedimentation of the antigen (resulting in the presence of a pellet) indicates a negative reaction. Agglutination of the antigen (resulting in a cloudy solution) indicates a positive reaction. Antibody titre is the reciprocal of the last dilution where a positive reaction is observed.

Further details on experimental methods

Indian yellow-nosed albatrosses being a protected, endangered species, permits were obtained to study no more than 30 nests per experimental group each year. The nests chosen to be included in the study were allocated to the vaccinated or the unmanipulated groups following a pre-determined order to which the person choosing the nests was blind. Those nests where eggs failed to hatch or chicks died before vaccination was started (none in the vaccinated group and six nests in the unmanipulated controls) were excluded from the analysis. As an un-vaccinated but sampled control group, we also used nests where parents had been injected with 0.9% NaCl in 2013 (see "Investigation into different vaccination strategies" below), but where neither parents nor the chick had received any other injection. Twelve of these nests had a chick at the time the 2015 survival analysis began and were thus included in the study. Staffs carrying out serological titrations were blind to treatment. Dead and missing chicks could unambiguously be identified; thus no specific blinding method was implemented for chick survival assessment.

Statistical models

All data were analysed using R (R Core Team 2015). Square brackets indicate 95% confidence intervals of the estimates throughout. All tests were two-sided. Computer codes are available upon request.

(i) Chick survival models. Survival of chicks subjected to different treatments was studied for the three reproductive seasons 2013-4, 2014-5 and 2015-6 (figure S1 and figure 2). Chick survival as a function of treatment in a given reproductive season was compared using a Cox proportional hazards model (Andersen & Gill 1982) of the form *survival ~ treatment* as implemented in the 'survival' package (Therneau 2015).

Cox models were chosen for statistical analyses of chick survival because:

- They are standard models to use in medical studies not involving extrapolation beyond the observation period (Crawley 2007), and there were sufficient numbers of events (chick mortality) per group
- Their AIC was substantially lower than those of parametric regressions fitted to the same data (including those allowing for mortality rates to vary with time)
- The constant hazard hypothesis was robust to specific statistical testing

Potential year-to-year differences in baseline chick survival were investigated for negative controls (mock-injected in 2013-4 and unmanipulated in 2014-5 and 2015-6) using a mixed-effect Cox model (Therneau et al. 2003). Nest identity was treated as a random effect since some nests were studied over different years. This model was *survival ~ year + (1|nest)* with a Gaussian distribution assumed for the random effects.

(ii) Adult serological prevalence model. As an indication of *P. multocida* circulation in the study colony during the three reproductive seasons, *P. multocida* antibody prevalence in non-vaccinated

adults was estimated each year using the same microagglutination test used for chicks. Variation in antibody prevalence between years (figure S2) was analysed by logistic regression, fitting a generalised linear mixed model using the 'lme4' package (Bates et al. 2015) with individual identity as random effect.

Supplementary Text

Investigation into different vaccination strategies to improve chick survival

In order to optimize the vaccination protocol, we assessed the effect of different vaccination strategies on chick survival. Chicks were directly vaccinated in December of 2013, 2014, and 2015, while adults were vaccinated in December of 2013 to assess any positive effect of maternal antibody transfer for the 2014-5 (n+1) and 2015-6 (n+2) breeding seasons. Figure S1 shows the effect of these different vaccination strategies on chick survival for the three consecutive breeding seasons (direct chick vaccination: top three panels, and parent vaccination: bottom two panels).

(i) Direct chick vaccination. In 2013-4, vaccinated chicks received a first vaccine injection within four days of hatching, followed by a booster injection 12 ± 3.3 days later (mean ± SD). In 2014-5, the protocol was comparable with vaccinated chicks receiving one injection two days after hatching followed by a second injection 12 ± 0.9 days later. For these two seasons, the vaccine adjuvant was based on a white mineral oil (purified mixture of liquid saturated hydrocarbons). These protocols failed to yield statistically significant improvements in chick survival in 2013-4 (figure S1, top left; relative death risk of 0.71 [CI: 0.29, 1.72], p = 0.454) and 2014-5 (top centre, relative risk of 0.89 [CI: 0.48, 1.58], p = 0.694). Seroconversion rates of vaccinated chicks were also low for these seasons (respectively 3/22 and 7/28 in 2013-4 and 2014-5). In 2015-6 by contrast, there was only one injection on December 18th, *i.e.* an estimated 14 ± 3 days (mean ± SD) after hatching, and a different adjuvant formula was used (with an enriched light mineral oil and an extremely refined surfactant system without any ingredient of animal origin). This proved more effective, with strong protection afforded by direct chick vaccination that year (top right panel; see article main text for details) and seroconversion of all vaccinated chicks within two months.

Of note, the death risk for control chicks in 2014-5 and 2015-6 was twice that of 2013-4 (figure S1, top row). Relative to 2013-4, estimated risks were 2.05 [CI: 1.56, 2.68] (p = 0.007) in 2014-5 and 2.07 [CI: 1.56, 2.74] (p = 0.009) in 2015-6. Antibody prevalence in non-vaccinated adults (figure S2) was also higher in 2014-5 and 2015-6 than 2013-4 ($p < 0.05$; figure S2). Taken together, these results suggest markedly more intense avian cholera episodes in 2014-5 and 2015-6 compared to 2013-4.

(ii) Adult vaccination. If antibodies were directly protective, an alternative to direct chick vaccination could be the vaccination of breeding females. This may have the potential to protect their offspring *via*

the transfer of maternal antibodies. In another Procellariiform species, maternal antibodies can persist at detectable levels for several weeks after hatching (Garnier et al. 2012).

Breeding females were vaccinated during the late egg incubation and early chick rearing period, between November 21st and December 16th of 2013. We detected no statistically significant effect of the 2013 vaccination of breeders (compared with 0.9% NaCl injection) on chick survival in 2014-5 (figure S1, bottom centre panel; relative death risk of 0.62 [CI: 0.33, 1.15], p = 0.13) or in 2015-16 (bottom right panel; relative risk of 0.81 [CI: 0.36, 1.85], p = 0.63). This could be due to low levels of persisting antibodies at the time of egg laying in females vaccinated in a previous year, resulting in low antibody transfer to chicks (Ramos et al. 2014) and shorter persistence (Grindstaff 2010). Booster injections in breeding females in the years following initial immunization could potentially ensure higher levels of circulating antibodies at the time of transfer.

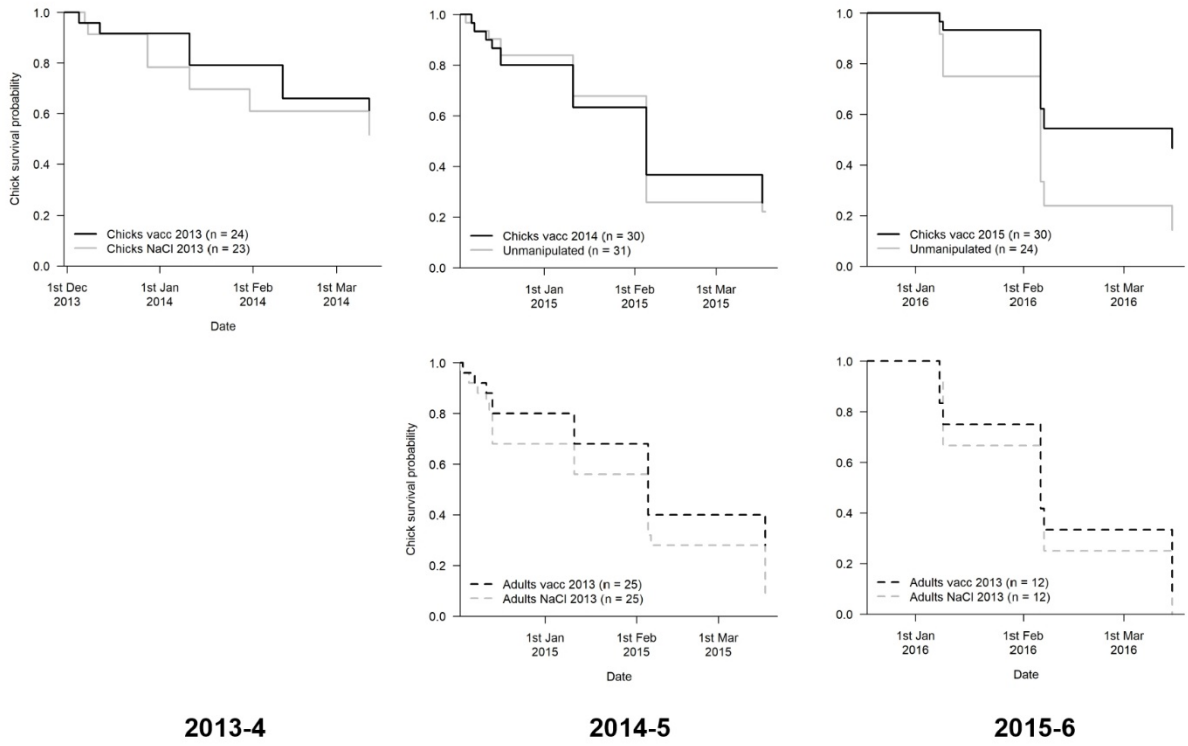

Fig. S1. Survival curves for chicks submitted to different treatments over the three reproductive seasons 2013-4 (left), 2014-5 (centre), 2015-6 (right). Chicks were either vaccinated directly (top row), or had their parents vaccinated in December 2013 (bottom row). Chicks were vaccinated at a later age and with a different adjuvant formula in 2015-6 than in the two previous years (detailed in text above).

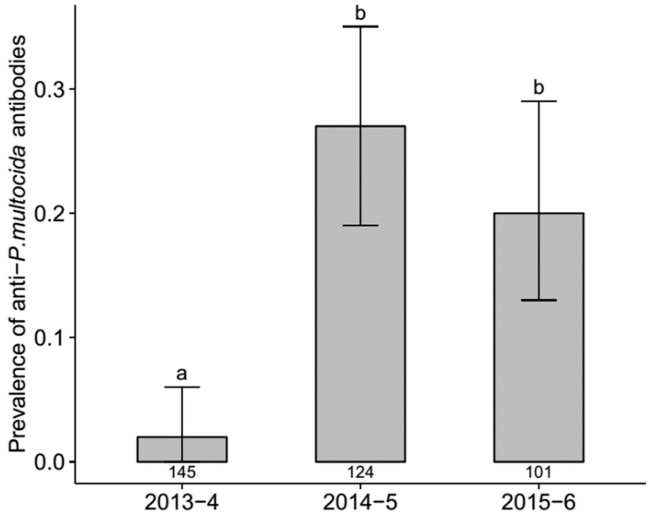

Fig. S2. Prevalence of antibodies to *P. multocida* in non-vaccinated adult Indian Yellow-nosed albatrosses sampled in November-December on Amsterdam Island over three reproductive seasons. Figures below bars indicate the number of individuals sampled and error bars show the Clopper-Pearson 95% confidence interval. In 2013 (a), seroprevalence was significantly lower than in 2014 and 2015 (b) ($p < 0.05$, mixed effect logistic regression).

Supplementary references

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