Supporting Information

Exposure of breeding albatrosses to the agent of avian cholera: dynamics of antibody levels and ecological implications

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Appendix S1. Study population and study design

Study site

Figure S1.1. Indian yellow-nosed albatrosses breed in high density in the Entrecasteaux cliffs, Amsterdam Island (left). In the monitored sub-colony, individuals are leg-banded and nests are tagged (right). Pictures: Thierry Boulinier/IPEV.

Nestling age

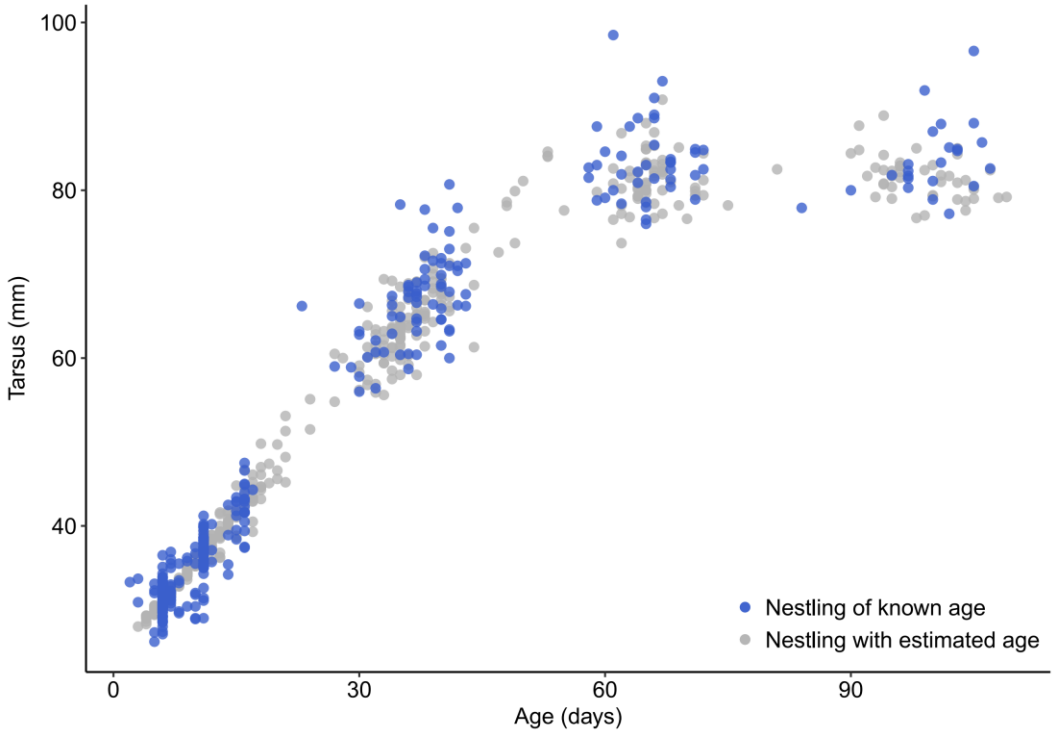

Figure S1.2. Tarsus length to age relationship in Indian yellow-nosed albatross nestlings. The relationship was determined using a linear mixed model linking tarsus length to the age of nestlings for which hatching date was known, accounting for an individual random effect. In order to increase the sample size, some of the nestlings integrated to this model were recruited in the study colony as part of another experiment (Bourret et al. 2018). As this relationship was stronger for younger individuals, only data from nestlings younger than 20 days were used. The determined relationship was [95% c.i.]: tarsus length = 24.3 [23.5; 25.0] + 1.13 [1.07; 1.19] × age (n = 178 measures). Hatching date was predicted for other nestlings based on this relationship and the first measurement of tarsus length. This relationship was checked by evaluating the correlation between true age and predicted age on one random observation per nestling for individuals for which hatching date was known (intercept [95% c.i.] = 0.56 [-0.71;1.86], slope [95% c.i.] = 0.96 [0.93;1.00], $r^2 = 0.97$, n = 96 individuals).

Biosecurity

Biosecurity measures were followed to avoid spreading infectious agents between handled birds. Whenever possible, single use material was favoured. Fieldworkers wore waterproof clothes allowing efficient cleaning between each individual. A hydrogen peroxide solution and disposable wipes were used to disinfect reusable material and handler clothes between each individual. Hands were cleaned using a hydroalcoholic gel.

Sample sizes

Table S1.1. Number of (re)captured individuals per treatment.

Breeding season and manipulation	Adults			Nestlings		
	NaCl-injected in 2013	Vaccinated in 2013	Vaccinated in 2013 and 2015	Parents NaCl-injected in 2013	Parents vaccinated in 2013	Parents vaccinated in 2013 and 2015
Sampled and injected in 2013-2014	67	67	-	-	-	-
Sampled the weeks following injection in 2013-2014	44	40	-	-	-	-
Sampled in 2014-2015	57	50	-	20	19	-
Sampled in 2015-2016	29	12	13	13	10	-
Sampled in 2016-2017	25	11	10	9	4	4

Appendix S2. Details of the immunoassays

Description of the procedures

The microagglutination tests (MAT; SEROPAST®, Ceva Biovac, France) was run following the procedure described by Bourret et al. (2018) and agglutination at the 1:10 dilution was used as the seropositivity threshold. Results are expressed as titre (log2[dilution/10]+1).

The enzyme-linked immunosorbent assay (ELISA; ID Screen® Pasteurella multocida Chicken and Turkey Indirect, IDvet, France) was run following the manufacturer instructions. Results are expressed as the optical density (OD) read at 450nm after correction for inter-plate variations (see Lobato et al. 2011). Because the ELISA kit used in this study was originally made for chicken samples and because the conjugate (anti-chicken IgY) affinity for albatross IgY may differ from the one from chicken IgY, the positivity threshold had to be adapted. For this, we use the method described by Garnier et al. (2017) relying on fitting a mixture of normal distributions to the values of ODs. In order to improve the calculation of the ELISA positivity threshold, ODs of additional samples collected from yellow-nosed albatrosses in the same colony as part of other studies (Bourret et al. 2018) were included in this analysis. As many individuals were sampled several times, we avoided data non-independence issues by using the mean threshold determined on 1000 data subsets including only one random sample per individual. The threshold value was estimated as the mean of the normal distribution of the negative samples + 2 standard deviations, corresponding to a 95% confident interval around the distribution of negative samples. The ELISA positivity threshold was thus set at an OD of 0.16 (0.16 ± 0.01, n = 687 samples from 247 individuals; Fig. S2.1).

Both the MAT and the ELISA proved repeatable. On a subset of samples, the MAT proved to be repeatable ($r^2 = 0.87$, n = 10). For the ELISA, high repeatability was observed both intra-plate ($r^2 = 0.98$, n = 10) and inter-plate (all $r^2 > 0.97$, n = 6 to 8 replicated samples on 10 plates) levels.

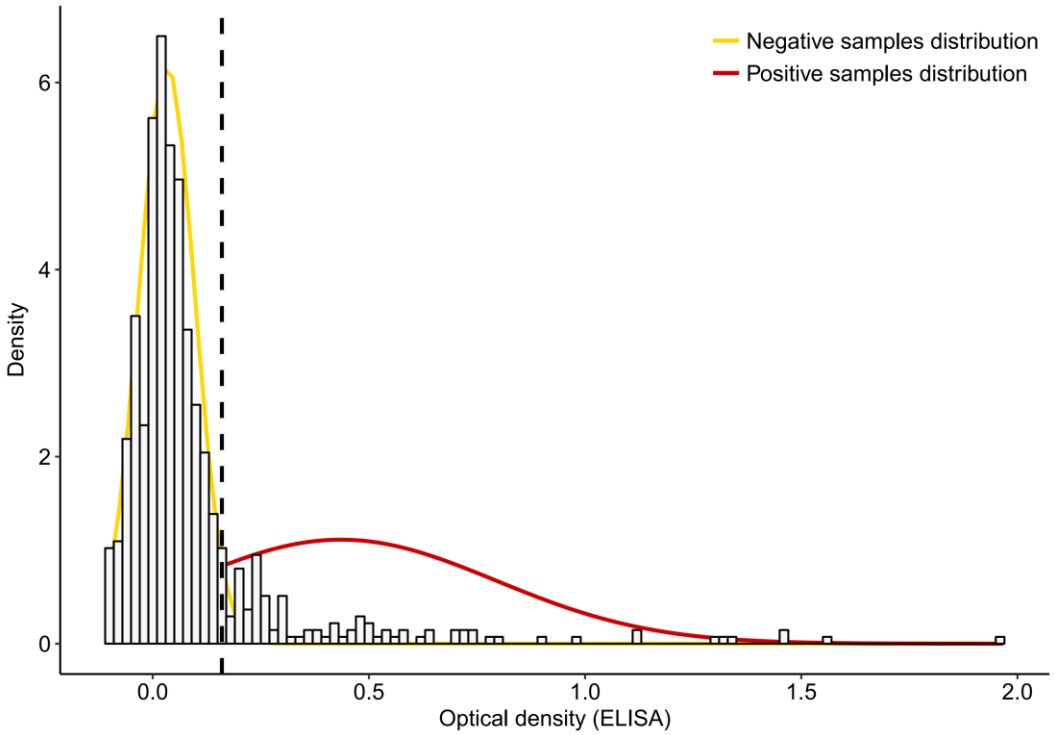

Figure S2.1. Distribution of ODs for Indian yellow-nosed albatrosses used to quantify the immune response against *Pasteurella multocida*. The histograms present the normalized counts of individuals and the curves correspond to the probability density function of the fitted two normal distributions (negative and positive samples). The threshold value (dashed line) was estimated as mean of the negative normal + 2 s.d.

Sample analyses

Most adult samples were analysed by both MAT and ELISA (n = 447, Table S1), although due to limited volumes of plasma, some were analysed only by MAT (n = 24). Similarly, most nestling samples were analysed using both methods (n = 131), except because of low volumes (n = 26) and in 2015-2016 when low antibody levels of parents suggested that no maternal transfer of immunity could have occurred (n = 69; see results); these samples were nevertheless analysed by MAT.

Table S2.1. Details of plasma sample analyses.

Immunoassay	Adults		Nestlings	
	Individuals	Samples	Individuals	Samples
MAT and ELISA	132	447	55	131
MAT only	2	24	28	95
ELISA only	0	0	0	0
Total	**134**	**471**	**83**	**266**

Individual CF45997

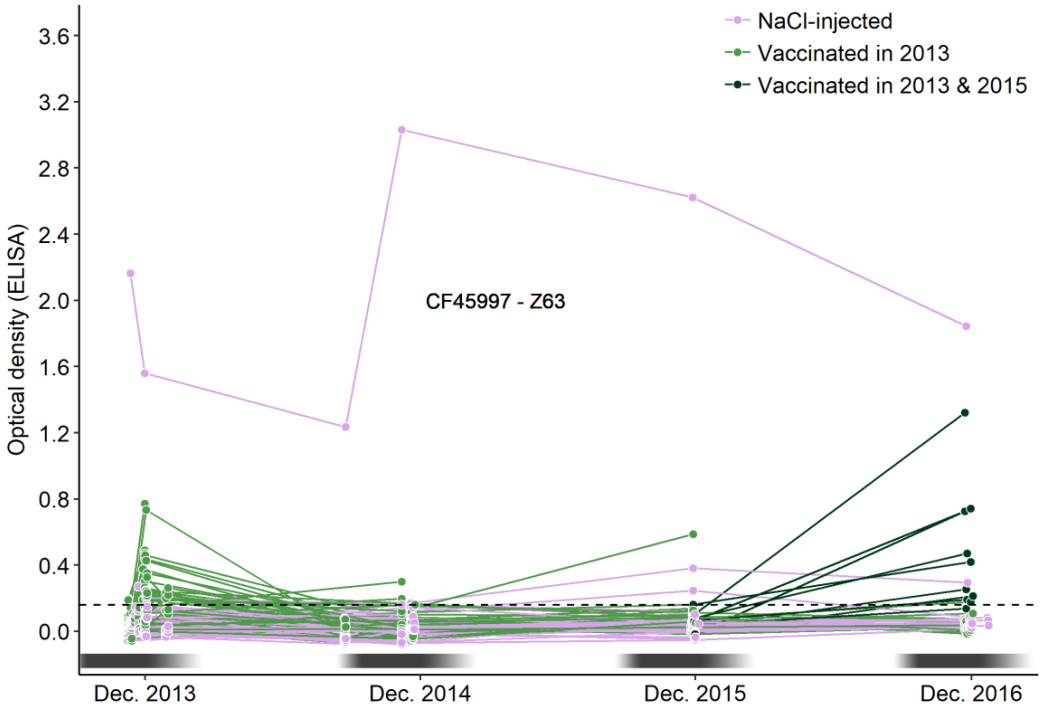

Figure S2.3. Anti-*P. multocida* antibody dynamics based on ELISA results in adult Indian yellow-nosed albatrosses after injection of NaCl or an autogenous vaccine between November and December 2013. Boosted individuals received a new vaccine injection in December 2015. The positivity threshold is denoted by a dashed line. Nest attendance by adults is schematized by the black and white line, the darker the line, the higher is the attendance. One NaCl-injected individual (metal ring "CF45997", Darvic® ring "Z63") showed surprisingly consistently high values (top curve) and was not considered in the analyses presented in this study; the same samples were negative or weakly positive by MAT (titers of 2 in September 2014 and 1 in December 2015, negative otherwise).

Comparison of the immunoassays

In the present study, we explored the use of a specifically designed MAT and of a commercially available ELISA to monitor *P. multocida* circulation in a wild population and response to vaccination. The results suggest a higher sensitivity of the MAT to detect natural exposure to *P. multocida* while both methods were efficient to detect response to vaccination. Closely related technics have already been validated in poultry and also highlighted important variations between assays (e.g., Solano, Giambrone & Panangala 1983, Liu *et al.* 2017). Such easily available methods, particularly concerning the ELISA, constitute useful tools for eco-epidemiological studies involving *P. multocida* in wild bird populations. Several possible reasons exist to explain the higher sensitivity of the MAT in the present case. First, the bacterial strain used to manufacture the ELISA may share less epitopes with the Amsterdam strain used

to develop the MAT: this would result in higher numbers of false negative individuals using the ELISA. However, if the MAT is indeed very strain-specific, it may prove less useful in other systems or to detect different strains. Second, the MAT and the ELISA detect different antibody isotypes produced with different kinetics in response to an exposure to *P. multocida*. MATs detect agglutinating antibodies, which are mostly IgM and less importantly IgY (Tizard 2004), while the used ELISA specifically measures IgY as it relies on labelled anti-IgY antibodies. Third, the MAT was calibrated on albatross plasma from the study site while the ELISA was on poultry. Although the use of anti-chicken IgY to detect albatross IgY has previously been validated (Garnier *et al.*, 2017), the protocol may need to be adapted to albatrosses to reach better performances, for instance by modifying various reagent concentrations. In any case, the results suggest that ELISAs and MATs may be complementary assays capturing different components of the immune response, potentially informing on the time since infection or repeated exposures (e.g., Peeling et al. 2010). Overall, both assays are highly repeatable but their ability to detect past exposure to *P. multocida* is less than one. This lack of sensitivity likely results from the fact that each assay only targets certain types of immunoglobulins binding only certain *P. multocida* antigens. Using repeated sampling designs, as suggested when using direct infectious agent detection data (McClintock *et al.* 2010), is thus not expected to improve the detection probability of past exposure events. In contrast, combining different assays should efficiently strengthen the inference of past exposure events.

While the results highlight that interpretation of serological data should be made cautiously (e.g., using only the ELISA could have led to an underestimation *P. multocida* circulation in the study colony), integrating complementary sources of information such as MATs and ELISAs may help gaining a better understanding of infectious disease dynamics (Borremans *et al.* 2016, Buzdugan *et al.* 2017).

Appendix S3. Additional results

Immune response after natural exposure

Table S3.1. Model selection for the prevalence of anti-*P. multocida* antibodies in non-vaccinated adult yellow-nosed albatrosses after egg-laying measured using a MAT and an ELISA. Annual variations in seroprevalences were explored using generalized linear mixed models with individual and nest identity as random effects in the model. Bold Akaike Information Criterions (AIC) indicate the selected models.

Fixed effects	Degrees of freedom	AIC	
		MAT	ELISA
.	3	172	**70**
Season	6	**157**	69

Table S3.2. Outputs from the generalized linear mixed model run on the data of anti-*P. multocida* antibody detection using a MAT. Individual and nest identity were included as random effects in the model.

	Estimate ± standard error	Odds ratio [95% confidence interval]
Season 2013/2014 (intercept)	-5.91 ± 1.85	-
Season 2014/2015	3.74 ± 1.11	42.26 [4.81; 371.16]
Season 2015/2016	3.73 ± 1.09	41.82 [4.96; 352.24]
Season 2016/2017	4.22 ± 1.31	68.04 [5.21; 888.29]

Short-term booster injection

In order to investigate the short term antibody response to vaccination and because repeated injections have been reported to enable better protection in poultry (e.g., Hofacre, Glisson & Kleven 1987, Perelman et al. 1990), an attempt was made to recapture all individuals within a two to three-week period following the first injection (received in November or December 2013) to blood sample them and perform a second vaccine or NaCl injection; 37 birds thus received the second injection of NaCl and 34 of vaccine 10 to 24 days after the first injection (mean ± s.d. = 19 ± 4 days). The new injection administrated in 2015-2016 was given irrespectively of the number of injections they had received in 2013-2014 (six had received two injection, and seven had received one).

A short-time booster effect was detected by MAT on antibody levels one year after vaccination (W = 415.5, p < 0.01, n = 27, 20). However, this effected was not detected by ELISA (W = 232, p = 1.00, n = 27, 20; Fig. S3.1 a and b). Two years after vaccination, both serological methods showed no effect of a short-term booster injection on antibody levels (MAT: W = 107.5, p = 0.35, n = 13, 12; ELISA: W = 95, p = 0.76, n = 13, 12; Fig. S3.1 c and d). Raw return rates did not suggest an effect of the number of injections one year after vaccination (28/34 for the birds that received two injections in 2013 *versus* 29/33 for the birds that received one). Vaccinated individuals were thus included in the vaccinated group irrespective of the number of injections they received in 2013-2014 in order to improve statistical power while being conservative with regards to the inter-annual persistence of antibody levels following vaccination. In addition, failure to complete vaccination protocols including several injections is likely to occur frequently in wild populations; our analyses thus present realistic results with regards to the potential implementation of vaccination programs in wild seabirds.

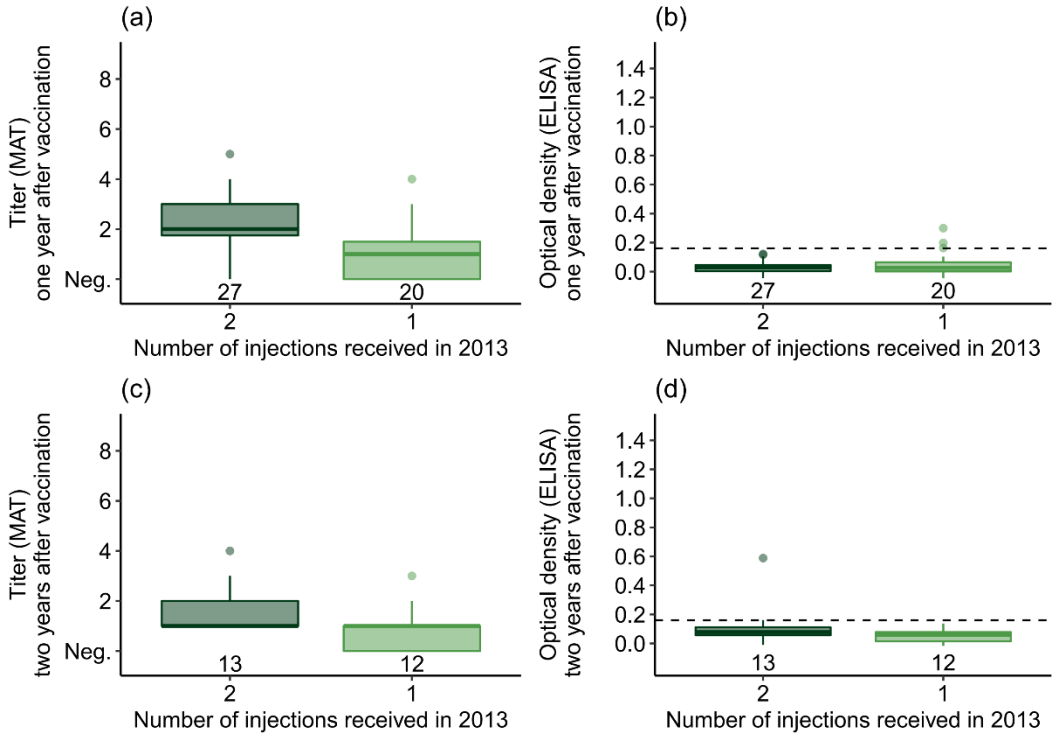

Figure S3.1. Quantification of anti-*P. multocida* antibodies by MAT (a, c) or ELISA (b, c) in adult Indian yellow-nosed albatrosses one (a, b) or two (c, d) years after the injection of an autogenous vaccine using a one or two-injection protocol. MAT non-null titers were considered positive; ELISA positivity threshold is denoted by a dashed line. Sample sizes are reported below the boxes.

Individual qualitative data

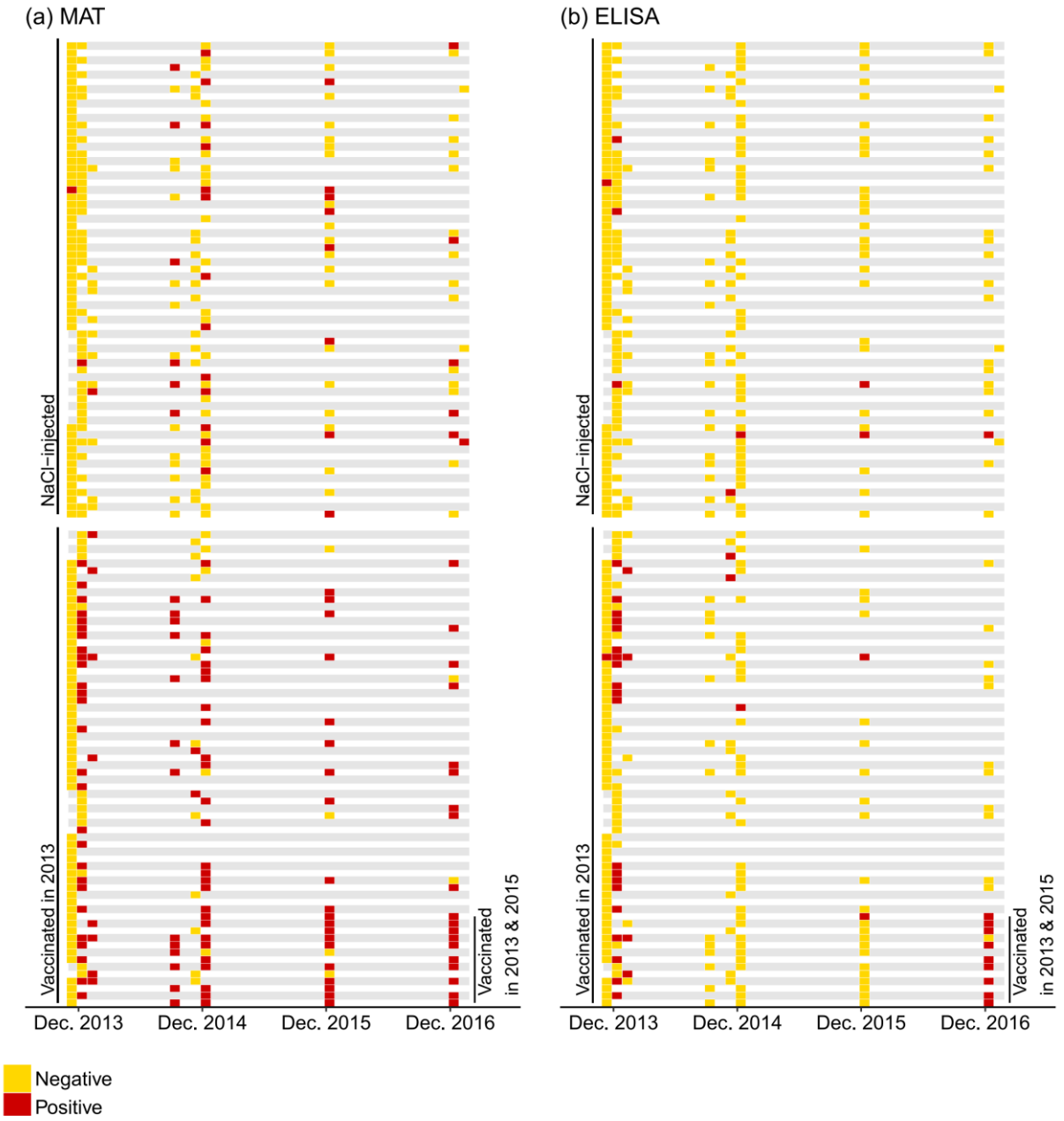

Figure S3.2. Individual histories of anti-*P. multocida* antibody detection by MAT (a) or ELISA (b) in adult Indian yellow-nosed albatrosses after injection of NaCl or of an autogenous vaccine between November and December 2013. Boosted individuals received a new vaccine injection in December 2015. Each line represents an individual and each column a month. Light yellow indicates a seronegative status and dark red a seropositive status.

Seroprevalences

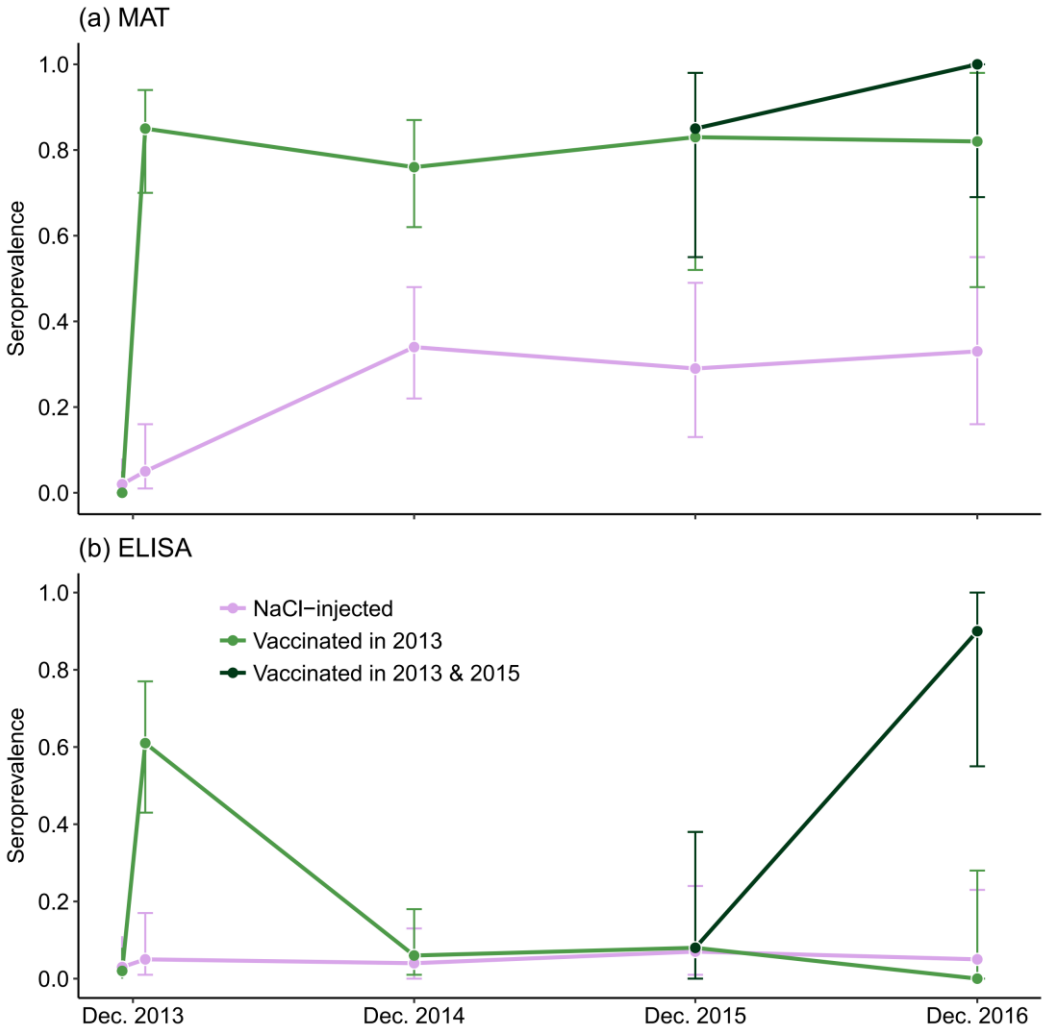

Figure S3.3. Prevalence of anti-*P. multocida* antibodies determined by MAT (a) or ELISA (b) in adult Indian yellow-nosed albatrosses after injection of NaCl or of an autogenous vaccine between November and December 2013. Boosted individuals received a new vaccine injection in December 2015. Bars represent the 95% c.i.

ELISA individual dynamics

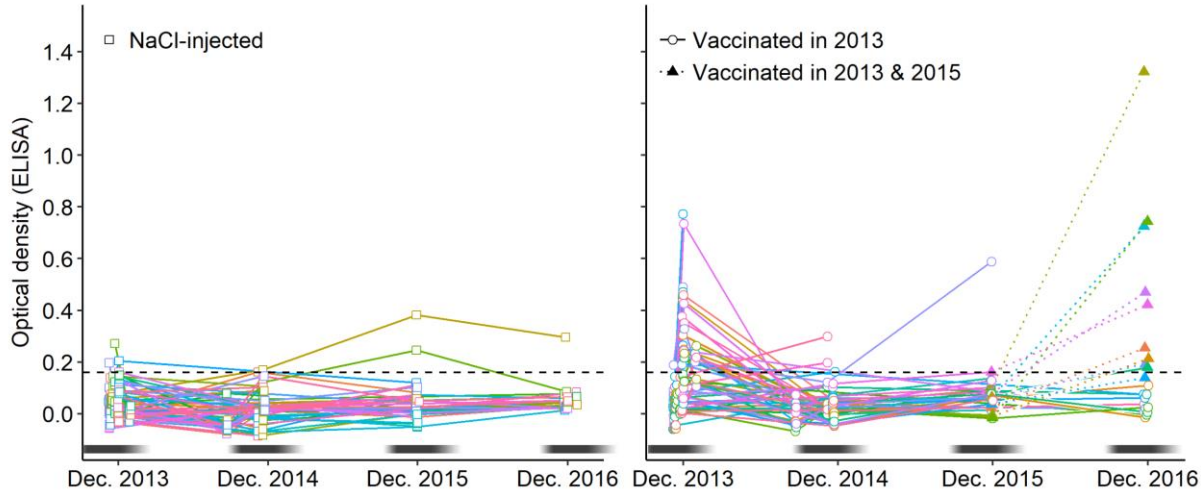

Figure S3.4. Anti-*P. multocida* antibody individual dynamics based on ELISA in adult Indian yellow-nosed albatrosses after injection of NaCl (left) or of an autogenous vaccine (right) between November and December 2013. Boosted individuals received a new vaccine injection in December 2015. Each line represents an individual. The positivity threshold is represented by a dashed line. Nest attendance by adults is schematized by the black and white line, the darker the line, the higher is the attendance. All vaccinated individuals receiving a new vaccine injection two years after the initial injection (*i.e.*, 2015-2016) mounted an immune response detectable by ELISA one year later (one individual being ELISA-negative but having an OD close to the positivity threshold and higher than before the new vaccine injection).

Modeled response to vaccination

Individual antibody level dynamics after vaccination was modeled using a mechanistic function inspired from Simonsen et al. 2009 and based on the codes supplied by Pepin et al. 2017. This model was fitted using the Levenberg-Marquardt nonlinear least-squares algorithm with the '*minpack.lm*' R package (Elzhov et al. 2010; see codes in Appendix S5 for more details). As many individuals were sampled several times, we avoided data non-independence issues by using the mean threshold determined on 1000 data subsets including only one random sample per individual. Only the response following the first injection (December 2013) was thus modeled because natural outbreaks occurred the following years, interfering with the vaccine induced response and we chose the ELISA data because their quantitative nature allows a more refined description of the dynamics (relatively to the MAT data that are semi-quantitative). A mechanistic model was chosen rather than a purely statistical model because of the limited possibility to capture seabirds outside of the breeding season (when they are at sea) leading to incomplete time series.

The model predicted a peak OD ± s.d. of 0.29 ± 0.08 reached 26 ± 9 days after vaccination and a return to seronegativity by 61 ± 23 days after vaccination (Fig. S3.6). These figures are close to the one reported by Samuel et al. (2003) in ducks following experimental infection and measurement of specific antibodies using an ELISA.

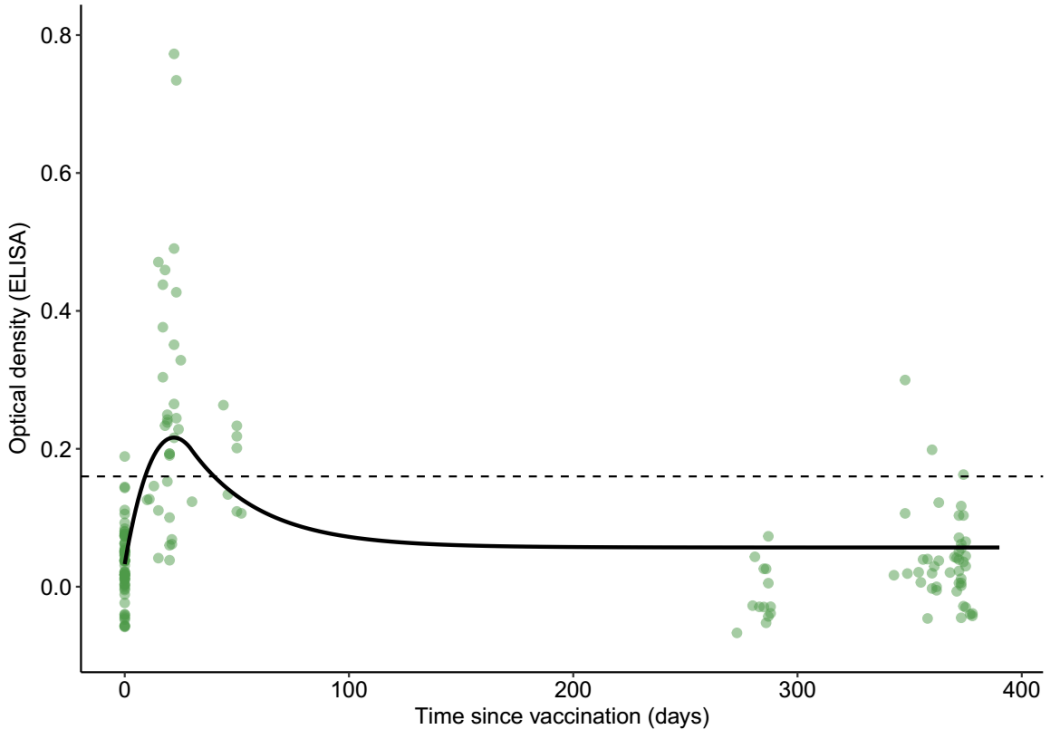

Figure S3.5. Modeled anti-*P. multocida* antibody dynamics based on ELISA results in adult Indian yellow-nosed albatrosses after injection of an autogenous vaccine using a segmented linear regression including a polynomial effect of time since injection and an individual random effect.

Relationship between sex and immune response to P. multocida *in adults*

Only females can transmit antibodies to their offspring through the egg yolk. Thus sex differences in the immune response following exposure to an infectious agent can have important implications regarding potential transgenerational effects. The relationship between sex and the probability to be seropositive across the four years of the study was assessed using a logistic regression with serological status (seronegative or seropositive) as the response variable and breeding season, treatment (non-vaccinated or vaccinated) and sex (male or female) as explanatory variables. As many individuals were sampled several times and a large proportion of individuals were partners, we used generalized linear mixed models in the '*lme4'* R package (Bates et al. 2015), with the individual and the nest as random effects. The model including sex and the one not including sex were compared using Akaike Information Criterion

(AIC; Burnham and Anderson 2002). Antibody levels one year after vaccination were compared between males and females by a Wilcoxon test. Both tests were run on both MAT and ELISA data.

No correlation between sex and the probability to be seropositive was detected (MAT: ΔAIC = 1.99, sex p = 0.94, n = 243 including 36 male and 38 female individuals; ELISA: ΔAIC = 0.20, sex p = 0.17, n = 231 including 36 male and 37 female individuals; with treatment and campaign as fixed effects and individual identity and nest as random effects). Similarly, no correlation between sex and antibody levels one year after vaccination was detected (MAT: W = 287.5, p = 0.79, n = 25, 22; ELISA: W =269.5, p = 0.91, n = 25, 22).

Modeled maternal antibody level decay

The decay of inherited maternal antibody level in nestlings was modeled for a visualisation purpose only. A generalized additive mixed model was fitted to individual antibody level dynamics after hatching using the '*gamm4*' R package (Wood and Scheipl 2014). Nestling antibody titers (measured by MAT) was included as the response variable, age as an explicative variable and individual identity as a random effect. Only nestlings with detectable levels of antibodies in their first blood sample were included in the analysis.

Serological status at egg-laying

Serological status at egg-laying (*i.e.*, September) was evaluated for a small proportion of females during the 2014-2015 breeding season (*i.e.*, one year after the initial injection) and was weakly related with that measured at egg-hatching (*i.e.*, December; MAT: Cohen's $\kappa = 0.27$ [-0.03; 0.56], p = 0.05, n = 34; Fig. S3.2). Hence, we did not correlate the serological status of females at hatching with that of their nestlings. However, seroprevalence according to MAT was higher in vaccinated adults than in NaCl-injected adults (NaCl: 35%, 7/20; vaccinated: 95%, 18/19; $\chi^2 = 13$, $p < 0.01$).

Appendix S4. Estimation of the seroconversion rate

To estimate the annual seroconversion rate, we calculated the probability to remain seropositive between two successive breeding seasons. The probability $P(R_{1\to2})$ to remain seropositive between time t_1 and time t_2 was approximated based on the probability $P(S^+_1)$ for an individual to be seropositive at time t_1 (corresponding to the seroprevalence at this time), the probability $P(S^+_2)$ to be seropositive at time t_2 and the probability $P(E_{1\to2})$ to have been naturally exposed between time t_1 and time t_2 (corresponding to the seroconversion probability in NaCl-injected individuals during the same period)

$$P(S^+_2) = P(S^+_1 \cap R_{1\to2}) \cup P(\overline{S^+_1} \cap E_{1\to2}) \cup P(S^+_1 \cap \overline{R_{1\to2}} \cap E_{1\to2})$$

calculated as

$$P(S^+_2) = P(S^+_1) \times P(R_{1\to2}) + \left(1 - P(S^+_1)\right) \times P(E_{1\to2}) + P(S^+_1) \times \left(1 - P(R_{1\to2})\right) \times P(E_{1\to2})$$

$$\Leftrightarrow P(R_{1\to2}) = \frac{P(S^+_2) - \left(1 - P(S^+_1)\right) \times P(E_{1\to2}) - P(S^+_1) \times P(E_{1\to2})}{P(S^+_1) \times \left(1 - P(E_{1\to2})\right)}$$

assuming that S^+_1 and $R_{1\to2}$ and S^-_1 and $E_{1\to2}$ are independent events.

For instance, based on 2013-2014 and 2014-2015 MAT data and assuming 100% seroconversion of vaccinated individuals, the probability to maintain detectable antibody levels one year after vaccination is

$$P(R_{1\to2}) = \frac{0.76 - (1 - 1.00) \times 0.30 - 0.30 \times 1.00}{1.00 \times (1 - 0.30)} = 0.66$$

Because the year of vaccination all the individuals captured three weeks or more after vaccination were seropositive (21/21 individuals), we considered that it was reasonable to assume that all vaccinated individuals eventually seroconverted within a few weeks.

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