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### 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](mailto:beatau@uow.edu.au)

Marcel Klaassen  
*Deakin University*

Bethany J. Hoye  
*University of Wollongong*, [bhoye@uow.edu.au](mailto:bhoye@uow.edu.au)

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## 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.

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

3 Alice Risely<sup>1\*</sup>, David Waite<sup>2</sup>, Beata Ujvari<sup>1</sup>, Marcel Klaassen<sup>1</sup> & Bethany Hoye<sup>1</sup>

4 <sup>1</sup> Centre for Integrative Ecology, Deakin University, Geelong, Australia

5 <sup>2</sup> Australian Centre for Ecogenomics, University of Queensland, Brisbane, Australia

6 \*Corresponding author: [riselya@gmail.com](mailto:riselya@gmail.com); Postal address: School of Life and  
7 Environmental Sciences, Deakin University, 75 Pigdons Road, Waurin Ponds, Geelong 3216,  
8 Victoria, Australia.

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## 21 ABSTRACT

22 Migratory animals encounter suites of novel microbes as they move between disparate sites  
23 during their migrations, and are frequently implicated in the global spread of pathogens.  
24 Although wild animals have been shown to source a proportion of their gut microbiota from  
25 their environment, the susceptibility of migrants to enteric infections as they move between  
26 sites may be dependent upon the capacity of their gut microbiota to resist incorporating  
27 encountered microbes. To evaluate migrants' susceptibility to microbial invasion, we  
28 determined the extent of microbial sourcing from the foraging environment, and examined  
29 how this influenced gut microbiota dynamics over time and space in a migratory shorebird,  
30 the Red-necked stint. Contrary to previous studies on wild, non-migratory hosts, we found  
31 that stint on their non-breeding grounds obtained very little of their microbiota from their  
32 environment, with most individuals sourcing only 0.1% of gut microbes from foraging  
33 sediment. This microbial resistance was reflected at the population level by only weak  
34 compositional differences between stint flocks occupying ecologically-distinct sites, and by  
35 our finding that stint that had recently migrated 10,000 km did not differ in diversity or  
36 taxonomy from those that had inhabited the same site for a full year. However, recent  
37 migrants had much greater abundances of the genus *Corynebacterium*, suggesting a potential  
38 inflammatory response to either migration or exposure to a novel environment. We conclude  
39 that the gut microbiota of stint is largely resistant to invasion from ingested microbes, and  
40 that this may have implications for their susceptibility to enteric infections during migration.

## 41 INTRODUCTION

42 The vast communities of microorganisms that make up the gastrointestinal ('gut') microbiota  
43 of animals are fundamental to host metabolism, nutrient acquisition, and immune function  
44 (Khosravi & Mazmanian 2013; Thaïss *et al.* 2016; Turnbaugh *et al.* 2006). The ecological

45 dynamics of this microbial community may be particularly important for migratory animals,  
46 because migrants face exceptional metabolic, nutritional, and immunological challenges as  
47 they traverse the globe during their migrations (Altizer *et al.* 2011; Wikelski *et al.* 2003).  
48 Notably, migrants are thought to encounter and ingest novel suites of microbes, including  
49 parasites and potential pathogens, as they forage at disparate locations along their migratory  
50 routes (Figuerola & Green 2000; Leung & Koprivnikar 2016). This increased risk of  
51 infection, in combination with their high mobility, has raised concerns that migratory animals  
52 may be of particular importance in the global transmission and dispersal of pathogenic  
53 microbes (Altizer *et al.* 2011; Waldenström *et al.* 2002). Critically, the risk of migrants  
54 dispersing enteric pathogens is, in part, dependent on the extent to which they incorporate and  
55 maintain novel microbes encountered at each location in their gut microbiota.

56 The susceptibility of hosts to enteric infection is linked to the capacity of their gut microbiota  
57 to resist invasion by foreign microbes ('colonization resistance'; Van der Waaij *et al.* 1971).  
58 This resilience may be achieved either via niche competition between native and foreign  
59 microbes, or by commensal bacteria actively inducing host immune responses when under  
60 invasion (Kamada *et al.* 2013; Round & Mazmanian 2009). Although young animals,  
61 including migratory shorebirds, have been shown to establish their gut microbiota at birth or  
62 hatching by incorporating microbes from their immediate environment (Brooks *et al.* 2014;  
63 Dominguez-Bello *et al.* 2010; Grond 2017), once established the healthy microbiota of  
64 humans and captive animals is generally associated with high levels of stability (Benskin *et*  
65 *al.* 2010; Caporaso *et al.* 2011; Wu *et al.* 2011). However, the microbiota may not be resilient  
66 to change when continually exposed to new bacterial assemblages. For example, microbes  
67 from soil sediment can successfully colonise and persist in the guts of germ-free mice, even  
68 outcompeting gut specialists (Seedorf *et al.* 2014). Moreover, laboratory rats challenged with  
69 the microbiota of other individuals develop a microbiota that is more diverse and resembles

70 that of donor rats (Manichanh *et al.* 2010). Indeed, fully-grown wild hosts have been shown  
71 to source a significant number of microbes from their environment, with wild woodrats and  
72 anole lizards estimated to source up to 25% and 47% of their gut microbiota community from  
73 ingested plant food, respectively (Kohl *et al.* 2016; Kohl & Dearing 2014). Whether such  
74 high levels of microbial sourcing from the environment is characteristic of all wild hosts,  
75 including those with migratory lifestyles, is unknown. However, if wild migrants have similar  
76 levels of environmental sourcing, then migratory hosts may increase their susceptibility to  
77 enteric infection through the continual incorporation of novel microbes ingested as they  
78 forage at multiple sites en route.

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

94 that elucidate microbiota dynamics and mechanisms in natural ecosystems (Amato 2013;  
95 Hird 2017).

96 In this study, we aimed to assess the invasion resistance of a long-distance migrant, the Red-  
97 necked stint (*Calidris ruficollis*), to ingested environmental microbes whilst controlling for  
98 host habitat and physiology. We achieved this by firstly determining the extent to which stint  
99 on their non-breeding grounds sourced microbes from their immediate foraging environment,  
100 and secondly by examining whether this translated into altered gut microbiota community  
101 structures across sites and over time. Importantly, the Red-necked stint provides an  
102 especially rare and insightful model species to investigate these questions for three reasons.  
103 Firstly, like many shorebird species, young birds remain on the non-breeding grounds for 1.5  
104 years following their first migration from their natal sites in Siberia. This allows comparisons  
105 between birds that have remained 'resident' on the non-breeding grounds for a full year (at  
106 this point 'second year' individuals that are 15 months old) and those that had recently  
107 migrated from Siberia, via multiple locations (those three or more years old), providing two  
108 conspecific groups that share diet and environment, but differ in how recently they completed  
109 a long distance, multi-stopover migration. Secondly, stint forage for prey by sifting through  
110 coastal sediment and biofilm with their bills, with sediment and biofilm making up the major  
111 component of the diet and stomach contents of closely related, and ecologically similar,  
112 *Calidris* species (Kuwae *et al.* 2008; Lourenço *et al.* 2017; Mathot *et al.* 2010). This creates  
113 direct and ongoing exposure to sediment microbiota. Thirdly, stint are site faithful, and make  
114 limited movements during the non-breeding seasons, often remaining on the same foraging  
115 site within the same flock for the entire season (Rogers *et al.* 2010). This not only provides  
116 opportunities to monitor the same individuals over time, but also provides reasonable  
117 certainty of foraging areas and movement patterns over the season.

118 Given this study system, if the gut microbiota of stint is not resistant to invasion from  
119 environmental microbes, then a series of predictions can be made. Firstly, we predicted that  
120 individuals will source a similar proportion of their gut microbiota from their immediate  
121 foraging sediment to that found in previous studies of other wild hosts (30-50%). This would  
122 be reflected in distinct gut microbiota community structures between flocks occupying  
123 different sites. Secondly, we predicted that newly arrived migrants that had recently been  
124 exposed to novel suites of microbes during migration (adults) would have a phylogenetically  
125 distinct, and more diverse gut microbiota from resident second year birds that had inhabited  
126 the site for a full year. Thirdly, the microbiota of newly arrived migrants should, through  
127 ongoing exposure to the same local microbes and other members of the flock, become more  
128 similar to that of resident birds with increasing time spent at the non-breeding site.  
129 Collectively, these analyses allow us to assess how resistant the gut microbiota of migratory  
130 stint are to invasion from novel environmental microbes during their non-breeding season.

## 131 MATERIALS AND METHODS

### 132 Sample collection

133 Red-necked stint from two non-breeding populations were captured using cannon nets in  
134 Victoria, Australia. One population occupied a coastal beach site, Flinders (-38°48 S, 145°00  
135 E), and was sampled at three time points during the non-breeding season (September 2015 –  
136 April 2016) in order to assess temporal changes in gut microbiota communities. Twelve out  
137 of a total of 71 individuals were recaptured at least once over the season (see below). Firstly,  
138 a single flock of recent migrants (3+ years old) and resident second years (15 months old)  
139 were captured on the 20th September (n = 29). Given that adult stint arrive at this site over the  
140 course of mid- to late- September, recent migrants captured on this day would have  
141 completed their post-breeding migration 1 - 14 days prior to capture. Although age



142 differences exist between the two groups, it is extremely unlikely that this would be the cause  
143 of differences in microbiota community structure. Age is an important factor determining gut  
144 microbiota composition when young, with chicks having different gut microbiota to adult  
145 birds in penguins, kittiwakes and barn swallows (Barbosa *et al.* 2016; Kreisinger *et al.* 2017;  
146 van Dongen *et al.* 2013). However, poultry studies suggest that gut microbiota structure  
147 resembles that of adults within 0.5 - 3 months after hatching (Oakley *et al.* 2014; Ranjitkar *et*  
148 *al.* 2016), and studies of two wild migratory shorebird species, Dunlin and Red phalarope,  
149 suggest that microbiota diversity stabilizes in 3-10 days old chicks (Grond 2017). On this  
150 basis, and given that both our resident and migrant groups consist of fully-grown birds that  
151 have completed at least one Siberia-to-Australia migration, we do not believe that  
152 differences in gut microbiota should exist between second year birds at 15 months old and  
153 birds that are 3+ years old due to age *per se*. The population was then targeted on the 23rd  
154 January (n = 13), and again prior to the pre-breeding migration, on the 11th March (n = 18).  
155 At this point in their moult cycle adults and second year birds could not be distinguished on  
156 the basis of their plumage, although juveniles (birds hatched in the 2015 breeding season, and  
157 which arrived on the site October-November, after the first September catch) were still  
158 distinguishable. However, using recapture history of banded birds we were able to distinguish  
159 between adults and second year birds for 61% of the individuals at this point in time. As a  
160 comparison site, a second population inhabiting the Werribee Western Treatment Plant  
161 (WTP; -37°99 S, 144°61 E), a sewage treatment works characterized by lagoons and  
162 estuaries, was also sampled. Birds were captured during two capture events on the 28th  
163 December 2015 (n = 25). Stint are site-faithful on the non-breeding grounds, with little  
164 connectivity between the sites: of 9,856 recaptures of the same individual stint across the  
165 wider region of our study site over the last 30 years, only 146 individuals (1.5%) were  
166 recaptured at a different site to where they were first caught (Rogers *et al.* 2010).

167 Cloacal swabs were taken from stints using sterile swabs (Copan 170KS01), placed in sterile  
168 plastic tubes without medium, and kept refrigerated for 3 - 5 hours before being stored at -  
169 80°C. Differences in bacterial composition resulting from storage conditions generally do not  
170 eclipse differences between samples (Dominianni *et al.* 2014; Lauber *et al.* 2010), therefore  
171 we assume differences in refrigeration time had minimal effect on our results. Environmental  
172 samples of mud or sand from where birds had been observed foraging were collected at each  
173 capture site immediately after each capture event, and handled in the same manner as the  
174 cloacal swabs. Six environmental samples from each site were pooled into two DNA samples  
175 (2 x 3) per site, because we deemed small-scale spatial variation within the foraging areas  
176 were not relevant to our study.

#### 177 DNA isolation, amplification and sequencing

178 DNA was isolated using the phenol-chloroform method (Green *et al.* 2012). Briefly, swabs  
179 were suspended individually in 400 µl cetrimonium bromide (CTAB) with 50 µl of proteinase  
180 K and 60 µl of 10% sodium dodecyl sulfate (SDS). This solution was briefly vortexed and  
181 incubated overnight at 56 °C. The next day, 50 µl of 5M NaCl and 500 µl of phenol was  
182 added to each solution, briefly vortexed and left at room temperature for 10 minutes. From  
183 here, DNA isolation and ethanol precipitation followed standard procedures outlined in Green  
184 *et al.* (2012). DNA was extracted from four sterile swabs as negative controls to correct for  
185 contaminants (Salter *et al.* 2014). DNA samples were sent to the Ramaciotti Centre for  
186 Genomics, Sydney, for amplification using paired 27F/519R primers that amplify a 500bp  
187 V1-V3 region of the 16S rRNA bacterial gene, and amplicons were then sequenced using  
188 Illumina MiSeq technology (Caporaso *et al.* 2012; full protocol for these primers available at  
189 [www.bioplatforms.com](http://www.bioplatforms.com)). A mock community provided by Zybiotics was included as a  
190 positive control in order to assess exact sequencing error rate. In addition, two technical  
191 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

217 oligotyping, using the minimum entropy decomposition pipeline (version 2.1) to reveal fine-  
218 scale diversity within the genus (Eren *et al.* 2014), to assess whether the increased  
219 abundances observed were representative of a single or multiple strains.

## 220 Data analysis

221 Analysis of OTU communities was conducted using the Phyloseq (McMurdie & Holmes  
222 2013) and vegan (Oksanen *et al.* 2007) packages in R. The negative control contained forty  
223 OTUs represented by at least 5 sequences, and these OTUs were removed from the dataset. A  
224 single sample with under 7000 reads was excluded, and all remaining samples were rarefied  
225 to 9795 reads (the minimum read count) for further analyses. Because rarefied data can lead  
226 to false positives (McMurdie & Holmes 2014), we repeated analyses without rarefying  
227 samples with no difference to overall results or conclusions. We applied MDS and NMDS  
228 ordinations and conducted ADONIS tests (Anderson 2001) to statistically test for differences  
229 between groups. Methods for accounting for repeated samples from the same individual in  
230 ordination analyses are not currently available. To make sure repeat samples did not affect  
231 results we reiterated analyses randomly excluding repeats, which did not affect overall  
232 results. Because primary components in the MDS analyses generally explained little variance,  
233 we present results from the NMDS ordination. We present both Bray-Curtis (based on  
234 abundance of OTUs) and unweighted Unifrac (based on evolutionary distance between  
235 OTUs; Hamady *et al.* 2010), distance measures. Unifrac distances were calculated using a  
236 16S alignment with SILVA. To identify which particular groups of bacteria were different  
237 between groups, we ran the analysis through LEFse, hosted by the Huttenhower galaxy server  
238 (<https://huttenhower.sph.harvard.edu/galaxy>). We analysed bacterial richness by calculating  
239 both observed OTU richness and the Shannon diversity index. When comparing bacterial  
240 diversity between the three capture events within the Flinders population, we applied a mixed  
241 effect regression model with stint ID as a random effect to account for repeated measures.

242 We estimated the proportion of OTUs sourced from sediment samples using a Bayesian  
243 approach within SourceTracker (Knights *et al.* 2011). This approach uses the relative  
244 abundance of each OTU within both the sediment and each host to calculate the probability  
245 that each OTU found in the host gut was sourced from the sediment microbiota. Thereby it  
246 provides an estimate for the proportion of OTUs sourced from local sediment. For this  
247 analysis, we excluded any OTU which was represented by a single sequence in the control  
248 sample, because analyses suggested that 3% of OTUs present in our samples were sourced  
249 from laboratory contamination, despite being present at extremely low abundances (and  
250 therefore not affecting previous community composition analyses). Therefore, we note that  
251 previous studies that did not account for contamination may have inflated levels of OTU  
252 sourcing. We repeated this analysis between all groups, and in both directions, to estimate  
253 common sources between groups (see Fig. 5a). However, one bird was excluded from these  
254 analyses because it was estimated to source 27% of its gut microbiota from the environment,  
255 whilst the median was 0.1% (see Fig. 2b). We therefore could not rule out that this was due to  
256 environmental contamination of this sample. Because the sediment microbiota of the two  
257 sites differed (see results), we carried out analyses within SourceTracker for each site  
258 separately. For birds at Flinders, we compared birds to sediment samples collected during the  
259 March capture only. Although microbial profiles of sediment may change to certain extent  
260 over time, there was no difference in levels of OTU sourcing from sediment between birds  
261 captured in September, January or March, indicating that this should not affect results.

## 262 RESULTS

263 A total of 2275 operational taxonomic units (OTUs) were identified from 85 cloacal samples  
264 from 71 individual stint, with 10 individuals from Flinders beach sampled twice over the non-  
265 breeding season, and two individuals sampled at all three time points. The majority of these  
266 OTUs had very low prevalence within the sampled stint population (Fig. S2). Only 12 OTUs

267 (0.5% of the total OTUs derived from bird samples) made up the sampled population's 'core'  
268 microbiota (defined here as the suite of OTUs that occur in over 80% of samples; Table 1),  
269 whilst 85% of OTUs were present in less than 5% of birds. On average, the core microbiota  
270 made up  $40 \pm 23$  (s.d.) % of the total microbial abundance for each individual, with the  
271 remainder being largely OTUs that were unique to the individual. Across stint samples, the  
272 most abundant bacterial phyla were Proteobacteria (33%), Fusobacteria (17%), Firmicutes  
273 (14%), Actinobacteria (11%), and Bacteroidetes (9%). Environmental samples taken from  
274 foraging sediment at each site showed a less diverse microbial community at the phylum  
275 level, consisting of mostly Proteobacteria and Bacteroidetes (Fig. 1a), but each sample  
276 contained a much richer suite of OTUs than present within the individual stints (Fig. 1b).  
277 Both non-breeding sites displayed a distinct sediment microbial profile (Fig. 1b) which was  
278 also distinct from the overall stint gut microbiota (Fig. 1c), with the most abundant OTUs for  
279 each site not overlapping with each other (Table 2).

#### 280 Microbial sourcing from sediment across sites

281 Bayesian analysis with SourceTracker estimated only 1.7% of sediment OTUs at each site  
282 shared a common source (Fig. 2a). However, stint did not source a significant proportion of  
283 their gut microbiota from their environment, with an average of 0.16 % ( $\pm 0.6$  SD) and 0.4 %  
284 ( $\pm 1.4$  SD) of gut microbiota estimated to be sourced from sediment for flocks occupying the  
285 Flinders and WTP non-breeding sites, respectively (Fig. 2b). Stint were estimated to share  
286 slightly more OTUs with their own foraging site than the alternative foraging site (Fig. 2a),  
287 but these differences were not significant ( $t = 1.22$ ,  $p = 0.23$ ). This low incorporation of  
288 sediment bacteria was reflected by the two flocks occupying different sites differing only  
289 weakly (but significantly) in their gut microbiota composition (Fig. 3a; Adonis test applying  
290 Bray Curtis distance matrix, which emphasises differences in abundance:  $R^2 = 0.02$ ,  $p = 0.04$ ;

291 Unifrac distance matrix, which takes into account phylogeny but only considers  
292 presence/absence rather than abundance:  $R^2 = 0.05$ ,  $p = 0.001$ ,  $n = 85$ ).

293 The weak differences in gut microbiota between the two flocks were attributed to a number  
294 of bacterial groups being slightly more prevalent in birds at the water treatment plant than  
295 birds at Flinders beach, including bacteria belonging to phylum Chloroflexi, family  
296 *Succinivibrionaceae* (phylum Proteobacteria), genera *Streptococcus* (phylum Firmicutes) and  
297 *Salinimicrobium* (phylum Bacteroidetes; Fig. 3b; Fig. S3 for abundance plots of each  
298 bacterial group). However, with the exception of three Chloroflexi OTUs that were found at  
299 very low abundances in one stint each, none of the strains that showed higher prevalence in  
300 birds occupying the water treatment plant were present in environmental samples.

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

#### 306 Differences between recently arrived migrants and resident birds

307 At the start of the non-breeding season at Flinders beach, the composition of the gut  
308 microbiota of stint that had just returned from migration was distinct from second-year  
309 individuals that had inhabited the site for a full year (Fig. 4a; adonis test based on Bray Curtis  
310 distances;  $R^2 = 0.10$ ,  $p = 0.01$ ,  $n = 29$ ). However, this difference disappeared when using  
311 unweighted unifrac distances (adonis test;  $R^2 = 0.04$ ,  $p = 0.14$ ). Together, these results  
312 indicate that at the start of the non-breeding season the microbiota of both recent migrants  
313 and residents consists of phylogenetically similar communities but with marked differences  
314 in abundance. These differences primarily resulted from much higher abundances of

315 Actinobacteria in recent migrants (Fig. 4b), particularly strains of the genus *Corynebacterium*  
316 (Fig. 4c), and in particular just one OTU that was present in 13 of the 15 migrants in high  
317 abundances (average relative abundance of 23%), yet in only six of 14 residents at extremely  
318 low abundance (average relative abundance of less than 1%; Fig. S4). Oligotyping of the  
319 whole genus suggested that the majority of these sequences belonged to just one bacterial  
320 strain, although the strains found in the two migrants with the highest abundances of  
321 *Corynebacterium* were assigned to a different group (Fig. S5). In addition, residents had  
322 higher relative abundances of *Flavobacteriaceae* and *Mollicutes* (Kruskal-Wallis test:  $p <$   
323  $0.05$ ; Fig. 4c; Fig. S4). These differences were not obviously linked to condition, with both  
324 recent migrants and residents having similar body mass ( $t = 1.04$ ,  $p = 0.31$ ,  $n = 29$ ). However,  
325 contrary to our predictions, migrants did not have a more diverse suite of gut bacteria in  
326 comparison to residents (Fig. 4d; migrants =  $86.6 \pm 37.4$  s.d.; residents =  $88.7 \pm 36.0$  s.d.;  $t =$   
327  $0.14$ ,  $p = 0.88$ ). This was reflected by similar levels of OTU sourcing from the environment  
328 between recent migrants and residents in September (Fig. 4d), suggesting that length of time  
329 spent at the site did not influence OTU sourcing from foraging sediment.

### 330 Changes over the non-breeding season

331 The gut microbiota of stint shifted weakly (but significantly) over the non-breeding season  
332 (Fig. 5a; Adonis test applying unifrac:  $R^2 = 0.07$ ,  $p = 0.001$ ; Bray curtis;  $R^2 = 0.07$ ,  $p =$   
333  $0.001$ ;  $n = 59$ ). Over time, the relative abundance of Actinobacteria declined across the  
334 population, and was at negligible levels by March (Fig. 5b). This was mostly attributed to a  
335 decrease in the abundance of the order *Corynebacteriales* in recent migrants over the season  
336 (Fig. 5b; Fig. S6 for plots across individuals), as well as an increase in Fusobacteria in some  
337 individuals (genus *Cetobacterium*; Fig. 5b; S6). Both migrants and residents shifted their  
338 microbiota substantially over the season (Fig. 5a; Fig. S7 for stacked barplot showing  
339 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 =  $78.4 \pm 4.4$ ; January =  $-6.3$   
342  $\pm 6.9$ ,  $p = 0.38$ ; March =  $11.7 \pm 8.3$ ,  $p = 0.19$ ).

## 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

389 assumed to be the primary reason for differences in the gut microbiota between host  
390 populations of the same species (Amato *et al.* 2016; Amato *et al.* 2013; Degnan *et al.* 2012;  
391 McCord *et al.* 2014), we suggest that host movement ecology should also be considered more  
392 explicitly in future studies.

393 High invasion resistance may also explain why recent migrants had similar gut microbiota  
394 communities to resident second year birds that had remained at the site for a full year.

395 Although stint may have arrived at the non-breeding site at Flinders up to two weeks prior to  
396 being sampled, potentially allowing enough time for rapid changes to the microbiota to have  
397 taken place before sampling, our results suggest that such changes were not driven by the  
398 incorporation of novel microbes. This was supported by both migrants and residents having  
399 similarly low levels of OTU sourcing from their environment (Fig. 2b). However, migrants  
400 notably differed in the abundances of some groups of bacteria, particularly the genus  
401 *Corynebacterium*. The role of *Corynebacterium* within the gut microbiota is not well studied.  
402 However, increased abundances of *Corynebacterium* have been associated with chronic  
403 inflammation of the nasal sinus (Abreu *et al.* 2012; Wagner Mackenzie *et al.* 2016), induced  
404 inflammation of the gut (Ribi ere *et al.* 2016), and viral infection in pandas (Zhao *et al.* 2017),  
405 collectively indicating these bacteria may be associated with inflammatory immune  
406 responses. Moreover, Rooks *et al.* (2014) found that abundances of *Corynebacterium* in the  
407 gut of mice increase in response to an experimental dose of TNF- $\alpha$  (a pro-inflammatory  
408 cytokine), suggesting that an immune response can trigger an increase in this bacterial genus.  
409 Considering almost all recently arrived migrants had a remarkably high abundance of the  
410 same OTU, this may indicate either a physiological change related to migration or an  
411 intestinal immune response, rather than an opportunistic infection. This is generally  
412 supported by the fact that recently arrived migrants did not display signs of intestinal disease,  
413 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

438 understand the relationship between invasion susceptibility and infection risk. However, we  
 439 suggest the high resistance to environmental microbes found in stints are likely to have  
 440 implications for the susceptibility of migratory hosts to infection as they visit novel locations  
 441 during their migrations.

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631

## 632 DATA ACCESSIBILITY

633 All sequence data is available on NCBI under project PRJNA385545 and SRA study

634 SRP106581. All analytical data and code are downloadable at

635 <https://github.com/Riselya/Migratory-shorebird-microbiota>.

## 636 AUTHOR CONTRIBUTIONS

637 BH and AR designed study. AR conducted field and laboratory work. AR and DW conducted  
638 sequence analysis. AR analysed data and wrote manuscript. DW independently analysed data.

639 All authors contributed intellectually to the study from its conception and contributed towards  
640 manuscript revisions.

641