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

Jaeger Audrey [1, 2], Gamble Amandine [3, 4], Lagadec Erwan [1, 5], Lebarbenchon Camille [1], Bourret Vincent [3], Tornos Jérémy [3, 6], Barbraud Christophe [7], Lemberger Karin [8], Delord Karine [7], Weimerskirch Henri [7], Thiebot Jean-Baptiste [5, 7], Boulinier Thierry [3], Tortosa Pablo [1, *]

[1] UMR PIMIT (Processus Infectieux en Milieu Insulaire Tropical), CNRS 9192, INSERM 1187, IRD 249, Plateforme Technologique CYROI, Université de La Réunion, Sainte-Clotilde, La Réunion, France
[2] UMR ENTROPIE (Écologie marine tropicale des océans Pacifique et Indien) CNRS, IRD 250, Université de La Réunion, 15 Avenue René Cassin, CS 92003, 97744, Saint Denis Cedex 9, La Réunion, France
[3] Centre d'Écologie Fonctionnelle et Évolutive (CEFE), CNRS, EPHE, IRD, Université de Montpellier, Université Paul Valéry Montpellier 3, Montpellier, France
[4] Department of Ecology and Evolutionary Biology, University of California, Los Angeles, CA, USA
[5] Réserve Naturelle Nationale des Terres Australes Françaises, Terres Australes et Antarctiques Françaises, rue Gabriel Dejean, Saint Pierre, La Réunion, France
[6] Ceva Biovac Campus, Beaucouzé, France
[7] Centre d'Études Biologiques de Chizé, UMR CNRS 7372, Université La Rochelle, Villiers en Bois, France
[8] Vet Diagnostics, 14, Avenue Rockefeller, 69008, Lyon, France

* Corresponding author : Pablo Tortosa, email address : pablo.tortosa@univ-reunion.fr

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 (≤ 20%). These results suggest important variations in 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

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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 km^2 volcanic island lying in the southern Indian Ocean, more than 3,000 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 (*Phoebetria fusca*), and the endemic Amsterdam (*Diomedea amsterdamensis*) albatrosses. 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

44 recent introduction of this agent (Jaeger et al. 2018), potentially as a consequence of poultry
45 and/or rodent introduction to the island (Micol and Jouventin 1995). The causal link between
46 *Pm* infection and nestling mortality has been further addressed through experimental
47 vaccination, which significantly increased nestling survival during an avian cholera outbreak
48 (Bourret et al. 2018).

49 Avian cholera is a worldwide infectious disease with major economic impact and
50 conservation concern for several host species (Crawford et al. 1992; Wobeser 1997;
51 Österblom et al. 2004; Leotta et al. 2006; Descamps et al. 2012; Singh et al. 2014; Wille et al.
52 2016; Iverson et al. 2016). However, *Pasteurella multocida* is notorious for exhibiting distinct
53 patterns of pathogenicity across hosts species (Blanchong et al. 2006a). For instance, avian
54 cholera seems to strongly affect adult survival in several systems such as eider ducks and
55 gulls in North America (Wille et al. 2016; Iverson et al. 2016), guillemots in Northern Europe
56 (Österblom et al. 2004), cormorants in South Africa (Crawford et al. 1992), or skuas and
57 Adelie penguins in Antarctica (Leotta et al. 2006). In contrast, some sparse evidence suggest
58 that some populations or species may exhibit lower mortality rates and support chronic
59 carrying of *Pm*, such as some geese species in North America (Samuel et al. 2005), or as
60 generally considered in poultry (Glisson 2013). This heterogeneity in host response to *Pm*
61 infection hence does not allow making predictions regarding the system investigated on
62 Amsterdam Island. Deciphering the epidemiological dynamics of this emerging infectious
63 disease is a first step towards understanding the dynamics of maintenance and transmission of
64 a pathogen likely introduced to this ecosystem.

65 Although a handful of commercial tools are available for the screening of this disease
66 (including ID-Vet ® indirect ELISA and a RT-PCR kit recently developed by Bioeksen ®), ,
67 we developed and validated a Real-Time PCR approach targeting the *Pm* strain isolated from
68 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 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 where thus generated from previous demographic, serological and experimental data collected in the study system rather that 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 colonisation 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

93 Fieldwork was conducted between December 2013 and March 2017, in the Entrecasteaux
94 cliffs (south-western coast of Amsterdam Island) where approximately 20,000 pairs of
95 yellow-nosed albatrosses nest from September to March (Rolland et al. 2009). Yellow-nosed
96 albatrosses lay a single egg in early September, that hatches between late November and mid-
97 December (Jouventin et al. 1989). Nest attendance by adults is high until January. Nestlings
98 are then mostly on their own in their nest until fledging in April, except during feeding visits
99 by their parents (Jaeger et al. 2018). We surveyed a naturally-delineated subcolony of
100 approximately 250 albatross pairs where bird exposure to *Pm* has been monitored since the
101 2013/2014 breeding season (Bourret et al. 2018; Gamble et al. 2019b). Monitored nests were
102 georeferenced and marked with alphanumeric tags to individually identify the nestlings within
103 a breeding season. Adults were marked with alphanumeric rings, allowing individual
104 identification.

105 During the four studied seasons, cloacal swabs were collected from adults (n = 197
106 individuals, including 100 with two samples or more) during the early chick-rearing period
107 (late November to early January) using sterile applicators (one to two samples per breeding
108 season, per individual) and from nestlings (n = 67 individuals, including 50 with two samples
109 or more) between hatching and fledging (up to five samples per breeding season, per
110 individual). Sample sizes per sampling occasion and per individual are detailed in Figures 2
111 and 3, and Table S2 of the Electronic Supplementary Material (ESM) respectively. Swabs
112 were conserved in 0.5 mL Longmire lysis buffer (Longmire et al. 1988) and kept at 0-4°C in
113 the field, and then at -20°C when brought back to the main station, and eventually stored at -
114 80°C at the research facilities until analysis. In addition, when apparently fresh and intact
115 dead birds were opportunistically found on the colony, necropsies were carried out and tissue
116 samples were collected (n = 21 individuals). Samples were stored in 4% formaldehyde,
117 routinely processed and stained with haematoxylin-and-eosin for histological examination

118 essentially as previously described (see https://www.protocols.io/view/haematoxylin-eosin-h-
119 e-staining-ihxcb7n). Additional Gram staining was used for bacterial characterization (see
120 ESM for additional details).
121 Concomitantly, nestling survival was monitored during four monthly visits to the subcolony
122 per year, between early December (following hatching peak) and late March (before
123 fledging). Survival monitoring was focused on the sampled nestlings, in addition to up to 30
124 unmanipulated (*i.e.*, visually monitored without any handling) nestlings in 2014-2015, 2015-
125 2016 and 2016-2017. Because sampling of the nestlings appeared to have no detectable effect
126 on their survival (Figure S1; Bourret et al. 2018), the two groups (sampled and
127 unmanipulated) were pooled together in the survival analyses. Additional details are given in
128 ESM.
129 *Nucleic acid extraction and PCR detection of* P. multocida
130 Nucleic acids were prepared from cloacal swab samples using manual QiaAmp cador
131 pathogen Mini kits (Qiagen, Courtaboeuf, France) following the manufacturer's protocol. A
132 Real-Time probe-based PCR protocol was developed using *Pm*-for (5'-
133 ACGGCGCAACTGATTGGACG-3') and *Pm*-rev (5'-
134 GGCCATAAGAAACGTAACTCAACA-3') primers allowing the amplification of a 116
135 nucleotides amplicon within KMT1 gene, a locus routinely used for the detection of *Pm*
136 through end-point PCR (Townsend et al. 1998). Amplification was monitored in a Stratagene
137 MX3005P (Agilent Technologies, Santa Clara, USA) thermocycler using the fluorescent *Pm*-
138 probe (5'FAM-TCAGCTTATTGTTATTTGCCGGT3'BHQ1). Amplifications were
139 performed in 25 µL final volume containing 12.5 µL of Absolute Blue real-time PCR Low
140 Rox Mix (Thermo Scientific, Waltham, MA, USA), 0.4 µM of each primer and 0.2 µM of
141 Pm-probe. The PCR conditions included a first Taq-Polymerase activation step (95°C for 15
142 min), followed by 40 cycles each composed of a denaturation (95°C for 15 sec.), an annealing

143 (54°C for 30 sec.) and an extension (72°C for 30 sec.) step. The sensitivity of the PCR was
144 measured by serially diluting genomic DNA prepared from the D2C *Pm* strain (Jaeger et al.
145 2018). The assay specificity was not addressed, as previous microbiological analyses
146 suggested the occurrence of a single *Pm* clone in albatrosses from Amsterdam Island (Jaeger
147 et al. 2018) and the seasonal dynamics of this clone were the focus of this study.
148 *Statistical analyses*
149 Prevalence (with 95% Clopper-Pearson confidence interval) was calculated as the proportion
150 of adults or nestlings testing positive among all sampled individuals during a given period.
151 Variation in prevalence was quantified using logistic regressions, with *Pm*-PCR status
152 (negative or positive) used as the response variable, and breeding season (categorical), day
153 within the season (continuous) and their interaction as potential explicative variables. The
154 best model was selected using Akaike Information Criterion (AIC) (Burnham and Anderson
155 2002). As many individuals were sampled several times and a large proportion of adult
156 individuals were partners, we used generalized linear mixed models in the "*lme4*" R package
157 (Bates et al. 2015), with the individual and nest (for adults only) as random effects.
158 Likelihood-ratio (LR) tests are reported between parentheses, and effect sizes, odds ratios and
159 AIC are reported in Tables S3-5. Because the timing of presence in the colony differed
160 between adults and nestlings, two distinct models were used. The potential effects of handling
161 and breeding season on nestling survival was investigated by fitting a Cox proportional
162 hazards model (Andersen and Gill 1982) to the data using the "*survival*" package (Therneau
163 and Lumley 2019). Finally, a potential association between *Pm*-PCR status and fledging
164 probability at the individual or nest level was investigated. To do so, we classified as PCR-
165 positive all the nestlings from which *Pm* DNA was detected in at least one sample (individual
166 level), or that belonged to a nest from which *Pm* DNA was detected in at least one sample
167 (*i.e.*, including its parents; nest level). We then fitted logistic regressions, with fledging

168 success used as the response variable, and *Pm*-PCR status (negative or positive), breeding
169 season (categorical) and their interaction as potential explicative variables. All statistical
170 analyses were conducted in R 3.3.3. Additional methods and scripts are provided as ESM.
171
172 **Results**
173 The sensitivity of the developed RT-PCR scheme showed a positivity threshold of 9 DNA
174 molecule templates per reaction. We screened 391 samples from 197 adults, and 192 samples
175 from 67 nestlings. *Pm* DNA was detected in 157/583 tested samples (Table S2).
176 In adults, which were sampled during the early chick-rearing period only, prevalence
177 varied significantly among breeding seasons (LR $\chi^2 = 79$, $p < 0.01$; Tables S3-4), reaching its
178 maximum in 2015 (0.60 [0.50; 0.69]) and minimum in 2017 (0.01 [0.00; 0.07]; Figure 1a). In
179 nestlings, the model with an effect of the breeding season (LR $\chi^2 = 35$, $p < 0.01$), day (LR $\chi^2 =$
180 13, $p < 0.01$) and their interaction (LR $\chi^2 = 7$, $p = 0.07$) on prevalence was selected, indicating
181 variations among and within breeding seasons (Figure 1a; Tables S3 and 5). Notably,
182 prevalence was generally low at the beginning of the chick-rearing periods (≤ 0.14), except in
183 2014/2015 when prevalence was high already in December (0.70 [0.46; 0.88]), mirroring
184 prevalence in adults during the same period. Each year, prevalence tended to increase
185 throughout the season (see Table S2 for numerical data). Remarkably, prevalence in nestlings
186 was maximal in 2014/2015, 2015/2016 and 2016/2017, corresponding to seasons with very
187 low fledging success (≤ 0.20) while fledging success reached 0.57 [0.40; 0.81] in 2013/2014
188 (Figure 1b).
189 The longitudinal survey of a subset of birds also revealed that some individuals testing
190 positive at a given time point may test negative at one following time point (47/191 adults,
191 and 9/50 nestlings; Figures 2 and 3), suggesting that a fraction of the birds could possibly
192 clear out the infection. In 2014/2015, 2015/2016 and 2016/2017, all the fledged nestlings

193 sampled longitudinally from hatching were detected as positive at least once although
194 we did not detect any association between infection status and survival at the considered
195 monthly sampling scale (Figure S6). We did not detect any spatial structuration in *Pm*
196 infection status among nests either (Figure S5). Lastly, a histological analyses carried out to
197 assess the extent of tissue damage resulting from *Pm* infection and to rule out other
198 underlying processes revealed necrotic lesions in the heart, spleen and/or liver together with
199 Gram-negative bacterial sepsis in nine of 21 necropsied albatross nestlings (Figures S2-4),
200 together with hepatic and pulmonary congestion as well as haemorrhagic myocarditis (not
201 shown).

202 **Discussion**

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

211 *Temporal variations of* P. multocida *prevalence*

212 Infection prevalence was overall high, but varied among and within breeding seasons. As
213 expected considering the high pathogenicity of *Pm* in the study system (Weimerskirch 2004;
214 Bourret et al. 2018; Jaeger et al. 2018; and this study), very few nestlings (≤ 20%) fledged
215 in 2014/2015, 2015/2016 and 2016/2017, corresponding to the years with highest *Pm*
216 prevalence. Interestingly, all the fledged nestlings sampled longitudinally during these
217 three breeding seasons from hatching were detected as positive at least once. In contrast,

218 in 2013/2014, prevalence was minimal (but non-null) and nestling survival exceeded 50%. A
219 salient pattern highlighted here is the temporality of the infection. Indeed, prevalence was
220 most often low at the beginning of the chick-rearing period, then increases onwards until
221 fledging. The estimates obtained for 2014/2015 do not follow this trend, though: prevalence
222 was already high upon the first sampling date (*i.e.*, at the time of egg hatching), with over
223 50% of the birds testing positive, and remained high throughout the season. During
224 2014/2015 season, the epizootic may thus have started earlier for reasons that remain to be
225 elucidated. The magnitude of the infection prevalence together with its temporal dynamics
226 suggest that epizootics occur quasi-annually in this subcolony.

227 *Enlightening mechanisms of pathogen maintenance*

228 The recurrence of avian cholera outbreaks on Amsterdam Island raises the question of
229 the compartments involved in pathogen maintenance between two successive breeding
230 seasons, when most seabirds are absent from the island. We can hypothesize (H1) that some
231 of the infected birds surviving the infection become chronic shedders, hence re-infecting the
232 island every year as suggested in other wild systems (Samuel et al. 2005). The role of chronic
233 shedders may be especially important to explain outbreak recurrence in a system such as
234 Amsterdam Island where a high proportion of individuals, adults in particular, seems to
235 survive exposure, contrasting with other systems where adult survival is strongly affected by
236 the disease (e.g., Crawford et al. 1992; Österblom et al. 2004; Leotta et al. 2006; Descamps et
237 al. 2012; Wille et al. 2016; Iverson et al. 2016). Alternatively, the pathogen may persist on the
238 island during winter in the absence of yellow-nosed albatrosses, either in the environment
239 (Samuel et al., 2004) or in one of the resident avian/rodent species (H2). These non-exclusive
240 hypotheses may be tested by screening (i) environmental samples, (ii) adult birds at their
241 return to the island in September, and (iii) the putative reservoir species that permanently
242 settle on the island. Indeed, *Pm* circulation on Amsterdam Island is not restricted to yellow-

243 nosed albatrosses. Notably, predators and scavengers such as brown skuas (*Stercorarius*
244 *antarcticus*) and introduced rodents (*Rattus norvegicus* and *Mus musculus*) may contribute to
245 the maintenance and/or circulation of the pathogen (Curtis 1983; Gamble et al. 2019a).
246 Screening the environment and the resident animal species, especially before the return of
247 yellow-nosed albatrosses, will improve our understanding of the whole epidemiological
248 network. The novel PCR protocol we developed here is pivotal in this perspective. Overall,
249 the results presented herein will help to design field studies in an iterative process, as future
250 sampling protocols designed to elucidate the transmission and maintenance cycles will need
251 to be updated (Restif et al. 2012; see below).

252 *Methodological implications*

253 Avian cholera outbreaks on Amsterdam Island are characterized by a rapid epidemiological
254 process, as highlighted by the steep increase of *Pm* prevalence and decrease of nestling
255 survival throughout the breeding season, although some inter-annual variation exists. Hence,
256 a finer temporal scale of sampling is needed to precisely quantify key epidemiological
257 parameters, such as the case fatality rate and the probability to clear out the infection, as well
258 as to assess the presence of chronic shedders, as suggested by the longitudinal survey. Indeed,
259 the discrepancy between the within-year temporal scales of the sampling (once a month) and
260 the epidemiological process (*Pm* being believed to cause death within a few days; Wobeser
261 1997) prevented us from quantifying these parameters in this study (see Figure S7 for detailed
262 explanation). In addition, future studies could include repeated sampling (e.g., successive
263 collection of several swabs from a given individual at a given time point) in order to account
264 for detection probability issues. Indeed, most molecular assays have a sensitivity below unity,
265 and repeated samples can allow to estimate the protocol detection probability, and ultimately
266 account for it analysing the data using the patch occupancy framework(McClintock et al.
267 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 of 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 the 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.

Authors' 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.

Conflict of interest

The authors declare that they have no conflict of interest.

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.

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 (license #04939.03).

302 **Figures**

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

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

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

318

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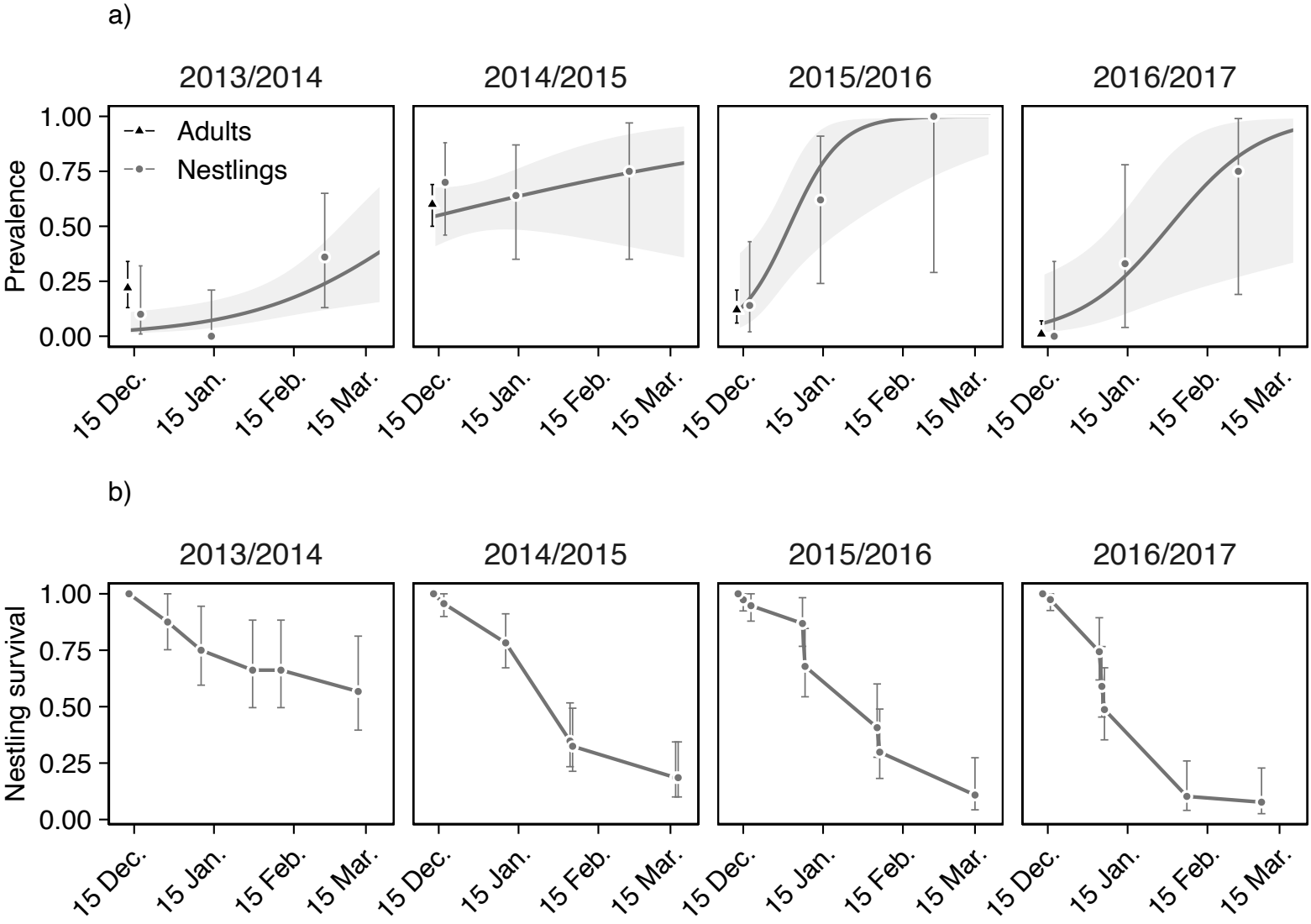
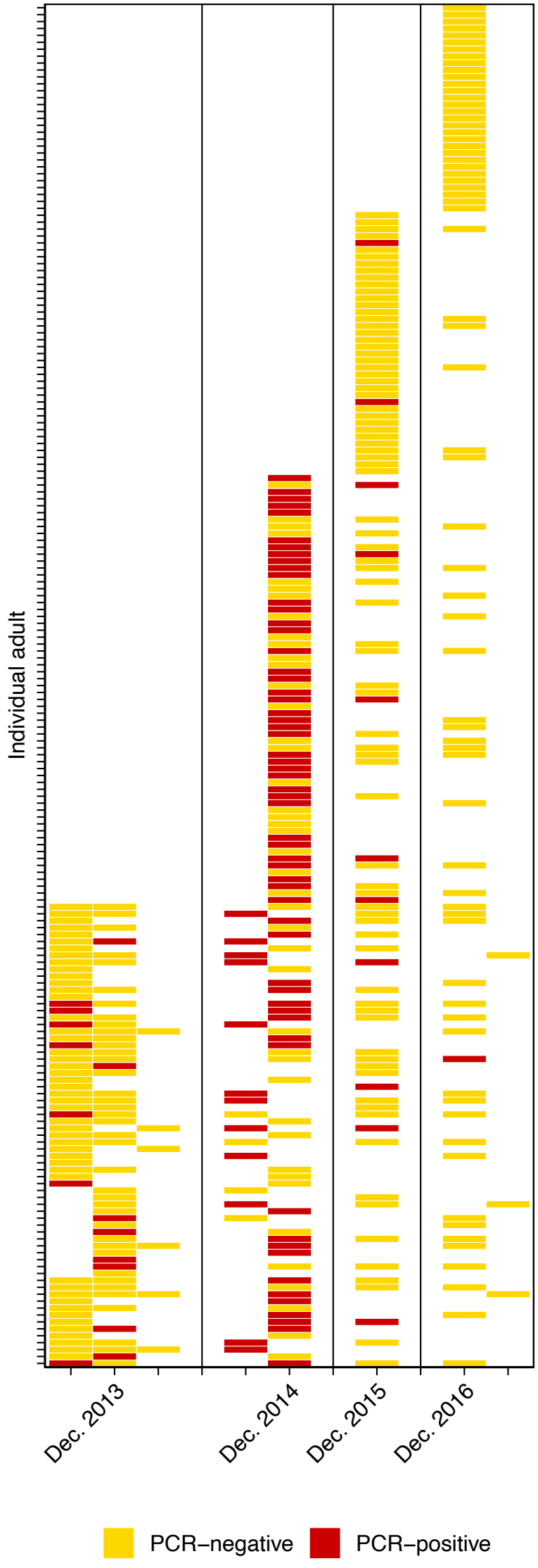
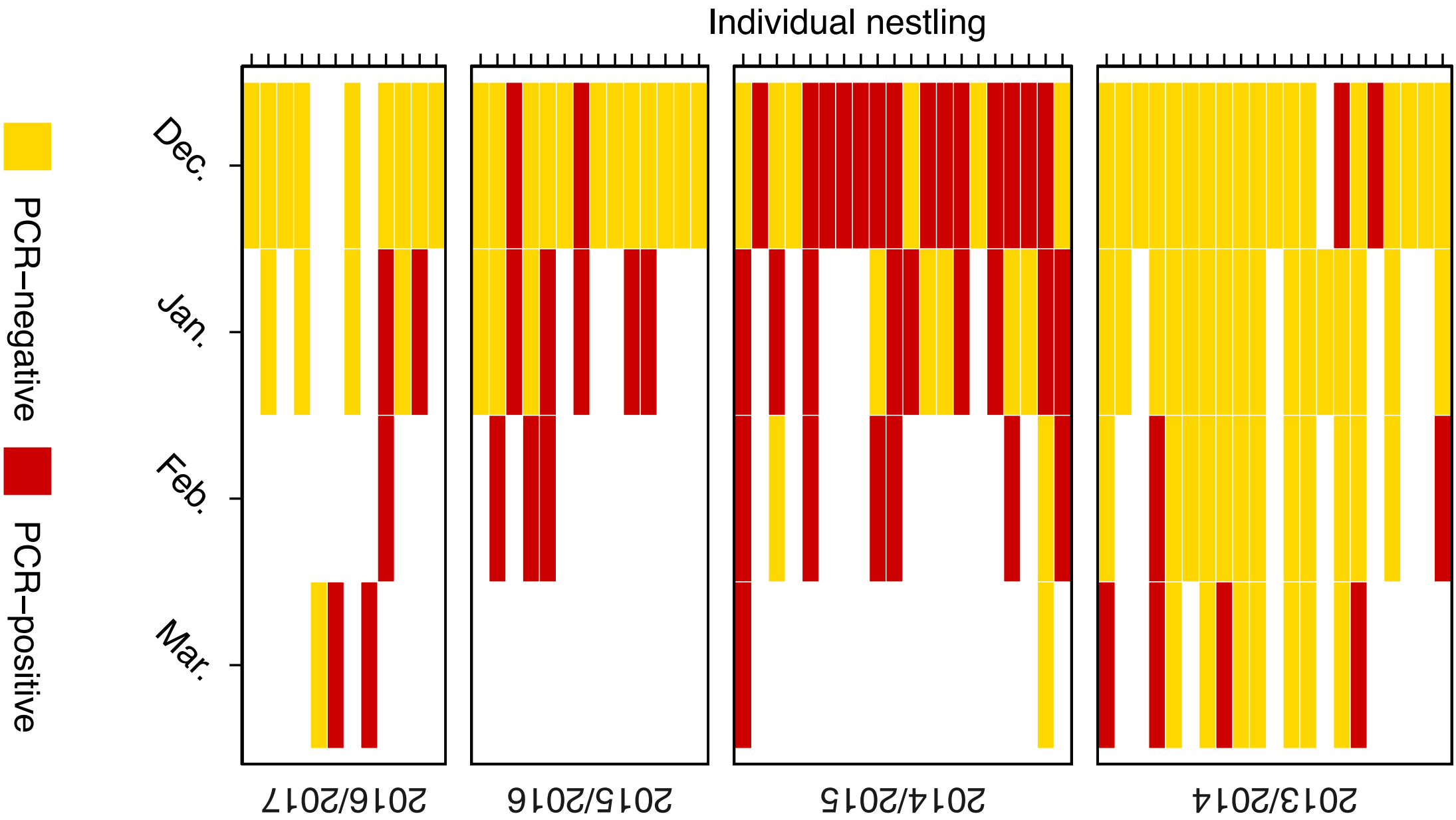

a)
2013/2014
2014/2015
2015/2016
2016/2017
Adults
Nestlings
Prevalence
1.00
0.75
0.50
0.25
0.00
15 Dec.
15 Jan.
15 Feb.
15 Mar.
b)
Nestling survival


Individual adult
Dec. 2013
Dec. 2014
Dec. 2015
Dec. 2016
PCR−negative
PCR−positive


Individual nestling
Dec.
Jan.
Feb.
Mar.
2016/2017
2015/2016
2014/2015
2013/2014
PCR−negative
PCR−positive