1-1-2017

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

Alice Risely
Deakin University

David Waite
University of Queensland

Beata Ujvari
Deakin University, beatau@uow.edu.au

Marcel Klaassen
Deakin University

Bethany J. Hoye
University of Wollongong, bhoye@uow.edu.au

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

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 over time and space in a migratory shorebird, the Red-necked stint Calidris ruficollis. Contrary to previous studies on wild, nonmigratory hosts, we found that stint on their nonbreeding 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.

Disciplines

Medicine and Health Sciences | Social and Behavioral Sciences

Publication Details

Risely, A., Waite, D., Ujvari, B., Klaassen, M. & Hoye, B. (2017). Gut microbiota of a long-distance migrant demonstrates resistance against environmental microbe incursions. Molecular Ecology, 26 (20), 5842-5854.

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

Alice Risely[1*], David Waite[2], Beata Ujvari[1], Marcel Klaassen[1] & Bethany Hoye[1]

[1] Centre for Integrative Ecology, Deakin University, Geelong, Australia

[2] Australian Centre for Ecogenomics, University of Queensland, Brisbane, Australia

*Corresponding author: riselya@gmail.com; Postal address: School of Life and Environmental Sciences, Deakin University, 75 Pigdons Road, Waurn Ponds, Geelong 3216, Victoria, Australia.

Running title: Microbial invasion susceptibility in migrants

Word count (excluding legends and references): 5690

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 as they move between sites 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 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 inflammatory 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), shifts 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, young birds 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 not resistant 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.

192 Sequence processing

193 Paired sequences were joined using UPARSE pipeline (Edgar 2013), and quality filtered
194 using USEARCH's maximum expected error method. Sequences were aligned and filtered in
195 mothur following their standard operating procedure (MiSeq SOP; Kozich *et al.* 2013;
196 accessed December 2016). We pre-clustered 2,066,515 unique sequences to allow four base
197 pair differences, resulting in 703,453 unique sequences. Chimeras were identified using the
198 UCHIME algorithm (Edgar *et al.* 2011), and 209,094 (29%) unique sequences were removed
199 from the dataset. Sequences were grouped into operational taxonomic units (OTUs) based on
200 a 97% similarity threshold. Taxonomic classification was performed using the SILVA
201 taxonomy (v123.1; Pruesse *et al.* 2007) trimmed to the alignment space of the amplicons
202 (Werner *et al.* 2012). OTUs that were identified as mitochondria, eukaryotic (including
203 chloroplast) or archaeal were removed from the data set. This created a total output of just
204 under 4 million sequences. Analysis of the mock community found an average sequencing
205 error rate of 0.2%. This is slightly higher than normal, and may explain the high proportion of
206 singleton OTUs found in the final dataset, with 90% of 77,000 OTUs being represented by a
207 single sequence (with a 'normal' proportion being between 5 - 40%, depending on sample
208 types). Inspection of the technical repeats indicated that these singletons were likely due to
209 sequencing error. We controlled for this error by excluding OTUs represented by 10
210 sequences or fewer to ensure sequencing error did not bias results. This excluded only 2% of
211 total sequences. To ensure data quality, we also reran sequence processing with stricter
212 quality control using a 50bp sliding window within mothur to discard reads that drop below
213 Q25, which did not change analytical results. Rarefaction curves for the OTU table used for
214 the study (i.e. excluding OTUs with total abundance of 10 or less) showed that almost all
215 OTUs were detectable by 5000 reads (Fig. S1). Sequences classified to the genus
216 *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 (Fig. 1b) 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). However, 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).

<u>Differences between recently arrived migrants and resident birds</u>

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 at the start of the non-breeding season the microbiota of both recent migrants and residents consists of phylogenetically similar communities but with marked differences in abundance. 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, and was at negligible levels by March (Fig. 5b). 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

340 significantly between months, with individuals both increasing and decreasing over time (Fig.
341 6; Mixed effect regression model: September baseline estimate = 78.4 ± 4.4; January = -6.3
342 ± 6.9, $p = 0.38$; March = 11.7 ± 8.3, $p = 0.19$).

343 DISCUSSION

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

359 We predicted that if migratory shorebirds incorporate environmental microbes into their gut
360 during foraging, then stints on their non-breeding grounds should source a proportion of their
361 gut bacteria from their foraging sediment. However, we found that stints were able to largely
362 resist the incorporation of sediment microorganisms, despite high exposure through their
363 feeding behaviour. This is in contrast to other studies that found relatively high levels of

364 OTU sourcing (up to 45%) between the gut microbiota of resident species, including wild
365 anoles and woodrats, and their ingested natural food (Kohl *et al.* 2016; Kohl & Dearing
366 2014), although it is unknown whether hosts sourced these microbes as adults or juveniles. It
367 is also in contrast to studies of migratory shorebird chicks on the breeding grounds, which
368 have been shown to share nearly 40% of their gut bacteria with their environment between
369 zero and ten days old (Grond 2017). This suggests that once the gut microbiota is established
370 from environmental sources, it is relatively resistant to further invasion once the migratory
371 host is fully grown.

372 High invasion resistance in stint may provide an explanation for why flocks inhabiting
373 ecologically-distinct sites differed only weakly in their gut microbiota, with site explaining
374 approximately 4% of variation in microbiota. This is considerably less than seen in studies of
375 largely sedentary species, with geographic site explaining an average of 30 – 70 % in
376 allopatric populations of Black howler monkeys (Amato *et al.* 2013), Red colobus monkeys
377 (McCord *et al.* 2014), and Galapagos land and marine iguanas (Lankau *et al.* 2012). In
378 contrast, differences in the gut microbiota of the migratory Greater white-fronted goose
379 inhabiting two lakes in China during the non-breeding season found that only 2% of variation
380 was explained by site (Yang *et al.* 2016). Similarly small but significant differences were
381 found between nearby colonies of migratory Barn swallows (Kreisinger *et al.* 2017), which
382 aligns closely with our findings in Red-necked stint. In light of our findings of minimal
383 uptake of environmental microbiota, and previous work suggesting that the environment
384 experienced during infancy has lasting effects on the gut microbiota into adulthood (Goedert
385 *et al.* 2014; Thompson *et al.* 2008), this difference in site-specific effects between migratory
386 (small effects of site) and sedentary species (large effects of site) may in part be a legacy
387 effect of the disparate natal sites of migratory individuals on their non-breeding (Finch *et al.*
388 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. 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

414 birds, although infections have variable effects on species diversity within the gut (e.g. de
415 Vos & de Vos 2012; Moeller *et al.* 2013; Newbold *et al.* 2016; Zhao *et al.* 2017). Therefore,
416 although we found significant differences in the composition of gut microbiota between
417 recent migrants and resident individuals, the causal mechanisms behind these differences
418 cannot be fully elucidated in this study. Considering the importance of the gut microbiota in
419 mediating host immune responses (Belkaid & Hand 2014), expanding our understanding of
420 the interactions between the gut microbiota, pathogenic infection, and host immune function
421 in migrants will be critical to fully understand the susceptibility and transmission potential of
422 migrants.

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

433 Conclusions

434 Overall, our results indicate that although the gut microbiota of Red-necked stint is subject to
435 fluctuations, it is relatively resistant to invasion from ingested environmental microbes, in
436 contrast to other studies on wild (non-migratory) hosts. Further research is required to assess
437 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.

ACKNOWLEDGEMENTS

We would like to thank the Victorian Wader Study Group, and in particular Penny Johns and Clive Minton, for organizing fieldwork. We would also like to thank four anonymous reviewers for their constructive comments that greatly helped to improve the manuscript. This work was funded by grants from the Holsworth Wildlife Endowment Fund, Birdlife Australia, and the Australian Research Council (DP1301041935). Catching and swabbing of Red-necked stint was approved by the Victorian Government's Department of Environment, Land, Water and Planning (DELWP) and the Australian Bird and Bat Banding Scheme (ABBBS). Ethics approval was obtained from Deakin University Animal Ethics Committee (B37-2013). No authors have any competing financial interests in the relation to this work.

REFERENCES

Abreu NA, Nagalingam NA, Song Y, *et al.* (2012) Sinus microbiome diversity depletion and Corynebacterium tuberculostearicum enrichment mediates rhinosinusitis. *Science translational medicine* **4**, 151ra124-151ra124.

Altizer S, Bartel R, Han BA (2011) Animal migration and infectious disease risk. *Science* **331**, 296-302.

Amato KR (2013) Co-evolution in context: the importance of studying gut microbiomes in wild animals. *Microbiome Science and Medicine* **1**.

Amato KR, Martinez-Mota R, Righini N, *et al.* (2016) Phylogenetic and ecological factors impact the gut microbiota of two Neotropical primate species. *Oecologia* **180**, 717-733.

Amato KR, Yeoman CJ, Kent A, *et al.* (2013) Habitat degradation impacts black howler monkey (Alouatta pigra) gastrointestinal microbiomes. *The ISME journal* **7**, 1344-1353.

Anderson MJ (2001) A new method for non-parametric multivariate analysis of variance. *Austral ecology* **26**, 32-46.

Barbosa A, Balagué V, Valera F, *et al.* (2016) Age-Related Differences in the Gastrointestinal Microbiota of Chinstrap Penguins (Pygoscelis antarctica). *PloS one* **11**, e0153215.

Belkaid Y, Hand TW (2014) Role of the microbiota in immunity and inflammation. *Cell* **157**, 121-141.

Benskin CM, Rhodes G, Pickup RW, Wilson K, Hartley IR (2010) Diversity and temporal stability of bacterial communities in a model passerine bird, the zebra finch. *Molecular ecology* **19**, 5531-5544.

Brooks B, Firek BA, Miller CS, *et al.* (2014) Microbes in the neonatal intensive care unit resemble those found in the gut of premature infants. *Microbiome* **2**, 1.

Caporaso JG, Lauber CL, Costello EK, *et al.* (2011) Moving pictures of the human microbiome. *Genome biology* **12**, 1.

Caporaso JG, Lauber CL, Walters WA, *et al.* (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal* **6**, 1621-1624.

David LA, Maurice CF, Carmody RN, *et al.* (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature* **505**, 559-563.

de Vos WM, de Vos EA (2012) Role of the intestinal microbiome in health and disease: from correlation to causation. *Nutrition reviews* **70**, S45-S56.

Degnan PH, Pusey AE, Lonsdorf EV, *et al.* (2012) Factors associated with the diversification of the gut microbial communities within chimpanzees from Gombe National Park. *Proceedings of the National Academy of Sciences* **109**, 13034-13039.

Dominguez-Bello MG, Costello EK, Contreras M, *et al.* (2010) Delivery mode shapes the acquisition and structure of the initial microbiota across multiple body habitats in newborns. *Proceedings of the National Academy of Sciences* **107**, 11971-11975.

Dominianni C, Wu J, Hayes RB, Ahn J (2014) Comparison of methods for fecal microbiome biospecimen collection. *BMC microbiology* **14**, 1.

Edgar RC (2013) UPARSE: highly accurate OTU sequences from microbial amplicon reads. *Nature methods* **10**, 996-998.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics* **27**, 2194-2200.

Eren AM, Borisy GG, Huse SM, Welch JLM (2014) Oligotyping analysis of the human oral microbiome. *Proceedings of the National Academy of Sciences* **111**, E2875-E2884.

Figuerola J, Green AJ (2000) Haematozoan parasites and migratory behaviour in waterfowl. *Evolutionary Ecology* **14**, 143-153.

Finch T, Saunders P, Avilés JM, *et al.* (2015) A pan-European, multipopulation assessment of migratory connectivity in a near-threatened migrant bird. *Diversity and Distributions* **21**, 1051-1062.

Fraser KC, Stutchbury BJ, Silverio C, *et al.* (2012) Continent-wide tracking to determine migratory connectivity and tropical habitat associations of a declining aerial insectivore. *Proceedings of the Royal Society of London B: Biological Sciences* **279**, 4901-4906.

Goedert JJ, Hua X, Yu G, Shi J (2014) Diversity and composition of the adult fecal microbiome associated with history of cesarean birth or appendectomy: analysis of the American Gut Project. *EBioMedicine* **1**, 167-172.

Green MR, Hughes H, Sambrook J, MacCallum P (2012) Molecular Cloning: a Laboratory Manual. In: *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor.

Grond K (2017) *Development and dynamics of gut microbial communities of migratory shorebirds in the Western Hemisphere*, Kansas State University.

Hamady M, Lozupone C, Knight R (2010) Fast UniFrac: facilitating high-throughput phylogenetic analyses of microbial communities including analysis of pyrosequencing and PhyloChip data. *The ISME journal* **4**, 17-27.

Hird SM (2017) Evolutionary Biology Needs Wild Microbiomes. *Frontiers in Microbiology* **8**.

Kamada N, Chen GY, Inohara N, Núñez G (2013) Control of pathogens and pathobionts by the gut microbiota. *Nature immunology* **14**, 685-690.

Khosravi A, Mazmanian SK (2013) Disruption of the gut microbiome as a risk factor for microbial infections. *Current Opinion in Microbiology* **16**, 221-227.

Knights D, Kuczynski J, Charlson ES, *et al.* (2011) Bayesian community-wide culture-independent microbial source tracking. *Nature methods* **8**, 761-763.

Kohl KD, Brun A, Magallanes M, *et al.* (2016) Gut microbial ecology of lizards: insights into diversity in the wild, effects of captivity, variation across gut regions, and transmission. *Molecular ecology*.

Kohl KD, Dearing MD (2014) Wild-caught rodents retain a majority of their natural gut microbiota upon entrance into captivity. *Environmental microbiology reports* **6**, 191-195.

Kozich JJ, Westcott SL, Baxter NT, Highlander SK, Schloss PD (2013) Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Applied and environmental microbiology* **79**, 5112-5120.

Kreisinger J, Kropáčková L, Petrželková A, *et al.* (2017) Temporal stability and the effect of transgenerational transfer on faecal microbiota structure in a long distance migratory bird. *Frontiers in Microbiology* **8**, 50.

Kuwae T, Beninger PG, Decottignies P, *et al.* (2008) Biofilm grazing in a higher vertebrate: the western sandpiper, Calidris mauri. *Ecology* **89**, 599-606.

Lankau EW, HONG PY, Mackie RI (2012) Ecological drift and local exposures drive enteric bacterial community differences within species of Galapagos iguanas. *Molecular ecology* **21**, 1779-1788.

Lauber CL, Zhou N, Gordon JI, Knight R, Fierer N (2010) Effect of storage conditions on the assessment of bacterial community structure in soil and human-associated samples. *FEMS microbiology letters* **307**, 80-86.

Leung TL, Koprivnikar J (2016) Nematode parasite diversity in birds: the role of host ecology, life history and migration. *Journal of Animal Ecology*.

Lewis WB, Moore FR, Wang S (2016) Changes in gut microbiota of migratory passerines during stopover after crossing an ecological barrier. *The Auk*.

Lourenço PM, Catry T, Lopes RJ, Piersma T, Granadeiro JP (2017) Invisible trophic links? Quantifying the importance of non-standard food sources for key intertidal avian predators in the Eastern Atlantic. *Marine Ecology Progress Series* **563**, 219-232.

Manichanh C, Reeder J, Gibert P, *et al.* (2010) Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. *Genome research* **20**, 1411-1419.

Mathot KJ, Lund DR, Elner RW (2010) Sediment in stomach contents of Western Sandpipers and Dunlin provide evidence of biofilm feeding. *Waterbirds* **33**, 300-306.

McCord AI, Chapman CA, Weny G, *et al.* (2014) Fecal microbiomes of non-human primates in Western Uganda reveal species-specific communities largely resistant to habitat perturbation. *American Journal of Primatology* **76**, 347-354.

McMurdie PJ, Holmes S (2013) phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PloS one* **8**, e61217.

McMurdie PJ, Holmes S (2014) Waste not, want not: why rarefying microbiome data is inadmissible. *PLoS Comput Biol* **10**, e1003531.

568 Moeller AH, Shilts M, Li Y, *et al.* (2013) SIV-induced instability of the chimpanzee gut
569 microbiome. *Cell host & microbe* **14**, 340-345.
570 Newbold LK, Burthe SJ, Oliver AE, *et al.* (2016) Helminth burden and ecological factors
571 associated with alterations in wild host gastrointestinal microbiota. *The ISME journal*.
572 Oakley BB, Buhr RJ, Ritz CW, *et al.* (2014) Successional changes in the chicken cecal
573 microbiome during 42 days of growth are independent of organic acid feed additives.
574 *BMC veterinary research* **10**, 282.
575 Oksanen J, Kindt R, Legendre P, *et al.* (2007) The vegan package. *Community ecology*
576 *package* **10**.
577 Pruesse E, Quast C, Knittel K, *et al.* (2007) SILVA: a comprehensive online resource for
578 quality checked and aligned ribosomal RNA sequence data compatible with ARB.
579 *Nucleic acids research* **35**, 7188-7196.
580 Ranjitkar S, Lawley B, Tannock G, Engberg RM (2016) Bacterial Succession in the Broiler
581 Gastrointestinal Tract. *Applied and environmental microbiology* **82**, 2399-2410.
582 Ren T, Grieneisen LE, Alberts SC, Archie EA, Wu M (2015) Development, diet and
583 dynamism: longitudinal and cross-sectional predictors of gut microbial communities
584 in wild baboons. *Environmental microbiology*.
585 Ren T, Kahrl AF, Wu M, Cox RM (2016) Does adaptive radiation of a host lineage promote
586 ecological diversity of its bacterial communities? A test using gut microbiota of
587 Anolis lizards. *Molecular ecology* **25**, 4793-4804.
588 Ribière C, Peyret P, Parisot N, *et al.* (2016) Oral exposure to environmental pollutant benzo
589 [a] pyrene impacts the intestinal epithelium and induces gut microbial shifts in murine
590 model. *Scientific Reports* **6**.
591 Rogers D, Herrod A, Menkhorst P, Loyn R (2010) Local movements of shorebirds and high
592 resolution mapping of shorebird habitat in the Port Phillip Bay (Western Shoreline)
593 and Bellarine Peninsula Ramsar Site. *Arthur Rylah Institute for Environmental*
594 *Research Technical Report Series*.
595 Round JL, Mazmanian SK (2009) The gut microbiota shapes intestinal immune responses
596 during health and disease. *Nature Reviews Immunology* **9**, 313-323.
597 Salter SJ, Cox MJ, Turek EM, *et al.* (2014) Reagent and laboratory contamination can
598 critically impact sequence-based microbiome analyses. *BMC biology* **12**, 87.
599 Seedorf H, Griffin NW, Ridaura VK, *et al.* (2014) Bacteria from diverse habitats colonize
600 and compete in the mouse gut. *Cell* **159**, 253-266.
601 Thaiss CA, Zmora N, Levy M, Elinav E (2016) The microbiome and innate immunity.
602 *Nature* **535**, 65-74.
603 Thompson CL, Wang B, Holmes AJ (2008) The immediate environment during postnatal
604 development has long-term impact on gut community structure in pigs. *The ISME*
605 *journal* **2**, 739-748.
606 Turnbaugh PJ, Ley RE, Mahowald MA, *et al.* (2006) An obesity-associated gut microbiome
607 with increased capacity for energy harvest. *Nature* **444**, 1027-1131.
608 Van der Waaij D, Berghuis-de Vries J, Lekkerkerk-Van der Wees J (1971) Colonization
609 resistance of the digestive tract in conventional and antibiotic-treated mice. *Journal of*
610 *Hygiene* **69**, 405-411.
611 van Dongen WF, White J, Brandl HB, *et al.* (2013) Age-related differences in the cloacal
612 microbiota of a wild bird species. *BMC ecology* **13**, 1.
613 Wagner Mackenzie B, Waite DW, Hoggard M, *et al.* (2016) Bacterial community collapse: A
614 meta-analysis of the sinonasal microbiota in chronic rhinosinusitis. *Environmental*
615 *microbiology*.

Waldenström J, Broman T, Carlsson I, *et al.* (2002) Prevalence of Campylobacter jejuni, Campylobacter lari, and Campylobacter coli in different ecological guilds and taxa of migrating birds. *Applied and environmental microbiology* **68**, 5911-5917.
Werner JJ, Koren O, Hugenholtz P, *et al.* (2012) Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *The ISME journal* **6**, 94-103.
Wikelski M, Tarlow EM, Raim A, *et al.* (2003) Avian metabolism: costs of migration in free-flying songbirds. *Nature* **423**, 704-704.
Wu GD, Chen J, Hoffmann C, *et al.* (2011) Linking long-term dietary patterns with gut microbial enterotypes. *Science* **334**, 105-108.
Yang Y, Deng Y, Cao L (2016) Characterising the interspecific variations and convergence of gut microbiota in Anseriformes herbivores at wintering areas. *Scientific Reports* **6**.
Yatsunenko T, Rey FE, Manary MJ, *et al.* (2012) Human gut microbiome viewed across age and geography. *Nature* **486**, 222-227.
Zhao N, Li M, Luo J, *et al.* (2017) Impacts of canine distemper virus infection on the giant panda population from the perspective of gut microbiota. *Scientific Reports* **7**.

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 and AR designed study. AR conducted field and laboratory work. AR and DW conducted sequence analysis. AR analysed data and wrote manuscript. DW independently analysed data. All authors contributed intellectually to the study from its conception and contributed towards manuscript revisions.