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Migratory patterns of two major influenza virus host species on tropical islands

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Animal migration is a major driver of infectious agent dispersal. Duck and seabird migrations, for instance, play a key role in the spatial transmission dynamics and gene flow of avian influenza viruses (AIV), worldwide. On tropical islands, brown and lesser noddies (*Anous stolidus* and *Anous tenuirostris*) may be important AIV hosts, but the lack of knowledge on their migratory behaviour limits our understanding of virus circulation in island networks. Here we show that high connectivity between islands generated by non-breeding dispersive behaviours may be a major driver in the spread and the maintenance of AIV among tropical islands of the western Indian Ocean. Tracking data highlight two types of dispersive behaviours during the non-breeding season: birds either staying in the vicinity of their breeding ground (on Bird Island, Seychelles), or moving to and roosting on other islands in the western Indian Ocean. Migrant birds used a wide range of roosting places from the Tanzanian coasts to the Maldives archipelago and Tromelin Island. Epidemiological data confirm that brown and lesser noddies are major hosts for AIV, although significant variations of seroprevalence between species suggest that other biological and ecological drivers could be involved in virus infection and transmission dynamics.

1. Introduction

Animal migration is a key mechanism for the dispersal of infectious agents over long distances [1] and may play a significant role in the spread of viruses to and among island ecosystems. Tropical oceanic islands might for instance be connected to the global avian influenza virus (AIV; *Alphainfluenzavirus*) epidemiology [2], but the frequency of virus exchanges between islands and neighbouring continents remains to be precisely assessed. Wild ducks and seabirds (mostly gulls and shorebirds) are natural hosts for AIV [3,4]. Gulls and terns are reservoir hosts for the H13 and H16 virus subtypes [5–7]. In these hosts, low prevalences of infected birds are usually reported [8–11], although high temporal variation is likely to characterize AIV transmission dynamics, in particular during the breeding season [12]. However, most investigations have focused on AIV transmission on continental habitats [13,14], and virus transmission dynamics and diversity in seabird populations on tropical oceanic islands is yet to be characterized.

The absence of wild ducks on most tropical islands could lead to major differences in AIV ecology and epidemiology, as compared to continental habitats. Tropical islands are major breeding sites for terns (order Charadriiformes), aggregating hundreds of thousands of birds at very high densities in breeding colonies [15]. High seabird breeding-site fidelity [16] could restrict virus dispersal between populations breeding on different islands. Because of the discrete geographical nature of oceanic islands, host population structure could also have major effects on virus transmission between islands as well as on the diversity of viruses circulating on each island. Spatial isolation may create an opportunity for the maintenance of viruses in wild bird communities inhabiting these islands, leading to the endemic circulation of certain AIV subtypes and genotypes (e.g. H15; [17]). Extensive seabird migrations may, however, counterbalance this effect by increasing virus dispersal between islands and homogenizing virus diversity between islands.

With an estimated breeding population size of 19 million individuals [18], seabirds are the most abundant and widespread avifauna in the western Indian Ocean [19]. Small oceanic islands in this region are major breeding sites for terns, with several species aggregating at very high densities in breeding colonies, involving hundreds of thousands, occasionally millions, of birds [15]. In a previous study, we identified the host range of AIV in seabirds in the islands of the western Indian Ocean, and further assessed the virus subtype diversity based on serological assays [2]. These findings suggested that terns may represent a major and neglected host on tropical oceanic islands. For instance, high prevalence of infection was estimated in lesser noddy (LN; *Anous tenuirostris*) on Reunion Island (up to 28% of birds shedding virus and 79% of seropositive birds). Such high prevalences contrast with the low level of AIV detection usually reported for terns [13], and are comparable to prevalences usually reported in ducks and gulls [3,20]. Virus gene flow between Eurasia and the western Indian Ocean was also demonstrated [2], highlighting that in spite of their spatial isolation, tropical oceanic islands are connected to global AIV epidemiology, and that tern migrations and behaviour may create opportunities for the maintenance of viruses in wild bird communities inhabiting these islands.

In this study, we investigated the dispersive behaviours of two seabird species (LN and brown noddy (BN), *Anous stolidus*). After breeding, tropical seabirds as their polar and temperate counterparts, can perform extensive migrations during their non-breeding period, although some of them stay at the vicinity of their breeding ground. Based on tracking data, we characterized their non-breeding distribution and activity in the western Indian Ocean. Serology and molecular detection were carried out to assess the prevalence of birds with AIV antibodies and shedding viruses, respectively. Our results highlight the high connectivity between islands, generated by non-breeding migrations, and the key role of LN and BN in the spread of AIV among tropical islands.

2. Material and methods

2.1. Study site

The study was conducted on Bird Island, the northernmost island of the Seychelles archipelago (3°43′ S, 55°12′ E). Bird Island is a 90 ha low-lying coral sand cay and a major breeding site for terns. Approximately 400 000 pairs of sooty terns (ST; *Onychoprion fuscatus*) nest annually on the ground in the northern part of the island [21]. The colony is a tourist attraction for visitors to the small hotel, located in the southern part of the island.

Woodland areas located in the centre of the island provide habitat for three tree-nesting seabird species: BN, LN, and the white tern (*Gygis alba*). During the breeding season (April to September), approximately 10 000 BN and 19 000 LN breeding pairs inhabit the island [21,22]. BN are mainly located in coconut trees and *Casuarina equisetifolia*, with some in decorative *Cordia subcordata* around the hotel and also in low *Scaevola taccada* around the coast and fringing the airstrip. BN also nest on the ground around the hotel. LN nest in the woodland on the western side of the island, mainly in *Pisonia grandis*, but also in *Ca. equisetifolia* and, around the hotel, in *Co. subcordata*. White terns nest mainly in *Casuarinas*, but some in smaller trees and bushes; the population slightly increased since the early 1970s when it was estimated at about 720 breeding pairs [22].

2.2. Research permits and ethic statements

Field work and collection of biological material in the Seychelles were approved by the Seychelles Bureau of Standards and the Seychelles Ministry of Agriculture, Climate Change and Environment. Bird capture, handling, and marking was also approved by the Center for Research on Bird Population Biology (National Museum of Natural History, Paris; Personal Program 616 of MLC) and the British Trust for Ornithology. All procedures were evaluated by an ethic committee (Comité d'éthique de La Réunion; agreement number A974001) and authorized by the French Ministry of Education and Research (APAFIS#3719-2016012110233597v2).

2.3. Seabird tracking

BN and LN at-sea distribution and activity were investigated with global location sensors (GLS). GLS were attached to stainless alloy leg rings with cable ties, on the tarsus of incubating adults. The combined mass of logger, leg band, and cable ties represented less than 3% of the body mass and thus was within acceptable mass limits for devices attached to seabirds [23]. In 2012, 25 GLS (MK18 model, British Antarctic Survey, United Kingdom) were deployed on BN as a preliminary study. Fifteen (60%) were recovered the following year (data were successfully downloaded from 13 GLS). Given the recovery rate, additional GLS were then deployed in 2014, on BN (17 MK3006, Biotrack Ltd., United Kingdom), and also on LN (17 MK5093, Biotrack Ltd., United Kingdom). Fifteen GLS were recovered from this second set of equipped birds, and data were successfully downloaded from 14 GLS (7 BN and 7 LN).

GLS devices record elapsed time and light level, allowing estimates of geographical position twice per day with an average spatial accuracy of 186 km for birds in flight [24]. Bird locations were estimated using the threshold-method with the GeoLight package [25]. We then removed unrealistic positions yielding unrealistic flight speed [26]. Fifty percent kernel density distributions (core areas) were calculated to examine the non-breeding at-sea distribution of the birds with the adehabitatHR package [27], using a smoothing factor of 200 km based on the magnitude of error in estimating locations from GLS [28]. The date and duration of migrations and long foraging trips were determined by identifying rapid shifts in distance from the colony for each bird. We designated roosting places as the closest island to the centroid of each non-breeding individual core area.

GLS also test for saltwater immersion every 3 s and record number of positive tests from 0 (continuously dry) to 200 (continuously wet) each 10 min. We thus estimated the percentage of time spent in contact with seawater [29], during both day and night (each 10 min block was categorized based on light data), and for both the breeding and non-breeding periods (classified with migrations dates derived from geolocation data). We reported the percentages of time spent in contact with seawater for the duration of the entire study (e.g. percentage during daylight hours across the breeding period was calculated by dividing the sum of all daylight 10 min blocks by the sum of all 10 min blocks during the breeding period). The fixed effects of sex, species, photoperiod (day and night data) and season (breeding and non-breeding data) on the percentage of time spent on water were investigated using a generalized linear mixed model (GLMM) with individuals as random effect. Analyses were performed with R 3.4.2 [30].

2.4. Bird sampling

Samples were collected to investigate species-related and temporal variation in AIV shedding and antibodies between the three most abundant tern species on Bird Island. In addition to tracked birds, samples were collected from a larger number of BN, LN, and also ST (details available in the electronic supplementary material, table S1). For each bird, faeces (cloacal swab) and saliva

(oropharyngeal swab) were collected with sterile rayon-tipped applicators (Puritan, Guilford, ME, USA). Both swabs were placed in a single tube, containing 1.5 ml of brain heart infusion media (Conda, Madrid, Spain) supplemented with penicillin G (1000 units ml^{-1}), streptomycin (1 mg ml^{-1}), kanamycin (0.5 mg ml^{-1}), gentamicin (0.25 mg ml^{-1}) and amphotericin B (0.025 mg ml^{-1}) [2,31]. Swabs were maintained at 4°C in the field, shipped to the laboratory within 48 h, and held at −80°C until tested. A small sample of blood (up to 1.0% of body weight) was collected from the medial metatarsal vein and centrifuged within 4 h after collection. Sera were transferred in cryotubes and stored at −20°C. Samples were shipped to the laboratory within 48 h and held at −20°C until tested.

2.5. Serology

Sera were tested with the IDvet ID Screen Influenza A Antibody Competition (IDvet, Montpellier, France) enzyme-linked immunosorbent assay, following an optimized protocol for the detection of antibodies specific to the AIV nucleoprotein (NP) in wild birds [32]. This protocol was also used for the detection of seropositive seabirds on Bird Island, in a previous study [2]. Sample absorbance was measured at 450 nm with a Sunrise microplate reader (TECAN, Grödig, Austria). Samples with a sample-to-negative control ratio (S/N) below 0.4 were considered positive for the presence of AIV NP antibodies; samples with S/N greater than or equal to 0.55 were considered negative. Samples that yielded S/N between 0.4 and 0.55 were re-tested and, following the S/N obtained in the second test, were considered either negative ($S/N > 0.4$) or positive ($S/N < 0.4$).

Previously published data were included in the statistical analysis ($n = 568$ sera collected in 2012 and 2013; electronic supplementary material, table S1). Chi square tests (χ^2) were performed to test the effect of the bird species (BN, LN, ST) and the breeding season (2012, 2013, 2014, 2015) on the probability of successful detection of AIV NP antibodies in bird serum, with R 3.4.2 [30]. Given the low number of sampled chicks ($n = 33$) and juveniles ($n = 4$), only adult birds were included in the statistical analysis ($n = 1246$; electronic supplementary material, table S2).

2.6. Molecular detection

Samples (swabs) were thawed overnight at 4°C, briefly vortexed and centrifuged at 1500*g* for 15 min. RNA extraction was performed with the QIAamp Viral RNA Mini Kit (QIAGEN, Valencia, CA, USA). Reverse-transcription was performed on 10 µl of RNA, with the ProtoScript II Reverse Transcriptase and Random Primer 6 (New England BioLabs, Ipswich, Massachusetts, USA), under the following thermal conditions: 70°C for 5 min, 25°C for 10 min, 42°C for 50 min and 65°C for 20 min. Complementary DNA were tested for the presence of the AIV Matrix (M) gene by real-time polymerase chain reaction (rt-PCR) [33] with a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA).

2.7. Molecular sexing

Molecular sexing was performed for birds equipped with GLS [34]. DNA was extracted from blood samples with the QIAmp Blood & Tissue kit (QIAGEN, Valencia, CA, USA). PCR reactions were performed with 7.5 µl of GoTaq G2 Hot Start Green Master Mix (Promega, Madison, WI, USA), 0.6 µl of each primer (10 µM) and 50 ng of DNA template (4 µl), in a final volume of 15 µl. Amplifications were performed using a GeneAmp PCR System 9700 (Thermo Fisher Scientific, Waltham, MA, USA) under the following thermal conditions: 94°C for 2 min, followed by 40 cycles at 94°C for 30 s, 50°C for 30 s and 72°C for 45 s, and by a final elongation at 72°C for 4 min. PCR products were size-fractioned in a 1.5% agarose gel stained with GelRed nucleic acid gel stain (FluoProbes).

3. Results

3.1. Non-breeding at-sea distribution

Two behaviours were highlighted in BN and LN during the non-breeding period: birds remaining in the vicinity of their breeding ground (residents: BN = 8, LN = 2) and birds moving to and roosting on another island (migrants: BN = 12, LN = 5) (figures 1 and 2). Proportions of residents and migrants were not statistically different between species (Fisher's exact test, $p = 0.678$), nor between females and males of

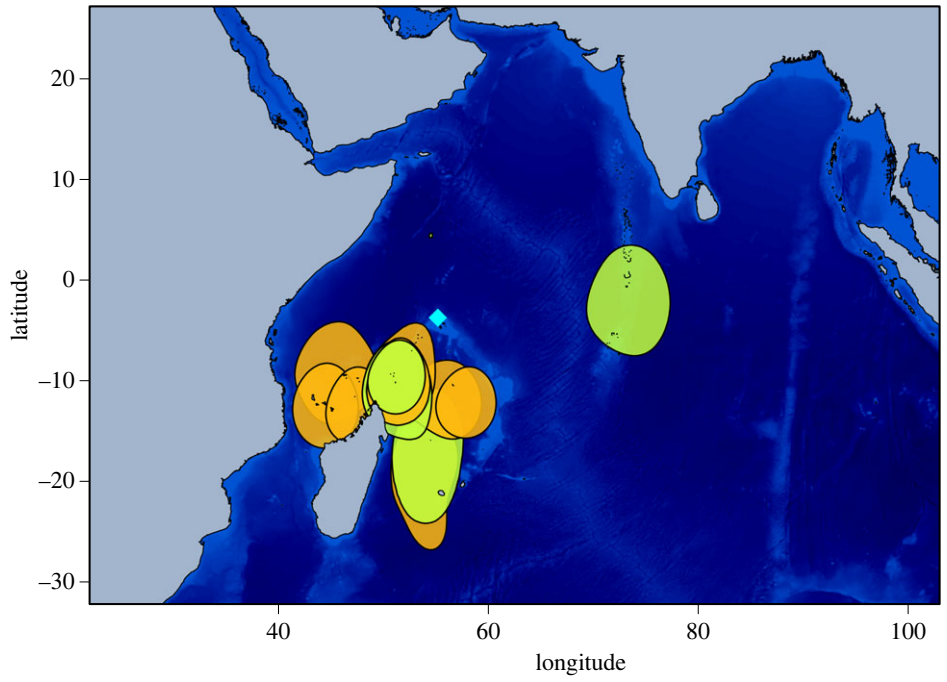

Figure 1. Brown noddy (BN; *Anous stolidus*) 50% kernel density distribution (migrant birds). The blue diamond indicates Bird Island (breeding colony) and black dots indicate roosting islands (i.e. closest island to the centroid of each non-breeding individual core area). Orange: females; green: males.

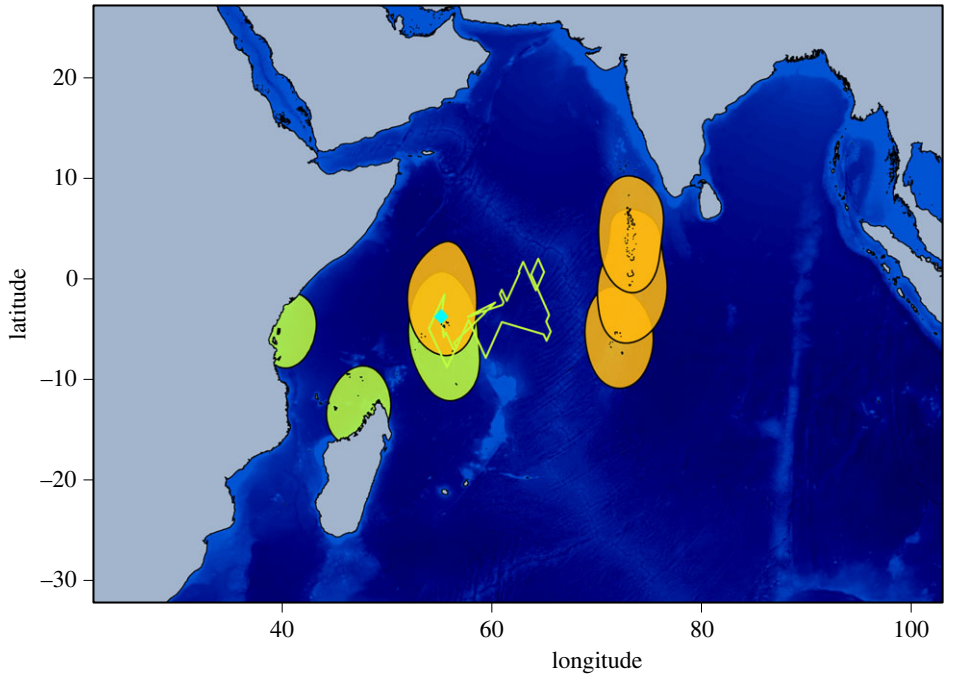

Figure 2. Lesser noddy (LN; *Anous tenuirostris*) 50% kernel density distribution (resident and migrant birds). The blue diamond indicates Bird Island (breeding colony) and black dots indicate roosting islands (i.e. closest island to the centroid of each non-breeding individual core area). The green track indicates a long foraging trip conducted by one resident bird. Orange: females; green: males.

the same species. Indeed, for BN, 80% of females and 40% of males were migrants (Fisher's exact test, $p = 0.17$), and for LN, 75% of females and 67% of males were migrants (Fisher's exact test, $p = 1$).

Migrant birds left the colony for 159.1 ± 35.3 and 174.5 ± 52.6 days for BN and LN, respectively (Mann-Whitney test, $p = 0.489$). All migrants used one specific roosting place except for one BN individual that visited two different places during the non-breeding period. Seven roosting places

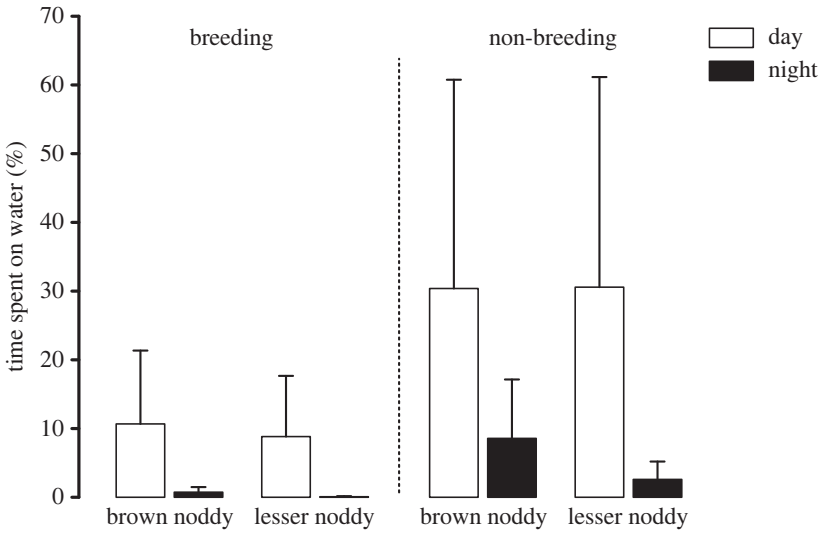

Figure 3. Proportion of time spent in contact with seawater for brown noddy (BN; *Anous stolidus*) and lesser noddy (LN; *Anous tenuirostris*). The proportion of time spent on water varied daily and seasonally (GLMM, both $p < 0.0001$), but no variation was observed between species (GLMM, $p = 0.072$).

were identified, mostly to the south of the Seychelles, on Tromelin Island ($n = 2$), Farquhar Group ($n = 5$), Agalega Island ($n = 2$), Aldabra Islands ($n = 1$), Comoro Islands ($n = 1$), and in the northwestern coast of Madagascar ($n = 1$); except one bird roosting to the east on the Maldives archipelago (figure 1). For LN, migration direction differed between males and females: all three migrant females roosted east of the Seychelles, on the Maldives ($n = 2$) and Chagos ($n = 1$) archipelagos, while both migrant males roosted to the west of the Seychelles on the Tanzanian coast ($n = 1$) and the northwest coast of Madagascar ($n = 1$) (figure 2).

Migrant BN, but not LN, performed long foraging trips from their roosting places (number of foraging trips per individual: 0.8 ± 1.0; average distance: 1 820.4 ± 1 075.3 km; average duration: 33.6 ± 23.2 days). Resident BN also performed long foraging trips (2.5 ± 0.8 per individuals; 2 244.2 ± 977.8 km; 64.4 ± 51.1 days). For LN, one resident bird performed a 17 days long foraging trip up to 1 206 km from its colony (figure 2, green track).

3.2. At-sea activity

The proportion of time spent on water varied daily and seasonally (GLMM, $F = 287.5$ and 189.1, respectively, both $p < 0.0001$), but no variation was observed between females and males (GLMM, $F = 0.6$, $p = 0.433$) nor between species (GLMM, $F = 3.6$, $p = 0.072$) (figure 3). Indeed, birds spent more time on water during the day (20.5 ± 10.6% for BN and 19.7 ± 11.8% for LN) than during the night (4.7 ± 5.6% for BN and 1.3 ± 2.0% for LN), and during the non-breeding period (19.5 ± 12.0% for BN and 16.5 ± 14.9% for LN) than during the breeding period (5.6 ± 5.4% for BN and 4.5 ± 4.9% for LN).

3.3. Molecular detection and seroprevalence of avian influenza virus

In total, cloacal and oropharyngeal swabs were obtained from 720 birds: 284 BN ($n = 151$ in 2014 and $n = 133$ in 2015), 274 LN ($n = 173$ in 2014 and $n = 101$ in 2015), and 162 ST ($n = 109$ in 2014 and $n = 53$ in 2015). None of the samples tested positive for the presence of the AIV M gene by rt-PCR.

Overall, significant differences in the prevalence of seropositive birds were detected between species ($\chi^2_2 = 241$; $p < 0.001$) and between years ($\chi^2_3 = 32$; $p < 0.001$) (figure 4). Seroprevalence was higher for LN (58 ± 4.9%) than for BN (27 ± 4.3%) and for ST (7.9 ± 2.4%); it was also higher in 2014 (36 ± 4.7%) than in 2012 (17 ± 4.3%), 2013 (32 ± 5.5%) and 2015 (31 ± 5.3%). When each species was considered independently, however, inter-annual variations were no longer significant (BN: $\chi^2_3 = 5.3$; $p = 0.15$; LN: $\chi^2_3 = 2.1$; $p = 0.55$; ST: $\chi^2_3 = 1.1$; $p = 0.77$).

Individual variation of AIV NP specific antibodies among years was investigated for recaptured birds. For BN, 63 birds were tested at two different breeding seasons, between 2012 and 2015. For LN, only 10 birds sampled in 2014 were recaptured and tested in 2015. Among those birds, 27% and 70%

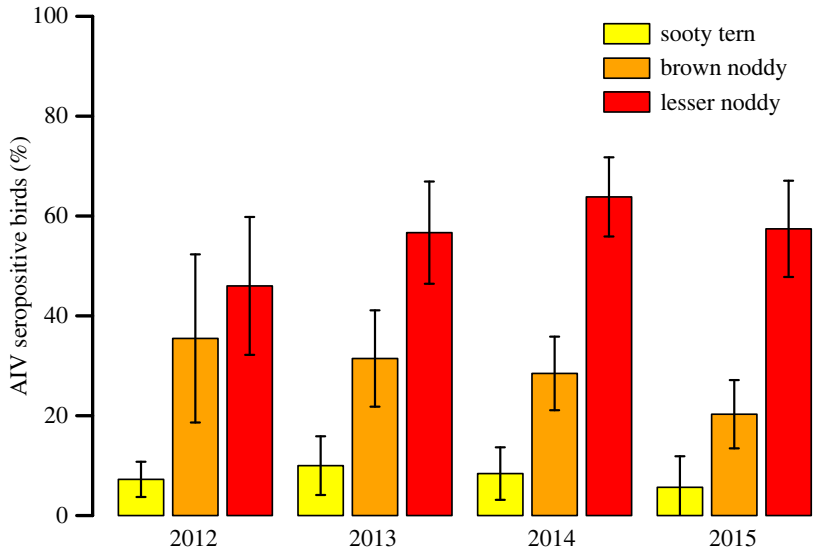

Figure 4. Prevalence of avian influenza virus (AIV) seropositive adult birds (± 95% confidence interval).

tested positive at least one year, for BN and LN, respectively. For BN, but not for LN, different seroconversion patterns were found (figure 5; electronic supplementary material, table S3). Although most seropositive birds remained positive for consecutive years, four birds acquired NP antibodies between the first and the second year (GE54841, GE54824, DE92952 and DE92989), and three birds tested negative after being previously positive (DE92967, DE95924, GE41756). One bird tested negative the first year, positive the following one, and turned seronegative again the third year (GE54837; figure 5).

4. Discussion

Host migrations favour the mixing of AIV between flyways [35] and intercontinental gene flow [36], in particular for seabirds [4,7]. Incorporating epidemiological data and animal movement analyses can provide critical insight for the understanding of virus transmission dynamics in island networks. In this study, we characterized the distribution and activity patterns of BN and LN, and assessed species-related and inter-annual variation in AIV shedding and seroprevalence. Our findings demonstrate the very diverse dispersive behaviours of BN and LN, and the high connectivity between islands generated by non-breeding migrations. Indeed, although based on a relatively limited number of tracked birds at a single breeding site, we detected a potential connectivity with 10 different roosting islands. AIV seroprevalence further support that these species are major hosts for AIV. At the scale of the western Indian Ocean, BN and LN migratory behaviours could therefore have major implications for AIV transmission between islands.

Specificity in the ecology of terns might account for the variation in virus infection and transmission between closely related species. For instance, species-related differences in life history such as colonial nesting, social behaviour, migration and foraging characteristics could affect opportunities for virus transmission. The extensive non-breeding migration of ST (average travelled distance > 50 000 km), together with their infrequent contact with seawater and time spent on the ground [37], may for instance limit infection opportunities. ST also never roosts on islands during the non-breeding migration, remaining airborne throughout the non-breeding period [37], and may therefore have a more limited role than BN and LN in AIV inter-island transmission. For both BN and LN, our data showed that tracked birds either stayed in the vicinity of their breeding ground, or moved to and roosted on another island, without major differences in the proportion of resident versus migrant birds between species. However, given the significant difference of AIV seroprevalence between BN and LN, other factors than migratory and activity patterns are likely to be involved in virus transmission.

Differences in the diversity and location of roosting sites selected by BN and LN may also account for the variation of AIV exposure. Indeed, one may hypothesize that virus maintenance and transmission could be heterogeneous at the scale of the western Indian ocean (i.e. between islands), therefore generating a higher proportion of seropositive birds for species migrating to AIV circulation areas.

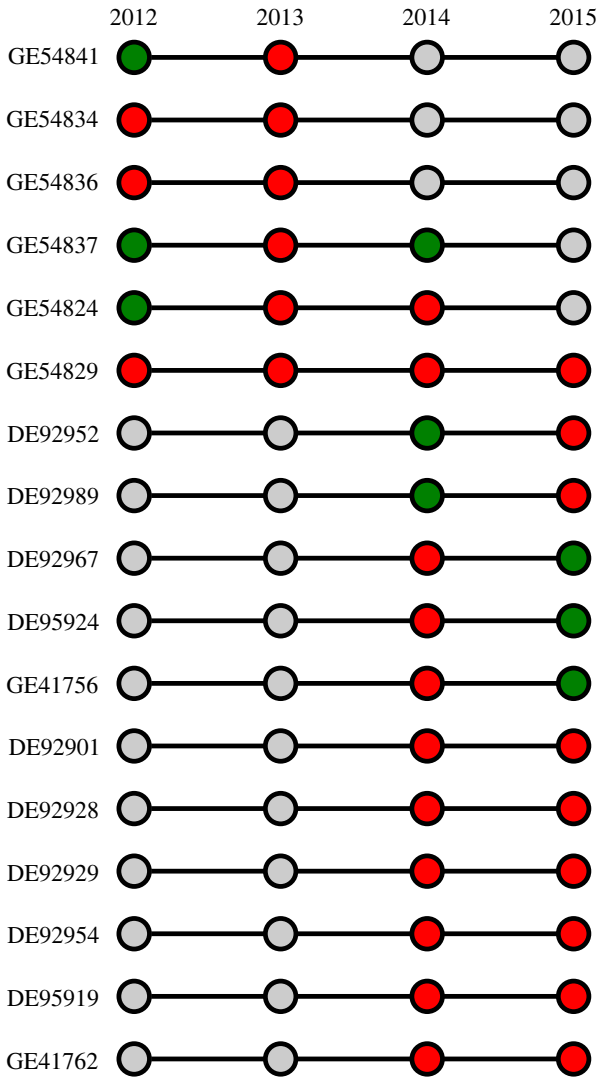

Figure 5. Individual patterns of influenza A virus nucleoprotein specific antibodies in brown noddies (BN; *Anous stolidus*). Only birds that tested positive at least one year are included. Green circle: seronegative bird; red circle: seropositive bird; grey circle: not sampled.

Our tracking data did not reveal species-related variation in bird migration but rather individual behaviours in the selection of roosting islands. Factors involved in the selection of roosting sites include nearby predictable availability of prey, which are often associated with the proximity of larger sub-surface predators [38] and oceanographic features including upwellings and counter-currents [39]. Conversely, seabirds might avoid regions prone to adverse weather, especially storms [40]. Our results also contrast with those reported in a study conducted in Western Australia, where BN undertook relatively short northward migrations during the non-breeding period (around 950 km north of the colony) and LN remained in vicinity of their breeding ground [41], and where AIV was also detected in LN and in ST in the 1970s [42]. In order to identify the links between tern migratory patterns and AIV transmission, future studies will have to focus on the drivers and repeatability of migratory decision making at the individual level, as well as inter-annual variation in the selection of roosting sites and associated infectious and immune status of migrant birds.

Beyond knowledge on tropical tern migrations, current understanding of AIV transmission dynamics in tropical seabirds remains limited. Although the seasonal increase of the number of fledglings at the end of the breeding season could drive virus transmission within seabird colonies [43], mechanisms involved in the inter-annual maintenance of these viruses remain to be identified. In this study, none of the samples tested positive for AIV RNA. This may be because sampling was conducted mostly on adult birds that already had mounted AIV-specific immunity, but also because sampling occurred mostly before hatching and the incoming of immunologically naive chicks into the population. Seroconversion was detected in recaptured adult BN, suggesting that infection occurred during the

course of the study. Differences in seroconversion patterns could be associated with AIV subtype-specific variation in the long-term immune response and protection, as demonstrated for H13 and H16 [44]. Longitudinal studies are needed to precisely assess the temporal variation and drivers of AIV transmission on tropical islands. Because for most species, seabirds leave their breeding colonies after chick fledging, virus detection after the breeding season is rarely feasible. Further epidemiological studies on resident BN and LN could, however, provide information on the ecological drivers of viral infection but also on the timing of virus introduction and transmission dynamics in highly isolated seabird populations.

Seabirds are highly sensitive to environmental changes and significant modifications of their biology in response to climatic [45] and anthropogenic changes, such as habitat modifications and alien predator introductions [46], have been described. Seabird numbers have declined by 70% over the past 60 years, with the highest decreases reported for terns [46]. Recently, the emergence and intercontinental spread of the highly pathogenic H5N1 AIV has been responsible for unprecedented mass mortality in seabirds, including terns [47]. Future studies focusing on AIV epidemiology on tropical oceanic islands will have to assess the consequences of the introduction of highly pathogenic viruses but will also need to consider the potential cascade effects of environmental changes, population decline and individual stress on virus transmission dynamics.

Ethics. All procedures were evaluated by an ethics committee (Comité d'éthique de La Réunion; agreement number A974001) and authorized by the French Ministry of Education and Research (APAFIS#3719-2016012110233597v2).

Data accessibility. Tracking data are available in the Seabird Tracking Database (dataset ID nos. 1943 and 1944), and epidemiological data in the electronic supplementary material [48].

Declaration of AI use. We have not used AI-assisted technologies in creating this article.

Authors' contributions. C.L.: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, visualization, writing—original draft; S.B.: formal analysis, investigation, visualization, writing—review and editing; C.F.: conceptualization, investigation, methodology, writing—review and editing; M.D.: investigation, writing—review and editing; C.L.: investigation; L.H.: investigation, methodology, writing—review and editing; M.L.C.: funding acquisition, writing—review and editing; A.J.: conceptualization, data curation, formal analysis, investigation, methodology, visualization, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. The authors declare that they have no competing interests.

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