



Original Contribution

Impact of Annual Bacterial Epizootics on Albatross Population on a Remote Island

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Abstract: The reduced species richness typical of oceanic islands provides an interesting environmental setup to examine *in natura* the epidemiological dynamics of infectious agents with potential implications for public health and/or conservation. On Amsterdam Island (Indian Ocean), recurrent die-offs of Indian yellow-nosed albatross (*Thalassarche carteri*) nestlings have been attributed to avian cholera, caused by the bacterium *Pasteurella multocida*. In order to help implementing efficient measures for the control of this disease, it is critical to better understand the local epidemiology of *P. multocida* and to examine its inter- and intra-annual infection dynamics. We evaluated the infection status of 264 yellow-nosed albatrosses over four successive breeding seasons using a real-time PCR targeting *P. multocida* DNA from cloacal swabs. Infection prevalence patterns revealed an intense circulation of *P. multocida* throughout the survey, with a steady but variable increase in infection prevalence within each breeding season. These epizootics were associated with massive nestling dies-offs, inducing very low fledging successes ($\leq 20\%$). These results suggest important variations in

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the transmission dynamics of this pathogen. These findings and the developed PCR protocol have direct applications to guide future research and refine conservation plans aiming at controlling the disease.

Keywords: Bacteria, Disease ecology, Eco-epidemiology, Molecular biology, Seabird, Wildlife

INTRODUCTION

Emerging infectious diseases are listed among the top five drivers of species extinction (Daszak 2000). However, investigating infection dynamics *in natura* and identifying emergence factors are challenged by the number of animal species potentially involved in the maintenance and transmission of such pathogens. Insular ecosystems are particularly appropriate for such studies because species richness on islands, especially young and remote oceanic ones, is generally lower than on mainland sites with comparable climate (MacArthur and Wilson 1967; Kier et al. 2009). Furthermore, geographic isolation facilitates the identification of migratory or introduced taxa that may add complexity to a given pathosystem (Tortosa et al. 2012).

Amsterdam Island (37° 49' S, 77° 33' E) is a 53 km² volcanic island lying in the southern Indian Ocean, more than 3000 km away from any continent. To help preserving its remarkable wildlife, the island has been designated as part of the National Nature Reserve of the French Southern Territories in 2006 and as part of the French Austral Lands and Seas UNESCO World Heritage site in 2019. Amsterdam Island notably hosts several endangered seabird species, including the northern rockhopper penguin (*Eudyptes moseleyi*) and three albatross species: the Indian yellow-nosed (*Thalassarche carteri*), the sooty (*Phoebastria fusca*), and the endemic Amsterdam (*Diomedea amsterdamensis*) albatrosses (IUCN 2018). Local numbers of yellow-nosed albatrosses have severely declined over the past 30 years, mirroring recurrent die-offs of the nestlings from infectious diseases (Jaeger et al. 2018), while adults do not appear to be affected (Rolland et al. 2009; Gamble et al. 2019b). Two microbiological studies have substantiated the link between nestling mortalities and infection by the Gram-negative bacterium *Pasteurella multocida* (hereafter *Pm*; Weimerskirch 2004; Jaeger et al. 2018). Isolation of the etiological agent from two distinct bird species on Amsterdam Island and identification of a single genotype support the hypothesis of a clonal infection, suggesting the recent introduction of this agent (Jaeger et al. 2018), potentially as a consequence of poultry and/or rodent introduction to the island (Micol and Jouventin 1995). The causal link between

Pm infection and nestling mortality has been further addressed through experimental vaccination, which significantly increased nestling survival during an avian cholera outbreak (Bourret et al. 2018).

Avian cholera is a worldwide infectious disease with major economic impact and conservation concern for several host species (Crawford et al. 1992; Wobeser 1997; Österblom et al. 2004; Leotta et al. 2006; Descamps et al. 2012; Singh et al. 2014; Wille et al. 2016; Iverson et al. 2016). However, *Pasteurella multocida* is notorious for exhibiting distinct patterns of pathogenicity across hosts species (Blanchong et al. 2006a). For instance, avian cholera seems to strongly affect adult survival in several systems such as eider ducks and gulls in North America (Wille et al. 2016; Iverson et al. 2016), guillemots in Northern Europe (Österblom et al. 2004), cormorants in South Africa (Crawford et al. 1992), or skuas and Adelie penguins in Antarctica (Leotta et al. 2006). In contrast, some sparse evidence suggests that some populations or species may exhibit lower mortality rates and support chronic carrying of *Pm*, such as some geese species in North America (Samuel et al. 2005), or as generally considered in poultry (Glisson 2013). This heterogeneity in host response to *Pm* infection hence does not allow making predictions regarding the system investigated on Amsterdam Island. Deciphering the epidemiological dynamics of this emerging infectious disease is a first step towards understanding the dynamics of maintenance and transmission of a pathogen likely introduced to this ecosystem.

Although a handful of commercial tools are available for the screening of this disease (including ID-Vet[®] indirect ELISA and a RT-PCR kit recently developed by Bioeksen[®]), we developed and validated a real-time PCR approach targeting the *Pm* strain isolated from Amsterdam Island (Gamble et al. 2019b). We used this PCR scheme to carry out high-throughput screening of *P. multocida* DNA and examine the infection dynamics of adult and nestling yellow-nosed albatrosses during four successive breeding seasons and in relation to the nestling survival. The screening further aimed at identifying the periods during which more intensive sampling should be conducted in order to quantify the case fatality rate of the disease and its

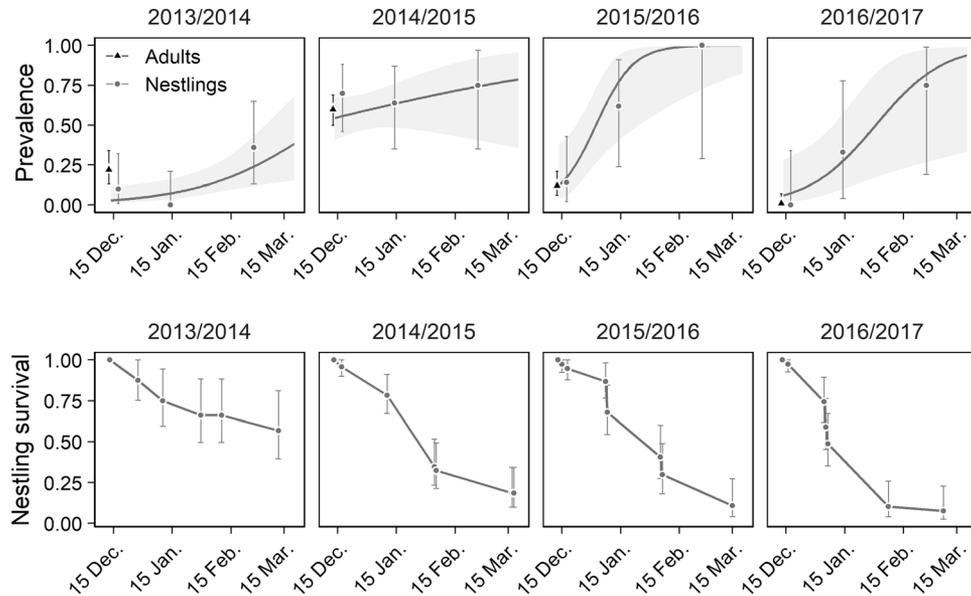


Figure 1. Proportion of yellow-nosed albatrosses shedding *Pm* DNA (a) and nestling survival (b) during the four breeding seasons on Amsterdam Island. Raw prevalence is represented by black triangles (adults) and grey dots (nestlings). The temporal variation in prevalence in nestlings (grey line) was predicted based on the generalized linear mixed model that fitted best to nestling data (prevalence \sim breeding season : day; Table S3). Bars (a, b) and shaded area (b) represent the corresponding 95% confidence intervals; sample sizes are given in Table S2.

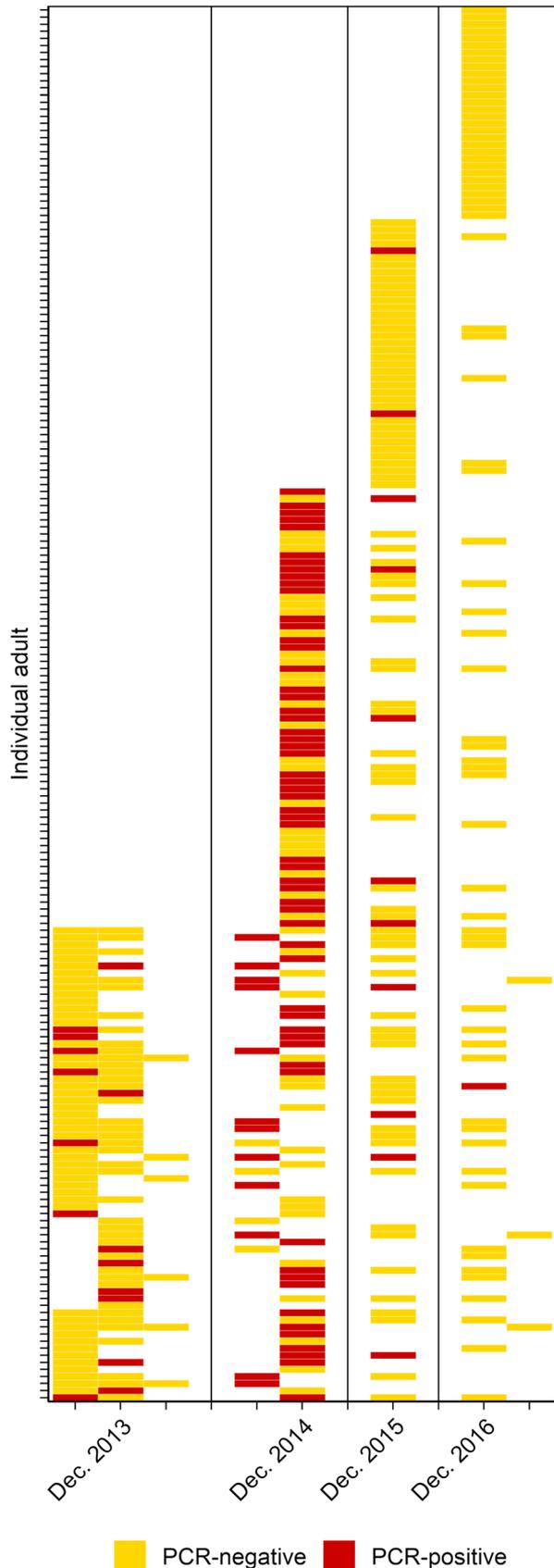
spatial progression in the colony. Lastly, this screening aimed at testing a number of hypotheses regarding transmission patterns. *Pm* infections are highly heterogeneous among bacterial strain and host species, and among individuals within a species (Wobeser 1997; Glisson 2013), some individuals developing acute and lethal diseases (e.g. Iverson et al. 2016), while some other develop chronic diseases or becoming asymptomatic carriers (e.g. Samuel et al. 2005). Our hypotheses were thus generated from previous demographic, serological, and experimental data collected in the study system rather than knowledge acquired in other systems. First, we predicted that *Pm* would be detected in both adults and nestlings, as a previous serosurvey revealed that adults were also exposed to the bacterium (Gamble et al. 2019b). Second, considering the high mortality rate of the disease in nestlings (Weimerskirch 2004; Bourret et al. 2018), we predicted that *Pm* would be detected at high prevalence years of large nestling die-offs, in addition to lesions suggestive of avian cholera such as necrosis and bacterial colonization of several organs following septicaemia, but not in years of high fledging success. Finally, nestlings were expected to die following infection, while adults were expected to either clear out the infection or chronically carry it, as suggested in other wild systems (Samuel et al. 2005) and as generally admitted in poultry (Glisson 2013).

METHODS

Bird Sampling

Fieldwork was conducted between December 2013 and March 2017, in the Entrecasteaux cliffs (south-western coast of Amsterdam Island) where approximately 20,000 pairs of yellow-nosed albatrosses nest from September to March (Rolland et al. 2009). Yellow-nosed albatrosses lay a single egg in early September that hatches between late November and mid-December (Jouventin et al. 1989). Nest attendance by adults is high until January. Nestlings are then mostly on their own in their nest until fledging in April, except during feeding visits by their parents (Jaeger et al. 2018). We surveyed a naturally delineated subcolony of approximately 250 albatross pairs where bird exposure to *Pm* has been monitored since the 2013/2014 breeding season (Bourret et al. 2018; Gamble et al. 2019b). Monitored nests were georeferenced and marked with alphanumeric tags to individually identify the nestlings within a breeding season. Adults were marked with alphanumeric rings, allowing individual identification.

During the four studied seasons, cloacal swabs were collected from adults ($n = 197$ individuals, including 100 with two samples or more) during the early chick-rearing period (late November to early January) using sterile



◀ **Figure 2.** Individual histories of *Pm* detection by PCR in cloacal swab samples of adult yellow-nosed albatrosses. Each line represents an individual and each column a month. Individuals sampled several times within a month were considered PCR-positive if at least one of these samples tested positive.

applicators (one to two samples per breeding season, per individual) and from nestlings ($n = 67$ individuals, including 50 with two samples or more) between hatching and fledging (up to five samples per breeding season, per individual). Sample sizes per sampling occasion and per individual are detailed in Figures 2 and 3, and Table S2 of the Electronic Supplementary Material (ESM), respectively. Swabs were conserved in 0.5 mL Longmire lysis buffer (Longmire et al. 1988) and kept at 0–4°C in the field, and then at – 20°C when brought back to the main station, and eventually stored at – 80°C at the research facilities until analysis. In addition, when apparently fresh and intact dead birds were opportunistically found on the colony, necropsies were carried out and tissue samples were collected ($n = 21$ individuals). Samples were stored in 4% formaldehyde, routinely processed, and stained with haematoxylin and eosin for histological examination essentially as previously described (see <https://www.protocols.io/view/haematoxylin-eosin-h-e-staining-ihxcb7n>). Additional Gram staining was used for bacterial characterization (see ESM for additional details).

Concomitantly, nestling survival was monitored during four monthly visits to the subcolony per year, between early December (following hatching peak) and late March (before fledging). Survival monitoring was focused on the sampled nestlings, in addition to up to 30 unmanipulated (i.e., visually monitored without any handling) nestlings in 2014–2015, 2015–2016, and 2016–2017. Because sampling of the nestlings appeared to have no detectable effect on their survival (Figure S1; Bourret et al. 2018), the two groups (sampled and unmanipulated) were pooled together in the survival analyses. Additional details are given in ESM.

Nucleic Acid Extraction and PCR Detection of *P. multocida*

Nucleic acids were prepared from cloacal swab samples using manual QiaAmp cadior pathogen Mini kits (Qiagen, Courtaboeuf, France) following the manufacturer's protocol. A real-time probe-based PCR protocol was developed using *Pm*-for (5'-ACGGCGCAACTGATTGGACG-3') and

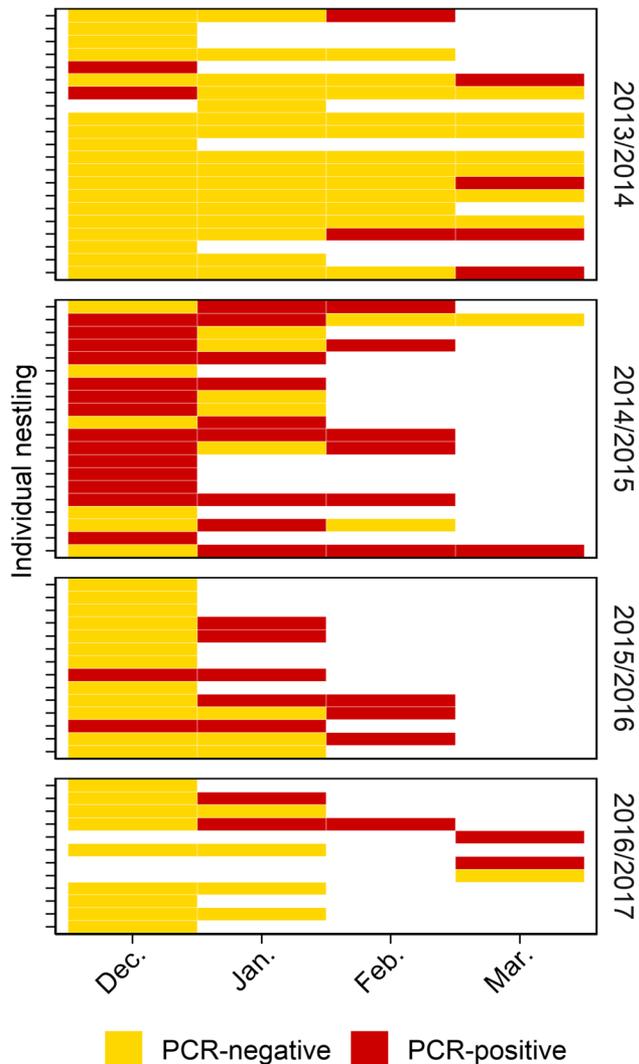


Figure 3. Individual histories of *Pm* detection by PCR in cloacal swab samples of yellow-nosed albatross nestlings. Each line represents an individual and each column a month. Individuals sampled several times within a month were considered PCR-positive if at least one of these samples tested positive.

Pm-rev (5'-GGCCATAAGAAACGTAACCTCAACA-3') primers allowing the amplification of a 116 nucleotides amplicon within KMT1 gene, a locus routinely used for the detection of *Pm* through end-point PCR (Townsend et al. 1998). Amplification was monitored in a Stratagene MX3005P (Agilent Technologies, Santa Clara, USA) thermocycler using the fluorescent *Pm*-probe (5'-FAM-TCAGCTTATTGTTATTTGCCGGT3'-BHQ1). Amplifications were performed in 25 μ L final volume containing 12.5 μ L of Absolute Blue real-time PCR Low Rox Mix (Thermo Scientific, Waltham, MA, USA), 0.4 μ M of each primer, and 0.2 μ M of *Pm*-probe. The PCR conditions included a first Taq polymerase activation step (95°C for 15 min),

followed by 40 cycles each composed of a denaturation (95°C for 15 s.), an annealing (54°C for 30 s.) and an extension (72°C for 30 s.) step. The sensitivity of the PCR was measured by serially diluting genomic DNA prepared from the D2C *Pm* strain (Jaeger et al. 2018). The assay specificity was not addressed, as previous microbiological analyses suggested the occurrence of a single *Pm* clone in albatrosses from Amsterdam Island (Jaeger et al. 2018) and the seasonal dynamics of this clone were the focus of this study.

Statistical Analyses

Prevalence (with 95% Clopper–Pearson confidence interval) was calculated as the proportion of adults or nestlings testing positive among all sampled individuals during a given period. Variation in prevalence was quantified using logistic regressions, with *Pm*-PCR status (negative or positive) used as the response variable, and breeding season (categorical), day within the season (continuous), and their interaction as potential explicative variables. The best model was selected using Akaike Information Criterion (AIC) (Burnham and Anderson 2002). As many individuals were sampled several times and a large proportion of adult individuals were partners, we used generalized linear mixed models in the “lme4” R package (Bates et al. 2015), with the individual and nest (for adults only) as random effects. Likelihood ratio (LR) tests are reported between parentheses, and effect sizes, odds ratios, and AIC are reported in Tables S3-5. Because the timing of presence in the colony differed between adults and nestlings, two distinct models were used. The potential effects of handling and breeding season on nestling survival were investigated by fitting a Cox proportional hazards model (Andersen and Gill 1982) to the data using the “survival” package (Therneau and Lumley 2019). Finally, a potential association between *Pm*-PCR status and fledging probability at the individual or nest level was investigated. To do so, we classified as PCR-positive all the nestlings from which *Pm* DNA was detected in at least one sample (individual level), or that belonged to a nest from which *Pm* DNA was detected in at least one sample (i.e. including its parents; nest level). We then fitted logistic regressions, with fledging success used as the response variable, and *Pm*-PCR status (negative or positive), breeding season (categorical), and their interaction as potential explicative variables. All statistical analyses were conducted in R 3.3.3. Additional methods and scripts are provided as ESM.

RESULTS

The sensitivity of the developed RT-PCR scheme showed a positivity threshold of 9 DNA molecule templates per reaction. We screened 391 samples from 197 adults and 192 samples from 67 nestlings. *Pm* DNA was detected in 157/583 tested samples (Table S2).

In adults, which were sampled during the early chick-rearing period only, prevalence varied significantly among breeding seasons (LR $\chi^2 = 79$, $P < 0.01$; Tables S3-4), reaching its maximum in 2015 (0.60 [0.50; 0.69]) and minimum in 2017 (0.01 [0.00; 0.07]; Fig. 1a). In nestlings, the model with an effect of the breeding season (LR $\chi^2 = 35$, $P < 0.01$), day (LR $\chi^2 = 13$, $P < 0.01$) and their interaction (LR $\chi^2 = 7$, $P = 0.07$) on prevalence was selected, indicating variations among and within breeding seasons (Fig. 1a; Tables S3 and 5). Notably, prevalence was generally low at the beginning of the chick-rearing periods (≤ 0.14), except in 2014/2015 when prevalence was high already in December (0.70 [0.46; 0.88]), mirroring prevalence in adults during the same period. Each year, prevalence tended to increase throughout the season (see Table S2 for numerical data). Remarkably, prevalence in nestlings was maximal in 2014/2015, 2015/2016, and 2016/2017, corresponding to seasons with very low fledging success (≤ 0.20) while fledging success reached 0.57 [0.40; 0.81] in 2013/2014 (Fig. 1b).

The longitudinal survey of a subset of birds also revealed that some individuals testing positive at a given time point may test negative at one following time point (47/191 adults, and 9/50 nestlings; Figs. 2, 3), suggesting that a fraction of the birds could possibly clear out the infection. In 2014/2015, 2015/2016, and 2016/2017, all the fledged nestlings sampled longitudinally from hatching were detected as positive at least once although we did not detect any association between infection status and survival at the considered monthly sampling scale (Figure S6). We did not detect any spatial structuration in *Pm* infection status among nests either (Figure S5). Lastly, a histological analysis carried out to assess the extent of tissue damage resulting from *Pm* infection and to rule out other underlying processes revealed necrotic lesions in the heart, spleen, and/or liver together with Gram-negative bacterial sepsis in nine of 21 necropsied albatross nestlings (Figures S2-4), together with hepatic and pulmonary congestion as well as haemorrhagic myocarditis (not shown).

DISCUSSION

This study reports the first multi-year investigation of *Pm* infection in an albatross population, hence contributing to fill the important knowledge gap regarding the impact of infectious diseases on one of the most rapidly declining bird taxa (Phillips et al. 2016). The results reveal that yellow-nosed albatrosses on Amsterdam Island have been facing *Pm* infection at their breeding sites every year throughout the study duration, even when the fledging success is relatively high. Such epizootics are in line with the massive nestling die-offs that have been recorded over the last three decades on the island, especially in yellow-nosed albatrosses (Weimerskirch 2004; Jaeger et al. 2018).

Temporal Variations in *P. multocida* Prevalence

Infection prevalence was overall high, but varied among and within breeding seasons. As expected considering the high pathogenicity of *Pm* in the study system (Weimerskirch 2004; Bourret et al. 2018; Jaeger et al. 2018; and this study), very few nestlings ($\leq 20\%$) fledged in 2014/2015, 2015/2016, and 2016/2017, corresponding to the years with highest *Pm* prevalence. Interestingly, all the fledged nestlings sampled longitudinally during these three breeding seasons from hatching were detected as positive at least once. In contrast, in 2013/2014, prevalence was minimal (but non-null) and nestling survival exceeded 50%. A salient pattern highlighted here is the temporality of the infection. Indeed, prevalence was most often low at the beginning of the chick-rearing period and then increased onwards until fledging. The estimates obtained for 2014/2015 do not follow this trend, though: prevalence was already high upon the first sampling date (*i.e.*, at the time of egg hatching), with over 50% of the birds testing positive, and remained high throughout the season. During 2014/2015 season, the epizootic may thus have started earlier for reasons that remain to be elucidated. The magnitude of the infection prevalence together with its temporal dynamics suggests that epizootics occur quasi-annually in this sub-colony.

Enlightening Mechanisms of Pathogen Maintenance

The recurrence of avian cholera outbreaks on Amsterdam Island raises the question of the compartments involved in pathogen maintenance between two successive breeding seasons, when most seabirds are absent from the island. We

can hypothesize (H1) that some of the infected birds surviving the infection become chronic shedders, hence re-infecting the island every year as suggested in other wild systems (Samuel et al. 2005). The role of chronic shedders may be especially important to explain outbreak recurrence in a system such as Amsterdam Island where a high proportion of individuals, adults in particular, seems to survive exposure, contrasting with other systems where adult survival is strongly affected by the disease (e.g. Crawford et al. 1992; Österblom et al. 2004; Leotta et al. 2006; Descamps et al. 2012; Wille et al. 2016; Iverson et al. 2016). Alternatively, the pathogen may persist on the island during winter in the absence of yellow-nosed albatrosses, either in the environment (Samuel et al. 2004) or in one of the resident avian/rodent species (H2). These non-exclusive hypotheses may be tested by screening (1) environmental samples, (2) adult birds at their return to the island in September, and (3) the putative reservoir species that permanently settle on the island. Indeed, *Pm* circulation on Amsterdam Island is not restricted to yellow-nosed albatrosses. Notably, predators and scavengers such as brown skuas (*Stercorarius antarcticus*) and introduced rodents (*Rattus norvegicus* and *Mus musculus*) may contribute to the maintenance and/or circulation of the pathogen (Curtis 1983; Boulinier et al. 2016; Gamble et al. 2019a). Screening the environment and the resident animal species, especially before the return of yellow-nosed albatrosses, will improve our understanding of the whole epidemiological network. The novel PCR protocol we developed here is pivotal in this perspective. Overall, the results presented herein will help to design field studies in an iterative process, as future sampling protocols designed to elucidate the transmission and maintenance cycles will need to be updated (Restif et al. 2012; see below).

Methodological Implications

Avian cholera outbreaks on Amsterdam Island are characterized by a rapid epidemiological process, as highlighted by the steep increase in *Pm* prevalence and decrease in nestling survival throughout the breeding season, although some inter-annual variation exists. Hence, a finer temporal scale of sampling is needed to precisely quantify key epidemiological parameters, such as the case fatality rate and the probability to clear out the infection, as well as to assess the presence of chronic shedders, as suggested by the longitudinal survey. Indeed, the discrepancy between the within-year temporal scales of the sampling (once a month) and the epidemiological process (*Pm* being believed to

cause death within a few days; Wobeser 1997) prevented us from quantifying these parameters in this study (see Figure S7 for detailed explanation). In addition, future studies could include repeated sampling (e.g. successive collection of several swabs from a given individual at a given time point) in order to account for detection probability issues. Indeed, most molecular assays have a sensitivity below unity, and repeated samples can allow to estimate the protocol detection probability and ultimately account for it by analysing the data using the patch occupancy framework (McClintock et al. 2010; DiRenzo et al. 2018). Designing the optimal sampling protocol requires preliminary knowledge on test sensitivity, but also infection prevalence (Mackenzie and Royle 2005), which our study brings here. Finally, the reported inter-annual variations in outbreak onset support the need of a monitoring scheme covering the full breeding season to identify the determinants of outbreak onset.

CONCLUSION

In this study, we showed the relevance of a multi-scale (inter- and intra-seasonal) approach to better understand the infection dynamics of *Pm* in endangered insular wildlife. Altogether, the results highlight the need for a comprehensive monitoring of *Pm* infection spanning different biotic and abiotic compartments possibly involved in pathogen maintenance on the Island. This study will guide future research on Amsterdam Island and other sites affected by avian cholera and comparable diseases (e.g. Crawford et al. 1992; Österblom et al. 2004; Blanchong et al. 2006b; Leotta et al. 2006; Descamps et al. 2012; Wille et al. 2016; Iverson et al. 2016). Eventually, it may also guide stakeholders to refine conservation measures and target the most relevant compartments of the pathosystem for the control of severe diseases affecting endangered wildlife populations.

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AUTHOR CONTRIBUTIONS

AJ, CL, and PT set up the quantitative PCR. TB, CB, and HW are responsible of the field research programs. AJ, TB, KD, JT, VB, JBT, and AG implemented the study in the field. AJ and EL implemented the molecular analyses. KL conducted the histological analyses. AG managed the data. AG and AJ conducted the data analyses. PT and AG led the writing of the manuscript. All authors contributed to the final version of the manuscript.

COMPLIANCE WITH ETHICAL STANDARDS

CONFLICT OF INTEREST The authors declare that they have no conflict of interest.

STATEMENT OF ANIMAL ETHICS The experimental design was approved by the Comité de l'Environnement Polaire and Comité National de Protection de la Nature (TAAF A-2013-71, A-2014-134, A-2015-107 and A-2016-80) and the French Ministry of Research (licence #04939.03).

DATA AVAILABILITY The datasets and R code used in the current study are available on the OSU OREME online repository: <https://data.oreme.org/doi/view/e34e5542-f819-4c88-a048-655f77e3668e>.

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