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## Vaccination protects endangered albatross chicks against avian cholera

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**Abstract:** Global change is contributing to unprecedented expansions of infectious diseases in wildlife. Recurrent avian cholera outbreaks are causing dramatic chick mortality and population decline in endangered albatross colonies on Amsterdam Island, a critical seabird breeding ground in the Southern Indian Ocean. We manufactured a killed vaccine using a *Pasteurella multocida* strain isolated from a dead albatross in the field. We used this same bacterial strain to establish a serological assay allowing the monitoring of antibody levels following bird vaccination. Using this vaccine on chicks 2 weeks post-hatching caused 100% seroconversion and reduced the death risk by a factor exceeding 2.5, raising fledging probability from 14% to 46%. These results suggest that using a specifically tailored vaccine could be a key tool to effectively protect endangered seabirds from disease outbreaks threatening them with extinction.

## Introduction

Emerging infectious diseases are a growing concern for both public health and wildlife conservation (Daszak et al. 2000; Smith et al. 2009). The globally connected marine environment is especially

sensitive to environmental change, and oceanic ecosystems are increasingly suspected to be “incubators and conveyors” of diseases (Harvell et al. 1999, 2002). Understanding and managing infectious diseases in marine ecosystems is thus a current priority (Lafferty & Hofmann 2016), especially at higher latitudes which include some of the places most dramatically impacted by climate change (Van Hemert et al. 2014).

Colonial seabirds are particularly sensitive to changes in their local, regional and global environments (Croxall et al. 2002) and infectious disease outbreaks. Given the high mortalities it provokes and its recurrent appearances in affected populations, avian cholera, caused by the bacterium *Pasteurella multocida*, has the potential to threaten several wild bird species (Botzler 1991; Samuel et al. 2007). This is notably true of birds aggregating at high densities at a time of their life cycle, such as Arctic eider ducks (Descamps et al. 2012), penguins (Cooper et al. 2009), Cape cormorants (Waller & Underhill 2007), and albatrosses (Weimerskirch 2004). Currently, because of the high mortality this disease may cause in these species, attention to albatrosses and large petrels affected by avian cholera is considered an “utmost priority” (reviewed in Uhart et al. 2017).

Amsterdam Island (37.8 °S, 77.6 °E) is a ~55 km<sup>2</sup> isolated volcanic island that hosts two thirds of the global population of the endangered Indian yellow-nosed albatross (*Thalassarche carteri*; figure 1) and a small sub-population of dark-mantled sooty albatrosses (*Phoebastria fusca*). Since the 1980’s, chicks of these two species experience recurrent and dramatic mortality episodes attributed to avian cholera (Weimerskirch 2004). By contrast, the disease does not seem to affect the survival of adults (Rolland et al. 2009). Although the exact case-fatality rate in chicks is not known, acute infection in chicks often results in rapid death, as in poultry exposed to virulent strains of *P. multocida*. These intense chick mortality episodes lead to near-zero chick productivity in some years, a major cause of overall population decline in Indian yellow-nosed albatrosses (Rolland et al. 2009). Importantly, Amsterdam Island is also home to the critically endangered and endemic Amsterdam

albatross (*Diomedea amsterdamensis*) (IUCN 2013). Although its population has increased over the past decades, the Amsterdam albatross has also suffered from marked chick mortality in 2000 and 2001 (Weimerskirch 2004). For these reasons, avian cholera is considered the most severe global infectious threat to albatrosses (Phillips et al. 2016; Uhart et al. 2017).

One approach to limit the impact of deadly epizootics in wildlife is to promote the resilience of host populations through vaccination (Groner et al. 2016). However the availability of vaccines combining safety and efficacy against wildlife pathogens is limited. Here, we manufactured an autogenous vaccine based on a field strain of *P. multocida* and used it in the wild to test its ability to protect albatross chicks from avian cholera mortality.

## Methods

### Vaccine preparation

*Pasteurella multocida* strain D2C was isolated from a dead dark-mantled sooty albatross chick collected at an Amsterdam Island colony during the 2011-2 breeding season, and typed using lipopolysaccharide (LPS, somatic) antibodies as Heddleston serotype 1 and Namioka serotype 7. The Heddleston test is widely used to differentiate *P. multocida* strains based on LPS type, although it should be noted that this test may yield relatively frequent ambiguous results, especially compared with a recently developed PCR typing test (Harper et al. 2015). Strain D2C was grown on solid soybean-casein digest agar with yeast extract in a 500 cm<sup>2</sup> tissue culture flask at 37°C for approximately 22 hours. The bacteria were then collected in 20 mL of phosphate buffered saline (PBS), their concentration was estimated using optical density at 580 nm, and they were inactivated (killed) with a 34-38% formaldehyde solution at room temperature ( $20 \pm 5$  °C). After centrifugation at  $2,934 \times g$  for 30 min, the bacterial pellets were resuspended in PBS. Mineral oil adjuvants were used to obtain water-in-oil emulsions containing the equivalent of  $3 \times 10^9$  colony-forming units (CFU) per

millilitre (2013 and 2014 vaccination campaigns with 50% v/v adjuvant) or  $1 \times 10^9$  CFU/mL (2015 campaign with 60% v/v adjuvant). Water-in-oil emulsions are extensively used for the formulation of inactivated avian vaccines, where they have proven to be strong immunity adjuvants (Hilgers et al. 1998; Jansen et al. 2005, 2006). Several innocuity and efficacy tests conducted on a series of avian species (chickens, turkeys, and ducks) had indicated that these were appropriate antigen doses to use with these killed vaccine formulations. Endotoxin content of the 2015 vaccine was less than 5000 EU/mL, as assessed using the kinetic chromogenic method of the European Pharmacopoeia.

The inactivated bacteria were also used to set up an agglutination assay (detailed in Supplementary Online Material, SOM) to detect antibodies directed against this or potential cross-reactive strains in bird plasma samples.

### **Field procedures**

Rigorous biosecurity measures were observed in the field to avoid spreading the disease between bird colonies and individual birds within the colonies. These involved the use of dedicated clothing consisting of a different set of waterproof leggings, jackets and rubber boots for each bird colony. Waterproof overalls, any other equipment (e.g. calliper, ruler) and hands were disinfected between contacts with any two birds, respectively using hydrogen peroxide, commercial disinfectant wipes, and hydro-alcoholic gel. All pieces of clothing were thoroughly cleaned and disinfected again at the end of each day.

Indian yellow-nosed albatrosses lay only one egg and thus raise at most one chick per nest each year. Seronegative chicks from 30 nests were injected subcutaneously with 0.5 mL of autogenous vaccine on December 18<sup>th</sup> 2015 (an estimated  $14 \pm 3$  days after hatching, mean  $\pm$  SD). Vaccination date was optimized based on the results from previous vaccination trials in 2013 and

2014 (detailed in SOM.) Two control groups were used to compare their survival with that of the *vaccinated* group. In the first control group, chicks were not vaccinated but were *sampled* to monitor potential seroconversion (n=12). In the second control group, chicks were left entirely *unmanipulated* to assess baseline reproductive success and any potential adverse effect of vaccination or handling on survival (n=24). The nests from the three groups were mixed and dispersed over the same area.

Following injection, chick survival was assessed on three occasions in January, February and March 2016, fledging occurring in late March. Survival of chicks was analysed using the Cox proportional hazards model as detailed in SOM. Blood samples were also taken monthly to investigate seroconversion. Plasma samples were anonymised and randomised before serological testing.

## Results

Serological analyses showed seroconversion of all vaccinated chicks by February 8<sup>th</sup> 2016 (table 1), with mean titres increasing until March 2016 (figure 2a). By contrast, none of the sampled unvaccinated chick seroconverted over that period. Importantly, chick vaccination provided a strong protection against mortality (figure 2b). End survival probability was 46% for the vaccinated chicks *versus* 14% for the unmanipulated controls, the death risk at a given time being divided by 2.6 [95% confidence intervals or CI: 1.31, 5.07] ( $p = 0.006$ ); Cox proportional hazards model. The death risk was also divided by 3.1 [CI: 1.44, 6.72] ( $p = 0.004$ ) when comparing vaccinated to unvaccinated sampled chicks. No survival difference was detected between the sampled (and unvaccinated) *versus* unmanipulated control groups (death risk divided by 0.82 [CI: 0.40, 1.69],  $p = 0.602$ ).

## Discussion

To our knowledge, this is the first immunization trial in albatrosses, and the first evaluation of an autogenous vaccine in a threatened animal population. Vaccination programmes targeting endangered species in the wild generally endeavour to maximize protection of the target population (López et al. 2009; Livingston et al. 2013; Malakoff 2016) and thus do not include negative control groups. The present study directly demonstrates the efficacy of an autogenous vaccine in protecting endangered albatrosses against lethal infection in a controlled field trial.

Vaccine production was inspired from a process initially developed for the poultry industry, with analogies to the production of a bacterin that had shown promising results in giant Canada geese (*Branta canadensis maxima*) (Price 1985) and was later used to investigate the effect of avian cholera on adult survival in lesser snow geese (*Anser caerulescens caerulescens*) (Samuel et al. 1999). Multi-locus sequence typing (MLST) using seven housekeeping genes (*adk, est, gdh, mdh, pgi, pmi, zwf*) (Subaaharan et al. 2010) showed no variation on *Pasteurella* isolated on Amsterdam Island from 3 albatross individuals sampled in 2011-2 and 3 individuals sampled in 2012-3. This analysis (carried out by AJ and colleagues) showed that the isolates all belonged to multi-locus sequence type 61. Only one strain was thus included in vaccine preparation. However, *P. multocida* strains can mutate in antigenic LPS regions that are not examined using MLST, and the potential for heterologous protection across different serotypes with this killed vaccine was not examined. Live mutant *P. multocida* vaccines have been shown to offer better protection against strains differing in their LPS structure compared to killed vaccine (Harper et al. 2016) and cross-serotype protection (Scott et al. 1999). However, the concern that live vaccines might revert to virulence, or be detrimental in immunocompromised hosts, precludes their use in endangered albatrosses and other protected wild animal populations including seabirds on Amsterdam Island. In the future, it will be feasible and might be advisable to manufacture multivalent killed vaccines including different strains of *P. multocida*, notably with different LPS structures to prevent the apparition of “vaccine escape mutants”



with, for instance, shorter LPS. Other bacterial species could also be included if deemed relevant. For the time being, the spectacular improvement in chick survival following immunization with a monovalent (anti-*Pasteurella* only) vaccine is one further confirmation of the role of *P. multocida* in chick die-offs.

Choosing an optimal date for chick vaccination is important. If vaccinating too early, the chick immune system may still be immature and unable to mount a protective response (Mast & Goddeeris 1999), as suggested by the negative results of the 2013 and 2014 early vaccination protocols (SOM). On the other hand, excessive delay in vaccination may result in an epidemic occurring before chicks are effectively protected. Vaccinating when most chicks were 10-15 days old (mid-December) proved an effective compromise. Complete seroconversion (in the sense of plateauing antibody titres) took well over a month following vaccination (figure 2a), but differential survival could be noted as early as January, when antibody titres in vaccinated chicks were still low. Thus even low antibody titres appear associated with protection, and the vaccine may also stimulate other arms of the immune system before antibody titres peak.

The worrying conservation status of numerous albatross species has prompted the Agreement on the Conservation of Albatrosses and Petrels (ACAP). Given the dramatic consequences of these infections in terms of chick mortality, which can severely impact population viability (Finkelstein et al. 2010), increase in chick survival through vaccination may be a key element in albatross conservation. Successfully vaccinated birds fledged in March 2016 have not yet returned to the colony to breed. Therefore, it is still unclear how long the benefits of vaccination of a given individual are likely to last, and whether there may be long-term benefits of chick vaccination beyond increasing the fledging probability. If the effect of vaccination is limited to improving the fledging rate, models developed in Rolland et al. (2009) suggest that the improvement observed in the present study might not be sufficient to stabilize the population, although slight differences in adult mortality or

emigration can strongly affect the outcome. Also, in this trial we vaccinated only about a tenth of the local study colony, itself accounting for about 1% of the Amsterdam Island population of yellow-nosed albatrosses (Rolland et al. 2009). Therefore, the trial likely stands well below the ‘population immunity threshold’ above which one infectious individual would not cause an outbreak (Feng et al. 2015). By contrast, vaccination programmes involving a high coverage of local albatross populations may benefit from substantial herd immunity effects, proportionally providing even greater protection to the chicks. The possible protection afforded to chicks via the vaccination of mothers and the recurrent transfer of antibodies via the egg yolk is another perspective to explore further (Garnier et al. 2012).

The suite of issues involved in the decision of vaccinating a wild threatened population is quite complex. Scaling up vaccination as a management tool in this system will also necessitate a refined understanding of factors affecting the frequency and timing of epizootics. Modelling studies comparing different vaccination scenarios (Haydon et al. 2006; Garnier et al. 2012) may then help anticipate the outcome of vaccinating yellow-nosed albatrosses or other species in the system. For instance, vaccination may directly benefit the minute population of critically endangered Amsterdam albatross. The vaccination of Brown skuas (*Catharacta antarctica lonnbergi*), whose predatory behaviour, substantial infection rates and high mobility could make them key epidemiological actors (Boulinier et al. 2016), might also prove helpful in controlling the negative effects of avian cholera on Amsterdam Island. Of note, without a much clearer understanding of the epidemiological reservoirs on the island, local extirpation of the pathogen through vaccination only is probably not a realistic perspective at this stage, and further investigations into disease dynamics are certainly warranted.

Finally, avian cholera affects wild bird populations in other very remote areas (Leotta et al. 2006; Iverson et al. 2016). The presence of such infections in these isolated systems raises the question of their origin. On Amsterdam Island, some hypotheses involve human activities, such as a

poultry pen that was active until 2006 (Weimerskirch 2004) or the presence of carrier rats introduced involuntarily about a century ago. In the past, other domesticated animals potentially acting as reservoirs (reviewed in Wilson & Ho 2013), such as cattle and dogs, were present on the island; a small number of feral cats still remain. Beyond the academic interest, understanding the origin of the diseases may also have ethical implications on the appropriateness of bold human intervention such as vaccination in wild ecosystems (Cleaveland 2009), where infectious diseases may be a natural component. However, the high density of birds on Amsterdam Island, representing a significant proportion of the global population of the endangered Indian yellow-nosed albatross, and the seemingly strong potential for this pathogen to drive some of these species of conservation concern closer to extinction (Phillips et al. 2016; Uhart et al. 2017) seems to provide an unusually compelling case for intervention with vaccination.

In conclusion, this report identifies a way of promoting resistance of endangered albatross populations to a major infectious threat. This tool will be relevant to the applied conservation of these and other species suffering high levels of avian cholera (and potentially other bacterial disease) mortality. As such, it should be of interest to governments, non-governmental organizations, and other sustainable development policy actors (such as the international Commission for the Conservation of Antarctic Marine Living Resources) involved in the conservation of wildlife threatened by emerging infectious diseases.

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### Figure legends

**Fig. 1.** Adult Indian yellow-nosed albatross over dead chick at the Entrecasteaux cliffs on Amsterdam Island (37.8°S, 77.6°E) where recurrent massive chick mortality events have been occurring.



**Fig. 2.** a) Seroconversion and b) Survival curves for Indian yellow-nosed albatross chicks that were vaccinated (red, n = 30), or unvaccinated (blue) and either left entirely *unmanipulated* (solid line, n = 24) or *sampled* (dotted line, n = 12) in the 2015-6 reproductive season. Error bars indicate standard error of the mean antibody titres (expressed as natural log(serial serum dilutions+1)).

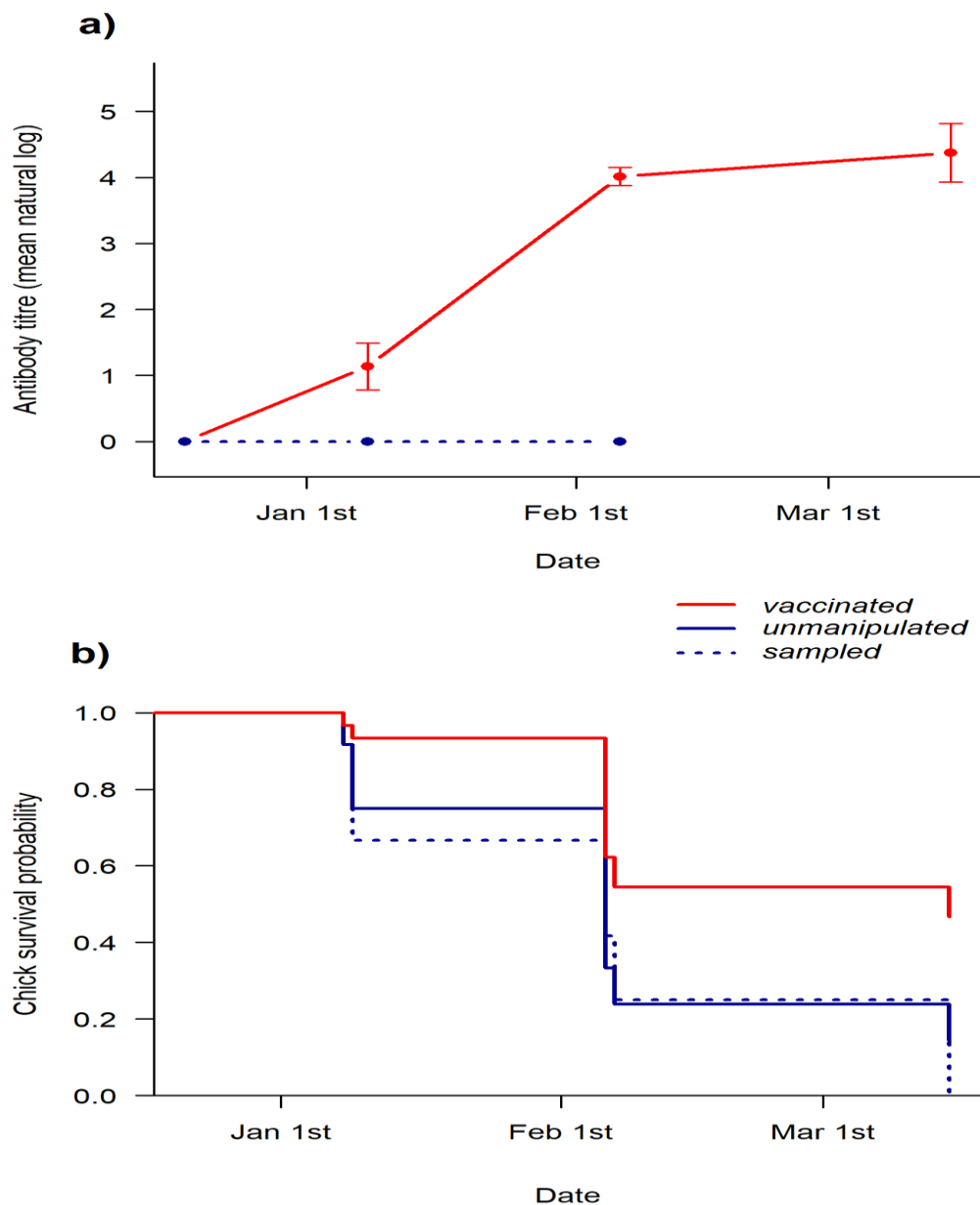


Table 1. Number of albatross chicks that were seropositive/sampled for serum during the 2015-6 reproductive season according to vaccination status.

Treatment group	December 2015	January 2016	February 2016	March 2016
Vaccinated	0/29*	8/28	16/16	11/12
Unvaccinated, sampled	0/11*	0/8	0/3	0/0

\*In December, one chick in each group could not be sampled