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# Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions

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ABSTRACT

Migratory animals encounter suites of novel microbes as they move between disparate sites during their migrations, and are frequently implicated in the global spread of pathogens. Although wild animals have been shown to source a proportion of their gut microbiota from their environment, the susceptibility of migrants to enteric infections may be dependent upon the capacity of their gut microbiota to resist incorporating encountered microbes. To evaluate migrants' susceptibility to microbial invasion, we determined the extent of microbial sourcing from the foraging environment, and examined how this influenced gut microbiota dynamics

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over time and space in a migratory shorebird, the Red-necked stint. Contrary to previous studies on wild, non-migratory hosts, we found that stint on their non-breeding grounds obtained very little of their microbiota from their environment, with most individuals sourcing only 0.1% of gut microbes from foraging sediment. This microbial resistance was reflected at the population level by only weak compositional differences between stint flocks occupying ecologically-distinct sites, and by our finding that stint that had recently migrated 10,000 km did not differ in diversity or taxonomy from those that had inhabited the same site for a full year. However, recent migrants had much greater abundances of the genus *Corynebacterium*, suggesting a potential microbial response to either migration or exposure to a novel environment. We conclude that the gut microbiota of stint is largely resistant to invasion from ingested microbes, and that this may have implications for their susceptibility to enteric infections during migration.

## INTRODUCTION

The vast communities of microorganisms that make up the gastrointestinal ('gut') microbiota of animals are fundamental to host metabolism, nutrient acquisition, and immune function (Khosravi & Mazmanian 2013; Thaiss *et al.* 2016; Turnbaugh *et al.* 2006). The ecological dynamics of this microbial community may be particularly important for migratory animals, because migrants face exceptional metabolic, nutritional, and immunological challenges as they traverse the globe during their migrations (Altizer *et al.* 2011; Wikelski *et al.* 2003). Notably, migrants are thought to encounter and ingest novel suites of microbes, including parasites and potential pathogens, as they forage at disparate locations along their migratory routes (Figuerola & Green 2000; Leung & Koprivnikar 2016). This increased risk of infection, in combination with their high mobility, has raised concerns that migratory animals may be of particular importance in the global transmission and dispersal of pathogenic microbes (Altizer *et al.* 2011; Waldenström *et al.* 2002). Critically, the risk of migrants

dispersing enteric pathogens is, in part, dependent on the extent to which they incorporate and maintain novel microbes encountered at each location in their gut microbiota.

The susceptibility of hosts to enteric infection is linked to the capacity of their gut microbiota to resist invasion by foreign microbes ('colonization resistance'; Van der Waaij *et al.* 1971). This resilience may be achieved either via niche competition between native and foreign microbes, or by commensal bacteria actively inducing host immune responses when under invasion (Kamada *et al.* 2013; Round & Mazmanian 2009). Although young animals, including migratory shorebirds, have been shown to establish their gut microbiota at birth or hatching by incorporating microbes from their immediate environment (Brooks *et al.* 2014; Dominguez-Bello *et al.* 2010; Grond 2017), once established the healthy microbiota of humans and captive animals is generally associated with high levels of stability (Benskin *et al.* 2010; Caporaso *et al.* 2011; Wu *et al.* 2011). However, the microbiota may not be resilient to change when continually exposed to new bacterial assemblages. For example, microbes from soil sediment can successfully colonise and persist in the guts of germ-free mice, even outcompeting gut specialists (Seedorf *et al.* 2014). Moreover, laboratory rats challenged with the microbiota of other individuals develop a microbiota that is more diverse and resembles that of donor rats (Manichanh *et al.* 2010). Indeed, fully-grown wild hosts have been shown to source a significant number of microbes from their environment, with wild woodrats and anole lizards estimated to source up to 25% and 47% of their gut microbiota community from ingested plant food, respectively (Kohl *et al.* 2016; Kohl & Dearing 2014). Whether such high levels of microbial sourcing from the environment is characteristic of all wild hosts, including those with migratory lifestyles, is unknown. However, if wild migrants have similar levels of environmental sourcing, then migratory hosts may increase their susceptibility to enteric infection through the continual incorporation of novel microbes ingested as they forage at multiple sites en route.

Understanding the mechanisms that drive gut microbiota composition in wild hosts is critical to understanding their susceptibility to enteric infections. This is particularly challenging for migratory animals, because migrants undergo simultaneous changes in geography, diet, and physiology, all of which may influence gut microbiota composition (David *et al.* 2014; Turnbaugh *et al.* 2006; Yatsunenko *et al.* 2012). Migratory birds have been shown to experience shifts in their gut microbiota composition over time, both during migration (Lewis *et al.* 2016), and over the breeding season (Kreisinger *et al.* 2017). However, the mechanisms behind these changes remain unclear. Whether they are driven by physiological requirements (e.g. a sudden physiological shift from sustained exercise to rapid mass gain in the case of refuelling migrants, or changes to reproductive hormones during breeding), alterations in diet, or represent the incorporation of novel microbes, is unknown, despite important implications for host susceptibility. Although laboratory based studies on wild hosts may help untangle these interactions, such studies may not truly reflect mechanisms acting in the wild. For example, bacterial sharing between gut and host environment decreased significantly in wild woodrats moved into captivity (25% to 6%; Kohl & Dearing 2014), highlighting the need for studies that elucidate microbiota dynamics and mechanisms in natural ecosystems (Amato 2013; Hird 2017).

In this study, we aimed to assess the invasion resistance of a long-distance migrant, the Red-necked stint (*Calidris ruficollis*), to ingested environmental microbes whilst controlling for host habitat and physiology. We achieved this by firstly determining the extent to which stint on their non-breeding grounds sourced microbes from their immediate foraging environment, and secondly by examining whether this translated into altered gut microbiota community structures across sites and over time. Importantly, the Red-necked stint provides an especially rare and insightful model species to investigate these questions for three reasons. Firstly, like many shorebird species, individuals remain on the non-breeding grounds for 1.5

years following their first migration from their natal sites in Siberia. This allows comparisons between birds that have remained 'resident' on the non-breeding grounds for a full year (at this point 'second year' individuals that are 15 months old) and those that had recently migrated from Siberia, via multiple locations (those three or more years old), providing two conspecific groups that share diet and environment, but differ in how recently they completed a long distance, multi-stopover migration. Secondly, stint forage for prey by sifting through coastal sediment and biofilm with their bills, with sediment and biofilm making up the major component of the diet and stomach contents of closely related, and ecologically similar, *Calidris* species (Kuwae *et al.* 2008; Lourenço *et al.* 2017; Mathot *et al.* 2010). This creates direct and ongoing exposure to sediment microbiota. Thirdly, stint are site faithful, and make limited movements during the non-breeding seasons, often remaining on the same foraging site within the same flock for the entire season (Rogers *et al.* 2010). This not only provides opportunities to monitor the same individuals over time, but also provides reasonable certainty of foraging areas and movement patterns over the season.

Given this study system, if the gut microbiota of stint is susceptible to invasion from environmental microbes, then a series of predictions can be made. Firstly, we predicted that individuals will source a similar proportion of their gut microbiota from their immediate foraging sediment to that found in previous studies of other wild hosts (30-50%). This would be reflected in distinct gut microbiota community structures between flocks occupying different sites. Secondly, we predicted that newly arrived migrants that had recently been exposed to novel suites of microbes during migration (adults) would have a phylogenetically distinct, and more diverse gut microbiota from resident second year birds that had inhabited the site for a full year. Thirdly, the microbiota of newly arrived migrants should, through ongoing exposure to the same local microbes and other members of the flock, become more similar to that of resident birds with increasing time spent at the non-breeding site.

Collectively, these analyses allow us to assess how resistant the gut microbiota of migratory stint are to invasion from novel environmental microbes during their non-breeding season.

## MATERIALS AND METHODS

### Sample collection

Red-necked stint from two non-breeding populations were captured using cannon nets in Victoria, Australia. One population occupied a coastal beach site, Flinders (-38°48 S, 145°00 E), and was sampled at three time points during the non-breeding season (September 2015 – April 2016) in order to assess temporal changes in gut microbiota communities. Twelve out of a total of 71 individuals were recaptured at least once over the season (see below). Firstly, a single flock of recent migrants (3+ years old) and resident second years (15 months old) were captured on the 20th September (n = 29). Given that adult stintarrive at this site over the course of mid- to late- September, recent migrants captured on this day would have completed their post-breeding migration 1 - 14 days prior to capture. Although age differences exist between the two groups, it is extremely unlikely that this would be the cause of differences in microbiota community structure. Age is an important factor determining gut microbiota composition when young, with chicks having different gut microbiota to adult birds in penguins, kittiwakes and barn swallows (Barbosa *et al.* 2016; Kreisinger *et al.* 2017; van Dongen *et al.* 2013). However, poultry studies suggest that gut microbiota structure resembles that of adults within 0.5 - 3 months after hatching (Oakley *et al.* 2014; Ranjitkar *et al.* 2016), and studies of two wild migratory shorebird species, Dunlin and Red phalarope, suggest that microbiota diversity stabilizes in 3-10 days old chicks (Grond 2017). On this basis, and given that both our resident and migrant groups consist of fully-grown birds that have completed at least one Siberia-to-Australia migration, we do not believe that differences in gut microbiota should exist between second year birds at 15 months old and

birds that are 3+ years old due to age *per se*. The population was then targeted on the 23rd January (n = 13), and again prior to the pre-breeding migration, on the 11th March (n = 18). At this point in their moult cycle adults and second year birds could not be distinguished on the basis of their plumage, although juveniles (birds hatched in the 2015 breeding season, and which arrived on the site October-November, after the first September catch) were still distinguishable. However, using recapture history of banded birds we were able to distinguish between adults and second year birds for 61% of the individuals at this point in time. As a comparison site, a second population inhabiting the Werribee Western Treatment Plant (WTP; -37°99 S, 144°61 E), a sewage treatment works characterized by lagoons and estuaries, was also sampled. Birds were captured during two capture events on the 28th December 2015 (n = 25). Stint are site-faithful on the non-breeding grounds, with little connectivity between the sites: of 9,856 recaptures of the same individual stint across the wider region of our study site over the last 30 years, only 146 individuals (1.5%) were recaptured at a different site to where they were first caught (Rogers *et al.* 2010).

Cloacal swabs were taken from stints using sterile swabs (Copan 170KS01), placed in sterile plastic tubes without medium, and kept refrigerated for 3 - 5 hours before being stored at -80°C. Differences in bacterial composition resulting from storage conditions generally do not eclipse differences between samples (Dominianni *et al.* 2014; Lauber *et al.* 2010), therefore we assume differences in refrigeration time had minimal effect on our results. Environmental samples of mud or sand from where birds had been observed foraging were collected at each capture site immediately after each capture event, and handled in the same manner as the cloacal swabs. Six environmental samples from each site were pooled into two DNA samples (2 x 3) per site, because we deemed small-scale spatial variation within the foraging areas were not relevant to our study.

DNA isolation, amplification and sequencing

DNA was isolated using the phenol-chloroform method (Green *et al.* 2012). Briefly, swabs were suspended individually in 400 µl cetrimonium bromide (CTAB) with 50 µl of proteinase K and 60 µl of 10% sodium dodecyl sulfate **(**SDS). This solution was briefly vortexed and incubated overnight at 56 °C. The next day, 50 µl of 5M NaCl and 500 µl of phenol was added to each solution, briefly vortexed and left at room temperature for 10 minutes. From here, DNA isolation and ethanol precipitation followed standard procedures outlined in Green et al. (2012). DNA was extracted from four sterile swabs as negative controls to correct for contaminants (Salter *et al.* 2014). DNA samples were sent to the Ramaciotti Centre for Genomics, Sydney, for amplification using paired 27F/519R primers that amplify a 500bp V1-V3 region of the 16S rRNA bacterial gene, and amplicons were then sequenced using Illumina MiSeq technology (Caporaso *et al.* 2012; full protocol for these primers available at www.bioplatforms.com). A mock community provided by Zybiotics was included as a positive control in order to assess exact sequencing error rate. In addition, two technical replicates were included as an additional data quality check.

Sequence processing

Paired sequences were joined using UPARSE pipeline (Edgar 2013), and quality filtered using USEARCH's maximum expected error method. Sequences were aligned and filtered in mothur following their standard operating procedure (MiSeq SOP; Kozich *et al.* 2013; accessed December 2016). We pre-clustered 2,066,515 unique sequences to allow four base pair differences, resulting in 703,453 unique sequences. Chimeras were identified using the UCHIME algorithm (Edgar *et al.* 2011), and 209,094 (29%) unique sequences were removed from the dataset. Sequences were grouped into operational taxonomic units (OTUs) based on a 97% similarity threshold. Taxonomic classification was performed using the SILVA

taxonomy (v123.1; Pruesse *et al.* 2007) trimmed to the alignment space of the amplicons (Werner *et al.* 2012). OTUs that were identified as mitochondria, eukaryotic (including chloroplast) or archaeal were removed from the data set. This created a total output of just under 4 million sequences. Analysis of the mock community found an average sequencing error rate of 0.2%. This is slightly higher than normal, and may explain the high proportion of singleton OTUs found in the final dataset, with 90% of 77,000 OTUs being represented by a single sequence (with a 'normal' proportion being between 5 - 40%, depending on sample types). Inspection of the technical repeats indicated that these singletons were likely due to sequencing error. We controlled for this error by excluding OTUs represented by 10 sequences or fewer to ensure sequencing error did not bias results. This excluded only 2% of total sequences. To ensure data quality, we also reran sequence processing with stricter quality control using a 50bp sliding window within mothur to discard reads that drop below Q25, which did not change analytical results. Rarefaction curves for the OTU table used for the study (i.e. excluding OTUs with total abundance of 10 or less) showed that almost all OTUs were detectable by 5000 reads (Fig. S1). Sequences classified to the genus *Corynebacterium* (see results) were extracted from the main data set and further analysed by oligotyping, using the minimum entropy decomposition pipeline (version 2.1) to reveal fine-scale diversity within the genus (Eren *et al.* 2014), to assess whether the increased abundances observed were representative of a single or multiple strains.

Data analysis

Analysis of OTU communities was conducted using the Phyloseq (McMurdie & Holmes 2013) and vegan (Oksanen *et al.* 2007) packages in R. The negative control contained forty OTUs represented by at least 5 sequences, and these OTUs were removed from the dataset. A single sample with under 7000 reads was excluded, and all remaining samples were rarefied to 9795 reads (the minimum read count) for further analyses. Because rarefied data can lead

to false positives (McMurdie & Holmes 2014), we repeated analyses without rarefying samples with no difference to overall results or conclusions. We applied MDS and NMDS ordinations and conducted ADONIS tests (Anderson 2001) to statistically test for differences between groups. Methods for accounting for repeated samples from the same individual in ordination analyses are not currently available. To make sure repeat samples did not affect results we reiterated analyses randomly excluding repeats, which did not affect overall results. Because primary components in the MDS analyses generally explained little variance, we present results from the NMDS ordination. We present both Bray-Curtis (based on abundance of OTUs) and unweighted Unifrac (based on evolutionary distance between OTUs; Hamady *et al.* 2010), distance measures. Unifrac distances were calculated using a 16S alignment with SILVA. To identify which particular groups of bacteria were different between groups, we ran the analysis through LEFse, hosted by the Huttenhower galaxy server (https://huttenhower.sph.harvard.edu/galaxy). We analysed bacterial richness by calculating both observed OTU richness and the Shannon diversity index. When comparing bacterial diversity between the three capture events within the Flinders population, we applied a mixed effect regression model with stint ID as a random effect to account for repeated measures.

We estimated the proportion of OTUs sourced from sediment samples using a Bayesian approach within SourceTracker (Knights *et al.* 2011). This approach uses the relative abundance of each OTU within both the sediment and each host to calculate the probability that each OTU found in the host gut was sourced from the sediment microbiota. Thereby it provides an estimate for the proportion of OTUs sourced from local sediment. For this analysis, we excluded any OTU which was represented by a single sequence in the control sample, because analyses suggested that 3% of OTUs present in our samples were sourced from laboratory contamination, despite being present at extremely low abundances (and therefore not affecting previous community composition analyses). Therefore, we note that

previous studies that did not account for contamination may have inflated levels of OTU sourcing. We repeated this analysis between all groups, and in both directions, to estimate common sources between groups (see Fig. 5a). However, one bird was excluded from these analyses because it was estimated to source 27% of its gut microbiota from the environment, whilst the median was 0.1% (see Fig. 2b). We therefore could not rule out that this was due to environmental contamination of this sample. Because the sediment microbiota of the two sites differed (see results), we carried out analyses within SourceTracker for each site separately. For birds at Flinders, we compared birds to sediment samples collected during the March capture only. Although microbial profiles of sediment may change to certain extent over time, there was no difference in levels of OTU sourcing from sediment between birds captured in September, January or March, indicating that this should not affect results.

RESULTS

A total of 2275 operational taxonomic units (OTUs) were identified from 85 cloacal samples from 71 individual stint, with 10 individuals from Flinders beach sampled twice over the non-breeding season, and two individuals sampled at all three time points. The majority of these OTUs had very low prevalence within the sampled stint population (Fig. S2). Only 12 OTUs (0.5% of the total OTUs derived from bird samples) made up the sampled population's 'core' microbiota (defined here as the suite of OTUs that occur in over 80% of samples; Table 1), whilst 85% of OTUs were present in less than 5% of birds. On average, the core microbiota made up 40 ± 23 (s.d.) % of the total microbial abundance for each individual, with the remainder being largely OTUs that were unique to the individual. Across stint samples, the most abundant bacterial phyla were Proteobacteria (33%), Fusobacteria (17%), Firmicutes (14%), Actinobacteria (11%), and Bacteroidetes (9%). Environmental samples taken from foraging sediment at each site showed a less diverse microbial community at the phylum level, consisting of mostly Proteobacteria and Bacteroidetes (Fig. 1a), but each sample

contained a much richer suite of OTUs than present within the individual stints (Fig. 1b). Both non-breeding sites displayed a distinct sediment microbial profile which was also distinct from the overall stint gut microbiota (Fig. 1c), with the most abundant OTUs for each site not overlapping with each other (Table 2).

Microbial sourcing from sediment across sites

Bayesian analysis with SourceTracker estimated only 1.7% of sediment OTUs at each site shared a common source (Fig. 2a). Stint did not source a significant proportion of their gut microbiota from their environment, with an average of 0.16 % (± 0.6 SD) and 0.4 % (± 1.4 SD) of gut microbiota estimated to be sourced from sediment for flocks occupying the Flinders and WTP non-breeding sites, respectively (Fig. 2b). Stint were estimated to share slightly more OTUs with their own foraging site than the alternative foraging site (Fig. 2a), but these differences were not significant ($t = 1.22$, $p = 0.23$). This low incorporation of sediment bacteria was reflected by the two flocks occupying different sites differing only weakly (but significantly) in their gut microbiota composition (Fig. 3a; Adonis test applying Bray Curtis distance matrix, which emphasises differences in abundance: $R^2 = 0.02$, $p = 0.04$; Unifrac distance matrix, which takes into account phylogeny but only considers presence/absence rather than abundance: $R^2 = 0.05$, $p = 0.001$, $n = 85$).

The weak differences in gut microbiota between the two flocks were attributed to a number of bacterial groups being slightly more prevalent in birds at the water treatment plant than birds at Flinders beach, including bacteria belonging to phylum Chloroflexi, family *Succinivibrionaceae* (phylum Proteobacteria), genera *Streptococcus* (phylum Firmicutes) and *Salinimicrobium* (phylum Bacterioidetes; Fig. 3b; Fig. S3 for abundance plots of each bacterial group). However, with the exception of three Chloroflexi OTUs that were found at

very low abundances in one stint each, none of the strains that showed higher prevalence in birds occupying the water treatment plant were present in environmental samples.

Despite the low levels of microbial sourcing from the environment, birds inhabiting the water treatment plant tended to have a richer suite of OTUs that those occupying Flinders beach (Observed richness: Flinders mean = 80.9 ± 32.6 s.d.; WTP mean = 142.5 ± 99.9 s.d.; t = 3.0, $p = 0.006$; Shannon index: t = 2.3, $p = 0.03$; Fig. 3c), although overall composition at the phyla level between populations was very similar (Fig. 3d).

Differences between recently arrived migrants and resident birds

At the start of the non-breeding season at Flinders beach, the composition of the gut microbiota of stint that had just returned from migration was distinct from second-year individuals that had inhabited the site for a full year (Fig. 4a; adonis test based on Bray Curtis distances; $R^2 = 0.10$, $p = 0.01$, n = 29). However, this difference disappeared when using unweighted unifrac distances (adonis test; $R^2 = 0.04$, $p = 0.14$). Together, these results indicate that both recent migrants and residents consisted of phylogenetically similar communities but with marked differences in abundances. These differences primarily resulted from much higher abundances of Actinobacteria in recent migrants (Fig. 4b), particularly strains of the genus *Corynebacterium* (Fig. 4c), and in particular just one OTU that was present in 13 of the 15 migrants in high abundances (average relative abundance of 23%), yet in only six of 14 residents at extremely low abundance (average relative abundance of less than 1%; Fig. S4). Oligotyping of the whole genus suggested that the majority of these sequences belonged to just one bacterial strain, although the strains found in the two migrants with the highest abundances of *Corynebacterium* were assigned to a different group (Fig. S5). In addition, residents had higher relative abundances of *Flavobacteriaceae* and *Mollicutes* (Kruskal-Wallis test: $p < 0.05$; Fig. 4c; Fig. S4). These differences were not obviously linked

to condition, with both recent migrants and residents having similar body mass (t = 1.04, $p$ = 0.31, n = 29). However, contrary to our predictions, migrants did not have a more diverse suite of gut bacteria in comparison to residents (Fig. 4d; migrants = 86.6 ± 37.4 s.d.; residents = 88.7 ± 36.0 s.d.; t = 0.14, $p$ = 0.88). This was reflected by similar levels of OTU sourcing from the environment between recent migrants and residents in September (Fig. 4d), suggesting that length of time spent at the site did not influence OTU sourcing from foraging sediment.

Changes over the non-breeding season

The gut microbiota of stint shifted weakly (but significantly) over the non-breeding season (Fig. 5a; Adonis test applying unifrac: $R^2$ = 0.07, $p$ = 0.001; Bray curtis; $R^2$ = 0.07, $p$ = 0.001; n = 59). Over time, the relative abundance of Actinobacteria declined across the population (Fig. 5b), and was at negligible levels by March. This was mostly attributed to a decrease in the abundance of the order *Corynebacteriales* in recent migrants over the season (Fig. 5b; Fig. S6 for plots across individuals), as well as an increase in Fusobacteria in some individuals (genus *Cetobacterium*; Fig. 5b; S6). Both migrants and residents shifted their microbiota substantially over the season (Fig. 5a; Fig. S7 for stacked barplot showing changes in composition at the phyla level per individual). Observed richness did not differ significantly between months, with individuals both increasing and decreasing over time (Fig. 6; Mixed effect regression model: September baseline estimate = 78.4 ± 4.4; January = -6.3 ± 6.9, $p$ = 0.38; March = 11.7 ± 8.3, $p$ = 0.19).

DISCUSSION

This study aimed to understand the susceptibility of the gut microbiota of migrants to sediment microbes by determining the extent of microbial sourcing from the environment, and examining the effect of environmental sourcing on gut microbiota dynamics over time

and space in the long-distance migrant, the Red-necked stint. Contrary to our predictions, we found very little sourcing of microbes from the local foraging sediment (<0.1%), which is much lower than previous studies of wild hosts. Correspondingly, we found only very weak differences between stint flocks occupying separate sites with distinct environmental microbial profiles. We found no difference in taxonomic composition or diversity of the gut microbiota between stint that had recently migrated and those that had remained resident at the site for a full year, suggesting migrants had not incorporated sediment microbes into their gut during their migration. However, recent migrants had much higher abundances of the genus *Corynebacterium* on arrival compared to residents, and this group of bacteria decreased in abundance within individuals over the non-breeding season. Over this same period, the gut microbiota of both migrants and residents remained highly diverse, with individuals experiencing large fluctuations in the composition of gut microbiota.

We predicted that if migratory shorebirds incorporate environmental microbes into their gut during foraging, then stints on their non-breeding grounds should source a proportion of their gut bacteria from their foraging sediment. However, we found that stint were able to largely resist the incorporation of sediment microorganisms, despite high exposure through their feeding behaviour. This is in contrast to other studies that found relatively high levels of OTU sourcing (up to 45%) between the gut microbiota of resident species, including wild anoles and woodrats, and their ingested natural food (Kohl *et al.* 2016; Kohl & Dearing 2014), although it is unknown whether hosts sourced these microbes as adults or juveniles. It is also in contrast to studies of migratory shorebird chicks on the breeding grounds, which have been shown to share nearly 40% of their gut bacteria with their environment between zero and ten days old (Grond 2017). This suggests that once the gut microbiota is established from environmental sources, it is relatively resistant to further invasion once the migratory host is fully grown.

High invasion resistance in stint may provide an explanation for why flocks inhabiting ecologically-distinct sites differed only weakly in their gut microbiota, with site explaining approximately 4% of variation in microbiota. This is considerably less than seen in studies of largely sedentary species, with geographic site explaining an average of 30 – 70 % in allopatric populations of Black howler monkeys (Amato *et al.* 2013), Red colobus monkeys (McCord *et al.* 2014), and Galapagos land and marine iguanas (Lankau *et al.* 2012). In contrast, differences in the gut microbiota of the migratory Greater white-fronted goose inhabiting two lakes in China during the non-breeding season found that only 2% of variation was explained by site (Yang *et al.* 2016). Similarly small but significant differences were found between nearby colonies of migratory Barn swallows (Kreisinger *et al.* 2017), which aligns closely with our findings in Red-necked stint. In light of our findings of minimal uptake of environmental microbiota, and previous work suggesting that the environment experienced during infancy has lasting effects on the gut microbiota into adulthood (Goedert *et al.* 2014; Thompson *et al.* 2008), this difference in site-specific effects between migratory (small effects of site) and sedentary species (large effects of site) may in part be a legacy effect of the disparate natal sites of migratory individuals on their non-breeding (Finch *et al.* 2015; Fraser *et al.* 2012). Although inter-population differences in diet are often shown or assumed to be the primary reason for differences in the gut microbiota between host populations of the same species (Amato *et al.* 2016; Amato *et al.* 2013; Degnan *et al.* 2012; McCord *et al.* 2014), we suggest that host movement ecology should also be considered more explicitly in future studies.

High invasion resistance may also explain why recent migrants had similar gut microbiota communities to resident second year birds that had remained at the site for a full year. Although stint may have arrived at the non-breeding site at Flinders up to two weeks prior to being sampled, potentially allowing enough time for rapid changes to the microbiota to have

taken place before sampling, our results suggest that such changes were not driven by the incorporation of novel microbes. This was supported by both migrants and residents having similarly low levels of OTU sourcing from their environment (Fig. 2b). However, migrants notably differed in the abundances of some groups of bacteria, particularly the genus *Corynebacterium*. The role of *Corynebacterium* within the gut microbiota is not well studied. However, increased abundances of *Corynebacterium* have been associated with chronic inflammation of the nasal sinus (Abreu *et al.* 2012; Wagner Mackenzie *et al.* 2016), induced inflammation of the gut (Ribière *et al.* 2016), and viral infection in pandas (Zhao *et al.* 2017), collectively indicating these bacteria may be associated with inflammatory immune responses. Moreover, Rooks *et al.* (2014) found that abundances of *Corynebacterium* in the gut of mice increase in response to an experimental dose of TFN-α (a pro-inflammatory cytokine), suggesting that an immune response can trigger an increase in this bacterial genus in some host taxa. Considering almost all recently arrived migrants had a remarkably high abundance of the same OTU, this may indicate either a physiological change related to migration or an intestinal immune response, rather than an opportunistic infection. This is generally supported by the fact that recently arrived migrants did not display signs of intestinal disease, with both body mass and gut microbial diversity maintained at a similar level to resident birds, although infections have variable effects on species diversity within the gut (e.g. de Vos & de Vos 2012; Moeller *et al.* 2013; Newbold *et al.* 2016; Zhao *et al.* 2017). Therefore, although we found significant differences in the composition of gut microbiota between recent migrants and resident individuals, the causal mechanisms behind these differences cannot be fully elucidated in this study. Considering the importance of the gut microbiota in mediating host immune responses (Belkaid & Hand 2014), expanding our understanding of the interactions between the gut microbiota, pathogenic infection, and host

immune function in migrants will be critical to fully understand the susceptibility and transmission potential of migrants.

Finally, we found only weak shifts in gut microbiota composition within the flock over the non-breeding season, and individual stints underwent large, seemingly random, fluctuations in their gut microbiota composition and diversity, demonstrating a remarkably changeable microbiota within individuals even during sedentary periods. Such dramatic shifts have also been found in other wild species such as anolis lizards (Ren *et al.* 2016) and baboons (Ren *et al.* 2015), suggesting microbial fluctuations in community composition, potentially in response to short-term shifts in host diet or physiology, may be the norm in wild animals, independent of being sedentary or migratory. However, our findings suggest these changes are likely to be due to short-term shifts in diet or physiology, rather than exposure to altered environmental microbiota.

## Conclusions

Overall, our results indicate that although the gut microbiota of Red-necked stint is subject to fluctuations, it is relatively resistant to invasion from ingested environmental microbes, in contrast to other studies on wild (non-migratory) hosts. Further research is required to assess whether this high resistance is characteristic of migratory hosts more generally, as well as understand the relationship between invasion susceptibility and infection risk. However, we suggest the high resistance to environmental microbes found in stint are likely to have implications for the susceptibility of migratory hosts to infection as they visit novel locations during their migrations.

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## DATA ACCESSIBILITY

All sequence data is available on NCBI under project PRJNA385545 and SRA study SRP106581. All analytical data and code are downloadable at https://github.com/Riselya/Migratory-shorebird-microbiota.

## AUTHOR CONTRIBUTIONS

BH, AR and MK designed study. AR conducted field and laboratory work. AR and DW processed sequences. AR analysed data and wrote manuscript. All authors contributed intellectually to the study from its conception and contributed towards manuscript revisions.

Table 1)

| **OTU** | **Prevalence (%)** | **Relative abundance (%)** | **Phylum** | **Family** | **Genus** |
|---|---|---|---|---|---|
| 1 | 95 | 8.7 | Proteobacteria | Helicobacteraceae | *Helicobacter* |
| 2 | 92 | 9.4 | Fusobacteria | Fusobacteriaceae | *Cetobacterium* |
| 3 | 91 | 5.2 | Proteobacteria | Campylobacteraceae | *Campylobacter* |
| 4 | 89 | 1.3 | Deferribacteres | Deferribacteraceae | *Mucispirillum* |
| 5 | 87 | 0.9 | Proteobacteria | Gammaproteobacteria | Unclassified |
| 6 | 87 | 2.5 | Fusobacteria | Fusobacteriaceae | *Cetobacterium* |
| 7 | 86 | 0.8 | Proteobacteria | Desulfovibrionaceae | *Desulfovibrio* |
| 8 | 84 | 3.0 | Proteobacteria | Succinivibrionaceae | *Anaerobiospirillum* |
| 9 | 84 | 2.5 | Spirochaetae | Brachyspiraceae | *Brachyspira* |
| 10 | 84 | 2.4 | Bacteroidetes | Bacteroidaceae | *Bacteroides* |
| 11 | 82 | 1.7 | Proteobacteria | Succinivibrionaceae | *Anaerobiospirillum* |
| 12 | 82 | 3.2 | Firmicutes | Peptostreptococcaceae | Unclassified |

Table 2)

| Site | OTU ID | Relative abundance (%) | Phylum | Family | Genus |
|---|---|---|---|---|---|
| Flinders | 1 | 5.6 | Proteobacteria | Rhodobacteraceae | *Celeribacter* |
| | 2 | 4.3 | Proteobacteria | Rhodobacteraceae | Unclassified |
| | 3 | 2.5 | Proteobacteria | JTB255 marine benthic group | Unclassified |
| | 4 | 2.4 | Bacteroidetes | Flavobacteriaceae | *Maribacter* |
| | 5 | 2.4 | Bacteroidetes | Flavobacteriaceae | *Winogradskyella* |
| | 6 | 2.3 | Proteobacteria | JTB255 marine benthic group | Unclassified |
| | 7 | 2.0 | Proteobacteria | Pseudomonadaceae | Unclassified |
| | 8 | 1.4 | Proteobacteria | Pseudomonadaceae | *Azomonas* |
| | 9 | 1.3 | Bacteroidetes | Flavobacteriaceae | *Maribacter* |
| | 10 | 1.3 | Bacteroidetes | Flavobacteriaceae | *Maribacter* |
| WTP | 11 | 2.7 | Bacteroidetes | Flavobacteriaceae | *Robiginitalea* |
| | 12 | 2.2 | Proteobacteria | Rhodobacteraceae | *Roseovarius* |
| | 13 | 2.0 | Proteobacteria | Rhodobacteraceae | Unclassified |
| | 14 | 1.9 | Proteobacteria | Rhodobacteraceae | Unclassified |
| | 15 | 1.7 | Proteobacteria | Halieaceae | *Pseudohaliea* |
| | 16 | 1.7 | Cyanobacteria | Family I | *Cylindrospermosis* |
| | 17 | 1.7 | Proteobacteria | Rhodobacteraceae | Unclassified |
| | 18 | 1.6 | Proteobacteria | Rhodobacteraceae | Unclassified |
| | 19 | 1.5 | Bacteroidetes | Flavobacteriaceae | *Psychroflexus* |
| | 20 | 1.3 | Proteobacteria | Rhodobacteraceae | Unclassified |