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Active migration is associated with specific and consistent changes to gut microbiota in *Calidris* shorebirds

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Active migration is associated with specific and consistent changes to gut microbiota in *Calidris* shorebirds

Abstract

Gut microbes are increasingly recognised for their role in regulating an animal's metabolism and immunity. However, identifying repeatable associations between host physiological processes and their gut microbiota has proved challenging, in part because microbial communities often respond stochastically to host physiological stress (e.g. fasting, forced exercise or infection). Migratory birds provide a valuable system in which to test host-microbe interactions under physiological extremes because these hosts are adapted to predictable metabolic and immunological challenges as they undergo seasonal migrations, including temporary gut atrophy during long-distance flights. These physiological challenges may either temporarily disrupt gut microbial ecosystems, or, alternatively, promote predictable host-microbe associations during migration. To determine the relationship between migration and gut microbiota, we compared gut microbiota composition between migrating and non-migrating ("resident") conspecific shorebirds sharing a flock. We performed this across two sandpiper species, *Calidris ferruginea* and *Calidris ruficollis*, in north-western Australia, and an additional *C. ruficollis* population 3,000 km away in southern Australia. We found that migrants consistently had higher abundances of the bacterial genus *Corynebacterium* (average 28% abundance) compared to conspecific residents (average < 1% abundance), with this effect holding across both species and sites. However, other than this specific association, community structure and diversity was almost identical between migrants and residents, with migration status accounting for only 1% of gut community variation when excluding *Corynebacterium*. Our findings suggest a consistent relationship between *Corynebacterium* and *Calidris* shorebirds during migration, with further research required to identify causal mechanisms behind the association, and to elucidate functionality to the host. However, outside this specific association, migrating shorebirds broadly maintained gut community structure, which may allow them to quickly recover gut function after a migratory flight. This study provides a rare example of a repeatable and specific response of the gut microbiota to a major physiological challenge across two species and two distant populations.

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1 **Active migration is associated with specific and consistent changes to gut microbiota in**
2 ***Calidris* shorebirds**

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21 ABSTRACT

- 22 1. Gut microbes are increasingly recognised for their role in regulating an animal's
23 metabolism and immunity. However, identifying repeatable associations between host
24 physiological processes and their gut microbiota has proved challenging, in part
25 because microbial communities often respond stochastically to host physiological
26 stress (e.g. fasting, forced exercise or infection).
- 27 2. Migratory birds provide a valuable system in which to test host-microbe interactions
28 under physiological extremes because these hosts are adapted to predictable metabolic
29 and immunological challenges as they undergo seasonal migrations, including
30 temporary gut atrophy during long-distance flights. These physiological challenges
31 may either temporarily disrupt gut microbial ecosystems, or, alternatively, promote
32 predictable host-microbe associations during migration.
- 33 3. To determine the relationship between migration and gut microbiota, we compared
34 gut microbiota composition between migrating and non-migrating ('resident')
35 conspecific shorebirds sharing a flock. We performed this across two sandpiper
36 species, *Calidris ferruginea* and *Calidris ruficollis*, in north-western Australia, and an
37 additional *C. ruficollis* population 3000 km away in southern Australia.
- 38 4. We found that migrants consistently had higher abundances of the bacterial genus
39 *Corynebacterium* (average 28% abundance) compared to conspecific residents
40 (average < 1% abundance), with this effect holding across both species and sites.
41 However, other than this specific association, community structure and diversity was
42 almost identical between migrants and residents, with migration status accounting for
43 only 1% of gut community variation when excluding *Corynebacterium*.
- 44 5. Our findings suggest a consistent relationship between *Corynebacterium* and *Calidris*
45 shorebirds during migration, with further research required to identify causal

46 mechanisms behind the association, and to elucidate functionality to the host.
47 However, outside this specific association, migrating shorebirds broadly maintained
48 gut community structure, which may allow them to quickly recover gut function after
49 a migratory flight. This study provides a rare example of a repeatable and specific
50 response of the gut microbiota to a major physiological challenge across two species
51 and two distant populations.

52

53 INTRODUCTION

54 Interactions between animals and their gut microbiota play an integral role in regulating host
55 physiological processes, including metabolism (Tremaroli & Bäckhed 2012) and immune
56 function (Round & Mazmanian 2009; Sommer & Bäckhed 2013a). Yet despite our increasing
57 understanding of these interactions, detecting consistent associations between the gut
58 microbiota and host physiology has proved challenging. Across vertebrates, both individuals
59 and species appear to demonstrate diverse microbial responses to experimental physiological
60 stressors such as food deprivation, infection, and forced exercise (e.g. de Vos & de Vos 2012;
61 Kohl *et al.* 2014; Allen *et al.* 2015; Lambert *et al.* 2015), with consistent and repeatable host-
62 microbe associations being rare. This has been attributed to hosts losing the ability to regulate
63 their gut microbiota when under physiological stress, generating stochastic microbial
64 responses to the same set of stressors (Zaneveld, McMinds & Vega 2017).

65 However, species that are adapted to predictable physiological challenges may provide
66 valuable study systems in which to investigate adaptive host-microbe interactions. For
67 example, hibernating bears and ground squirrels undergo highly specific and consistent
68 changes in gut microbiota composition between summer, when they must deposit body
69 stores, and winter, when they must conserve energy during hibernation (Carey, Walters &

70 Knight 2013; Dill-McFarland *et al.* 2014; Sommer *et al.* 2016). These changes in gut
71 microbiota trigger the accumulation of body fat in summer (Sommer *et al.* 2016), and are
72 linked to decreased levels of inflammation during hibernation (Dill-McFarland *et al.* 2014)
73 when metabolism is greatly reduced (Carey, Andrews & Martin 2003). Migratory animals
74 face comparable seasonal physiological challenges to hibernators, but provide a contrasting
75 study system whereby hosts gain body stores extremely rapidly in order to perform extended
76 bouts of exercise, with both phases requiring very high metabolic rates (Wikelski *et al.* 2003).
77 However, responses of the gut microbiota to migration, and whether these are comparable to
78 those found in hibernators, remain unknown.

79 Out of all migratory species, shorebirds perform some of the longest and fastest migrations
80 ever recorded (Gill *et al.* 2009), posing specific physiological challenges for migrant
81 nutrition, metabolism and immunity (Wikelski *et al.* 2003; Buehler & Piersma 2008; Weber
82 2009). For example, migrants must regain body stores quickly after completing a migratory
83 leg, during which they can lose up to 50% of their body mass (Piersma, Gudmundsson &
84 Lilliendahl 1999). Moreover, partial atrophy of the gastrointestinal tract during long-distance
85 flights is common, both for shorebirds and passerines (Piersma & Gill Jr 1998; McWilliams
86 & Karasov 2001). Such extreme physiological challenges may alter host-microbe
87 interactions to generate shifts in gut microbiota composition during active migration in
88 comparison to non-migratory periods. For example, migrants may form predictable
89 associations with specific bacterial assemblages during active migration, such as those that
90 increase energy harvest from food (e.g. Bäckhed *et al.* 2004; Caesar *et al.* 2012).

91 Alternatively, migrants may maintain broad gut community structure, similar to that of non-
92 migratory periods, in order to preserve critical gut functions, such as nutrient metabolism and
93 pathogen resistance, as they move between sites during migration. This may benefit the host
94 because a resilient gut microbial community decreases host susceptibility to infection by

95 excluding pathogens via niche competition, whereby commensal bacteria outcompete
96 potential pathogens (Kamada *et al.* 2013; Sommer *et al.* 2017). On the other hand, the
97 extreme physiological challenges faced by migrating shorebirds may feasibly disrupt gut
98 microbial ecosystems, potentially leading to stochastic and unpredictable alterations in the
99 gut microbiota during active migration.

100 Although a small number of studies have assessed gut microbial composition in migrating
101 birds, the absence of conspecific non-migrating controls has not allowed for the identification
102 of migration-specific gut microbiota profiles (e.g. Grond *et al.* 2014; Lewis, Moore & Wang
103 2016). In order to identify gut microbes associated with migration whilst controlling for
104 potential confounding variables (e.g. diet or location), actively migrating individuals should
105 ideally be compared to non-migrating ('resident') conspecifics inhabiting the same site at the
106 same time, yet examples of such study systems are rare.

107 In this study we aimed to identify gut microbiota profiles associated with active migration in
108 two closely related long-distance migratory *Calidris* shorebirds, the Red-necked stint
109 (*Calidris ruficollis*) and the Curlew sandpiper (*Calidris ferruginea*). Long-distance migratory
110 shorebirds provide an especially rare and insightful system to investigate these questions
111 because individuals remain on the non-breeding grounds for 1.5 years following their first
112 migration from their natal sites in Siberia. This allows comparisons between birds that have
113 remained 'resident' on the non-breeding grounds for a full year (at this point 15 months old)
114 and those that have just arrived after a long-distance migratory leg, providing two conspecific
115 groups that share the same flock, diet and environment, but differ in migratory physiology.

116 We compared individuals that had recently arrived at a globally important migratory fuelling
117 site in northern Western Australia to conspecifics that had remained in the area for a full year.
118 We repeated this comparison for Red-necked stint at a site over 3000 km away on the south

119 coast of Australia, where stint had recently arrived to their final non-breeding site, providing
120 three migrant-resident comparison groups across two species and two sites.

121 Our analyses focused on exploring three hypotheses that assume distinct major drivers of gut
122 microbiota diversity and composition. Firstly, if migrants form predictable associations with
123 the gut microbiota, we would predict repeated differences in specific bacterial taxa between
124 migrants and residents across the three migrant-resident comparison groups. Secondly, if
125 migrants benefit from maintaining gut function and pathogen resistance, migrating
126 individuals may be expected to maintain similar community structure and species diversity to
127 residents. Thirdly, if the physiological challenges posed by migration negatively affect gut
128 microbe ecosystem dynamics, then migrants may display reduced species diversity and
129 evidence of ecosystem dysregulation, whereby opportunistic bacterial taxa outcompete
130 typical community members (Sommer *et al.* 2017).

131 To test these hypotheses, we assessed how migrants and residents differ with respect to
132 specific bacterial taxa, community-wide differences in abundance and phylogeny, and species
133 diversity. Collectively, these analyses allowed us to elucidate the relationship between the gut
134 microbiota and long-distance migration in shorebirds.

135 METHODS

136 Sample collection

137 Microbiota samples were collected across three migrant-resident conspecific comparison
138 groups: 1) Curlew sandpiper in NW Western Australia (12 migrants and 6 residents); 2) Red-
139 necked stint in NW Western Australia (13 migrants and 16 residents); and 3) Red-necked
140 stint in SE Victoria (15 migrants and 15 residents). All migrants were adults (i.e. just
141 completed their second or more southward migration), and all residents were ‘overwintering’
142 second year birds (i.e. had completed their first southward migration a year previously). The

143 sex of the birds was unknown, although sex differences in gut microbiota of birds is thought
144 to be absent or minimal (Kreisinger *et al.* 2015). Red-necked stint and Curlew sandpiper were
145 captured using cannon nets at an internationally important migratory fuelling site in Broome,
146 Western Australia (17°97 S, 122°32 E), during two capture events on 22nd and 29th August
147 2015. Birds captured on the 22nd were largely resident second years of both species, because
148 at that point migrating adults had not yet arrived at the site from post-breeding migration.
149 Birds captured a week later were a mix of newly arrived migrants that had arrived within a
150 few days of capture, and resident second year individuals. Red-necked stint were also
151 captured during one capture event on 20th September 2015 at a coastal beach site in Victoria
152 (38°48 S, 145°00 E), 3000 km south east of Broome. Both study sites consisted of tidal beach
153 habitat. Given that adult stint arrive at the Victorian site over the course of mid- to late-
154 September, recent migrants captured at this site would have completed their post-breeding
155 migration 1 - 14 days prior to capture. These birds may therefore have had a longer period of
156 time between completing a migratory leg and being sampled in comparison to birds captured
157 in Broome, which were captured within 1-3 days of arrival. Although age differences exist
158 between the two groups, it is unlikely that this would be the cause of differences in
159 microbiota community structure. Age is an important factor determining gut microbiota
160 composition when young, with chicks having different gut microbiota to adult birds in
161 penguins, kittiwakes and barn swallows (van Dongen *et al.* 2013; Barbosa *et al.* 2016;
162 Kreisinger *et al.* 2017). However, poultry studies suggest that gut microbiota structure
163 resembles that of adults within 0.5 - 3 months after hatching (Oakley *et al.* 2014; Ranjitkar *et*
164 *al.* 2016), and studies of two wild migratory shorebird species, Dunlin (*Calidris alpina*) and
165 Red phalarope (*Phalaropus fulicarius*), suggest that microbiota diversity stabilizes in 3-10
166 days old chicks (Grond 2017). On this basis, and given that both our resident and migrant
167 groups consist of fully-grown birds that have completed at least one Siberia-to-Australia

168 migration, we do not believe that differences in gut microbiota should exist between second
169 year birds at 15 months old and birds that are 3+ years old due to age *per se*.

170 In Broome, cloacal samples were taken from stints using sterile swabs (Copan 170KS01),
171 placed in sterile plastic tubes without medium, and kept refrigerated for 3 - 5 hours before
172 being stored at -20°C. After one week, they were transported from the field facility to a
173 laboratory where they were stored at -80°C. Cloacal samples collected in Victoria were
174 treated in the same manner but stored at -80°C directly after 3-5 hours refrigeration.

175 Differences in bacterial composition resulting from storage conditions have been shown to
176 not eclipse differences between samples, even when left at ambient temperatures for two
177 weeks (Lauber *et al.* 2010; Dominianni *et al.* 2014; Song *et al.* 2016). Therefore we assumed
178 that differences in time spent at -20°C had minimal effect on bacterial composition of our
179 samples. Moreover, our analyses focused on comparisons made between samples treated
180 identically and treatment of samples is therefore not expected to impact our conclusions.

181 DNA isolation, amplification and sequencing

182 DNA was isolated using a phenol-chloroform method and washed in ethanol (Green *et al.*
183 2012). DNA samples were sent to the Ramaciotti Centre for Genomics, Sydney, for
184 amplification using paired 27F/519R primers that amplify a 500bp V1-V3 region of the 16S
185 rRNA bacterial gene, and amplicons were then sequenced using Illumina MiSeq technology
186 (Caporaso *et al.* 2012; full protocol for these primers available at www.bioplatforms.com).

187 Two technical replicates within each plate, as well as two technical replicates between plates,
188 were included as an additional data quality check.

189 Sequence processing

190 Paired sequences for 77 bird samples and two negative controls were joined, aligned and
191 filtered in mothur version 1.39.1 following their standard operating procedure (MiSeq SOP;

192 Kozich *et al.* 2013; accessed April 2017). Chimeras were identified using the UCHIME
193 algorithm (Edgar *et al.* 2011) and were removed from the dataset. Sequences were grouped
194 into operational taxonomic units (OTUs) based on a 97% similarity threshold. Taxonomic
195 classification was performed using the SILVA taxonomy (v123.1; Pruesse *et al.* 2007)
196 trimmed to the alignment space of the amplicons (Werner *et al.* 2012). OTUs that were
197 identified as mitochondrial or eukaryotic (including chloroplast) were removed from the data
198 set. Archaeal sequences were also removed, because they are not well represented by non-
199 specific primers (Baker, Smith & Cowan 2003). Representative OTU sequences were aligned
200 to the SILVA reference within mothur, then a maximum likelihood tree was inferred using
201 FastTree (v2.1; Price, Dehal & Arkin 2009) and used to calculate UniFrac distances.
202 Sequences belonging to abundant OTUs (outlined in Table S2) that were not classified to
203 genus within Mothur were aligned using the SINA web aligner (Pruesse, Peplies & Glöckner
204 2012) and then imported into the SILVA non-redundant, small subunit database release 128
205 using the ARB software package (Ludwig *et al.* 2004). Amplicon sequences were masked
206 using the ssu_ref:bacteria column filter and inserted into the tree using the ARB Parsimony
207 method. From 23 common OTUs that were not originally classified to the genus level, 21
208 OTUs were placed into well-defined genus-level clades which was inferred as the final
209 taxonomy of the OTU. Sequences were assigned reference genes within PICRUSt (Langille
210 *et al.* 2013) to predict functionality. However, only 35-45% of sequences were matched to a
211 reference genome (when applying 97 and 95% similarity, respectively). Moreover, key
212 sequences belonging to *Corynebacterium* (see results) were not assigned reference genomes,
213 and therefore we deemed this analysis to have limited meaning and we do not present its
214 results here.

215 Count data processing

216 We retained only OTUs represented by over 10 sequences (97% of all sequences), because
217 examination of technical repeats suggested rare OTUs were likely to be due to error rather
218 than rare bacterial strains. Removal of rare OTUs reduces error whilst maintaining statistical
219 power (Allen *et al.* 2016). The negative controls contained 97 OTUs represented by at least 5
220 sequences, and these OTUs were removed from the dataset to reduce any effect of
221 contamination. To identify OTUs that were differentially abundant in migrants and residents,
222 we rlog transformed raw count data in DESeq2 package (Love, Huber & Anders 2014). This
223 procedure allowed us to assess fold differences in OTUs whilst accounting for variation in
224 library size between samples. For all other analyses, count data were rarefied to the minimum
225 read count (5815; random seed = 3). This reduced the total number of OTUs from 5262 to
226 4406. Because rarefied data can lead to false positives (McMurdie & Holmes 2014), we
227 repeated these analyses without rarefying samples, but no differences in overall results or
228 conclusions were observed, and we therefore present results from rarefied data.

229 Data analysis

230 We analysed bacterial communities in three ways. 1) To identify which OTUs significantly
231 differed in abundance between migrants and residents, we fitted negative binomial
232 generalized linear models to each of the three comparison groups separately (using the rlog
233 transformed data), with migration status set as the test group, using the DESeq function in the
234 DESeq2 package. We present only OTUs that differed significantly between groups (adjusted
235 p value < 0.01); 2) To examine community-wide differences in phylogeny and abundance we
236 applied MDS and NMDS ordinations to rarefied count data, and conducted ADONIS tests
237 (Anderson 2001) to statistically test for differences between groups. Because primary
238 components in the MDS analyses generally explained little variance, we present results from
239 the NMDS ordination. We present results based on both Bray-Curtis (based on abundance of
240 OTUs) and unweighted Unifrac (based on evolutionary distance between OTUs; Hamady,

241 Lozupone & Knight 2010), distance measures. 3) We analysed community diversity by
242 calculating both observed OTU richness and the Shannon diversity index, which takes into
243 account species abundance (i.e. the evenness of species' abundances) and penalizes highly
244 uneven distributions. All analyses were conducted using the DESeq2, Phyloseq (McMurdie
245 & Holmes 2013) and vegan (Oksanen *et al.* 2007) packages in R.

246 RESULTS

247 High-throughput amplicon sequencing from 77 biological samples yielded a total of
248 2,556,822 good quality sequences. After rarefying, a total of 4406 operational taxonomic
249 units (OTUs) were identified from cloacal samples of eighteen Curlew sandpipers (12
250 migrants and 6 residents) and twenty nine Red-necked stints (13 migrants and 16 residents)
251 sampled in NW Western Australia and 30 Red-necked stints (15 migrants and 15 residents)
252 from SE Victoria. The majority of OTUs had low prevalence (mean prevalence = 4.6%) when
253 pooled across all birds (Fig. S1).

254 Differences in specific bacteria taxa

255 Across the three comparison groups, migrants consistently displayed higher abundances of
256 Actinobacteria than residents (Fig. 1a). This difference was primarily comprised of OTUs
257 within the family *Corynebacteriaceae* (Fig. 1b) and specifically the genus *Corynebacterium*
258 (see Table S2 for most abundant OTUs per group), which made up an average of 28% of the
259 microbiota of migrants and less than 1% in residents across all birds. One OTU in particular
260 was abundant in migrants across both species and both sites (OTU13; Table S2). A total of 38
261 OTUs differed significantly (adjusted $p < 0.01$) between migrants and residents (Fig. 2; see
262 Table S3 for OTU list and statistics). Across both species and sites, *Corynebacterium* OTUs
263 had 5 – 25-fold increases in migrants compared to residents. In contrast, there was less
264 consistency across residents, with a much broader range of OTUs being more common in this

265 group. Resident curlew sandpipers had the largest range of significantly inflated OTUs, in
266 particular those belonging to Firmicutes, such as *Lachnospiraceae*, *Ruminococcaceae*, and
267 *Peptostreptococcaceae* (Fig. 2). Red-necked stint in Victoria, which may have had the
268 longest interval between arrival and sampling, demonstrated the fewest differences between
269 migrants and residents.

270 Differences in phylogeny and abundance

271 Across species, sites and migration status, all individuals had relatively similar and
272 overlapping gut microbial communities (Fig. 3a). However, all three factors significantly
273 predicted weak effects on Bray-Curtis distances (based on abundance of OTUs) in a
274 multivariate ADONIS model (migration status: $F_{77,1} = 3.5$, $R^2 = 0.04$, $p < 0.001$, Fig. 3b;
275 species: $F_{77,1} = 2.8$, $R^2 = 0.03$, $p < 0.001$; site: $F_{77,1} = 3.4$, $R^2 = 0.04$, $p < 0.001$). When
276 applying a Unifrac distance matrix (based on evolutionary distance between OTUs),
277 differences in community composition were less pronounced for migration status and species,
278 but similar for site (migration status: $F_{77,1} = 2.3$, $R^2 = 0.03$, $p < 0.001$; species: $F_{77,1} = 2.2$, R^2
279 $= 0.03$, $p < 0.001$; site: $F_{77,1} = 3.4$, $R^2 = 0.04$, $p < 0.001$; Fig. S4 for ordination plot). If taxa
280 belonging to *Corynebacteriaceae* were excluded, weak differences between migrants and
281 residents still remained, whilst controlling for species and site (Bray-Curtis: $F_{77,1} = 1.5$, $R^2 =$
282 0.02 , $p = 0.04$; Unifrac: $F_{77,1} = 2.3$, $R^2 = 0.03$, $p < 0.001$).

283 Differences in species diversity

284 For birds staging in NW Australia, there was a tendency for migrants to have fewer OTUs
285 compared to resident conspecifics (Curlew sandpiper: migrants = 152 ± 57 s.d., residents =
286 212 ± 62 s.d, $t_{18,1} = 2.2$, $p = 0.05$; Red-necked stint: migrants = 179 ± 53 s.d., residents = 218
287 ± 64 s.d., $t_{30,1} = 1.8$, $p = 0.09$; Fig. 3c). There was, however, no difference in Shannon
288 diversity indices, indicating differences are attributable to fewer rare species in migrants (Fig.

289 3d). For Red-necked stint in SE Victoria there was no difference in either measure of
290 diversity between migrants and residents (Red-necked stint: migrants = 143 ± 62 s.d.,
291 residents = 140 ± 62 s.d., $t_{30,1} = 1.8$, $p = 0.88$).

292 DISCUSSION

293 Long-distance migratory birds have evolved numerous physiological adaptations that enable
294 them to perform some of the longest and fastest migrations found within the animal kingdom
295 (Piersma *et al.* 2005; Hedenström 2008). Identifying whether these adaptations encompass
296 alterations to the gut microbiota offers unique insights into the relationship between hosts and
297 their microbes under specific physiological challenges. We found that *Calidris* shorebirds
298 that had just completed a long-distance migratory leg had considerably higher abundances of
299 bacterial taxa belonging to the genus *Corynebacterium* in comparison to conspecifics that had
300 occupied the same site for a whole year (Fig. 1). This effect was consistent across three
301 migrant-resident comparison groups that spanned two shorebird species and two distant sites.
302 No other repeated differences in specific bacterial taxa were found between migrants and
303 residents across comparison groups, suggesting the majority of bacterial taxa were not
304 affected by migration. This was reflected by only weak community-wide differences between
305 migrants and residents, with migration accounting for only 2-4% of total variation with
306 respect to both bacterial abundance and phylogeny.

307 The consistency and specificity of the link between migration and *Corynebacterium* may
308 indicate an adaptive association between *Calidris* shorebirds and this bacterial genus,
309 although causality and functionality of this relationship remains to be tested. This association
310 is likely to be temporary, with another study finding *Corynebacterium* decreased over the
311 non-breeding season for Red-necked stint sampled over time (Risely *et al.* 2017). Functional
312 interactions between animals and their gut microbiota are highly complex, and our current

313 understanding of such interactions are largely based on human or mouse models (Tremaroli
314 & Bäckhed 2012; Sommer & Bäckhed 2013b). However, a powerful study on the
315 relationship between gut microbiota and hibernation experimentally demonstrated functional
316 links between these microbial changes and seasonal host fat deposition (Sommer *et al.* 2016).
317 Correspondingly, *Corynebacterium* may conceivably be involved in functional host-microbe
318 interactions that enable migrating shorebirds to maximise fat deposition and/or energy
319 harvest during migration. Such mechanisms are proposed to be triggered by bacterial
320 endotoxins (produced by gram-negative bacteria) or exotoxins (produced by some gram-
321 positive bacteria), which lead to host inflammatory responses that increase host energy
322 harvest and fat deposition (Tremaroli & Bäckhed 2012; Zhao 2013; Boulangé *et al.* 2016).
323 These mechanisms have been experimentally demonstrated by increased fat deposition in
324 mice inoculated with pathogenic gram-negative bacteria or their associated endotoxins (Cani
325 *et al.* 2007; Schertzer *et al.* 2011; Fei & Zhao 2013). Such associations may potentially
326 explain the unusually high abundances of pathogen-associated bacteria in migrating birds,
327 such as *Corynebacterium* (this study), *Campylobacter* in American shorebird species (Grond
328 *et al.* 2014), and *Escherichia* and *Paracoccus* in passerines (Lewis, Moore & Wang 2016).
329 In addition to functional interactions between migrants and their gut microbes, gut microbial
330 composition is also influenced by short-term changes to host diet, physiology, and
331 environment (Candela *et al.* 2012; David *et al.* 2014; Carmody *et al.* 2015). Differences in
332 composition between migrant and resident conspecifics may therefore also stem from the
333 presumably distinct range of diets and habitats experienced by migrants in the days or weeks
334 prior to sampling, as well as to physiological effects of exercise and gut atrophy experienced
335 during migration. Although the specificity and repeatability of increased abundances of
336 *Corynebacterium* in migrants suggest a shared physiological response to migration, other
337 weak differences in bacterial abundance and phylogeny still remained when this genus was

338 excluded from analyses. These may reflect differences in recent diet between migrants and
339 residents, and may explain some of the other group-specific differences found, such as
340 increased Firmicutes taxa in resident Curlew sandpiper. Differences may also reflect the
341 incorporation of distinct bacterial taxa from the environment during migration. However,
342 migratory shorebirds have been shown to be relatively resistant to microbial invasions from
343 the environment (Risely *et al.* 2017), suggesting differences in recent diet may potentially
344 explain some of the small amount of remaining variation in gut microbiota composition
345 between migrants and residents.

346 Migrating shorebirds maintained similar community diversity to resident conspecifics,
347 although they tended to have fewer rare species. The broad maintenance of gut community
348 structure despite the considerable physiological challenges faced by long-distance migrants is
349 noteworthy. Blood flow to the gut is reduced during long-distance migratory flights, causing
350 partial atrophy of the gut and cessation of digestion (Piersma 1998; Battley *et al.* 2000;
351 McWilliams & Karasov 2001). Such dramatic physiological changes may be expected to
352 disrupt gut function and potentially facilitate the invasion of opportunistic species (Khosravi
353 & Mazmanian 2013). In this light, *Corynebacterium* may be interpreted to behave like an
354 opportunistic pathogen: dominating an ecosystem under stress. Indeed, this genus comprises
355 an unusually high proportion of opportunistic pathogens due to cellular properties similar to
356 gram-negative bacteria (Burkovski 2013). However, if ecological disruption promotes
357 invasion from opportunists, then considering the vast variation within and amongst
358 individuals, one would expect a range of opportunistic strains to dominate, yet this was not
359 the case.

360 Conclusions

361 This study provides a rare example of a consistent and highly specific response of the gut
362 microbiota to a host physiological challenge, suggesting a consistent interaction between
363 *Corynebacterium* bacteria and *Calidris* shorebirds during migration. The nature of this
364 relationship, including functionality and causality, remains to be tested. However, the effect
365 of migration on overall gut community diversity and composition was relatively small. The
366 preservation of broad community structure may allow migrants to maintain gut function
367 during critical stopover periods, and reduce their susceptibility to enteric infections as they
368 move between sites.

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380 DATA ACCESSIBILITY

381 Data and code are available to download at <https://doi.org/10.5281/zenodo.1036852> (Risely
382 2017). All amplicon sequences are available at NCBI BioProject PRJNA385545.

383 AUTHOR CONTRIBUTIONS

384 AR, BH & MK designed study, AR collected data, AR & DW processed sequences, AR
385 analysed data and lead on writing MS. All authors contributed conceptually to study and MS
386 drafts.

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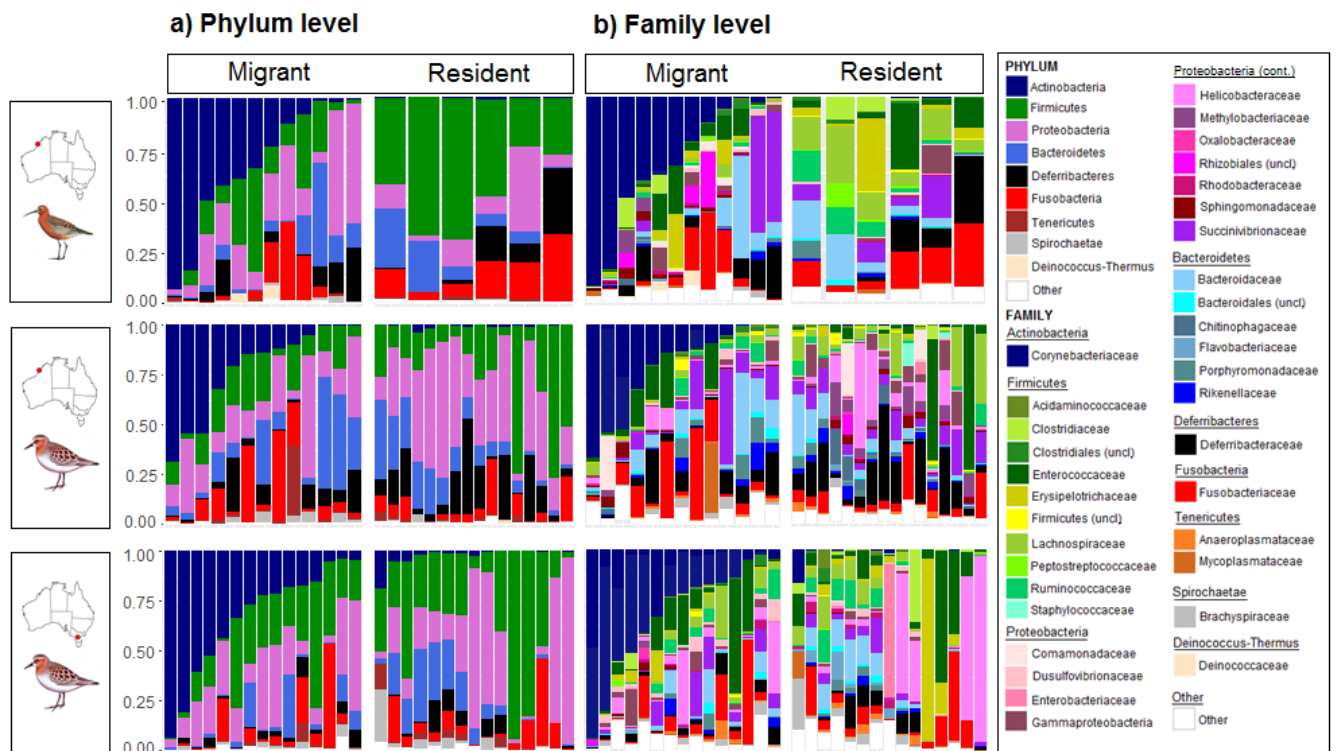
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609 FIGURES

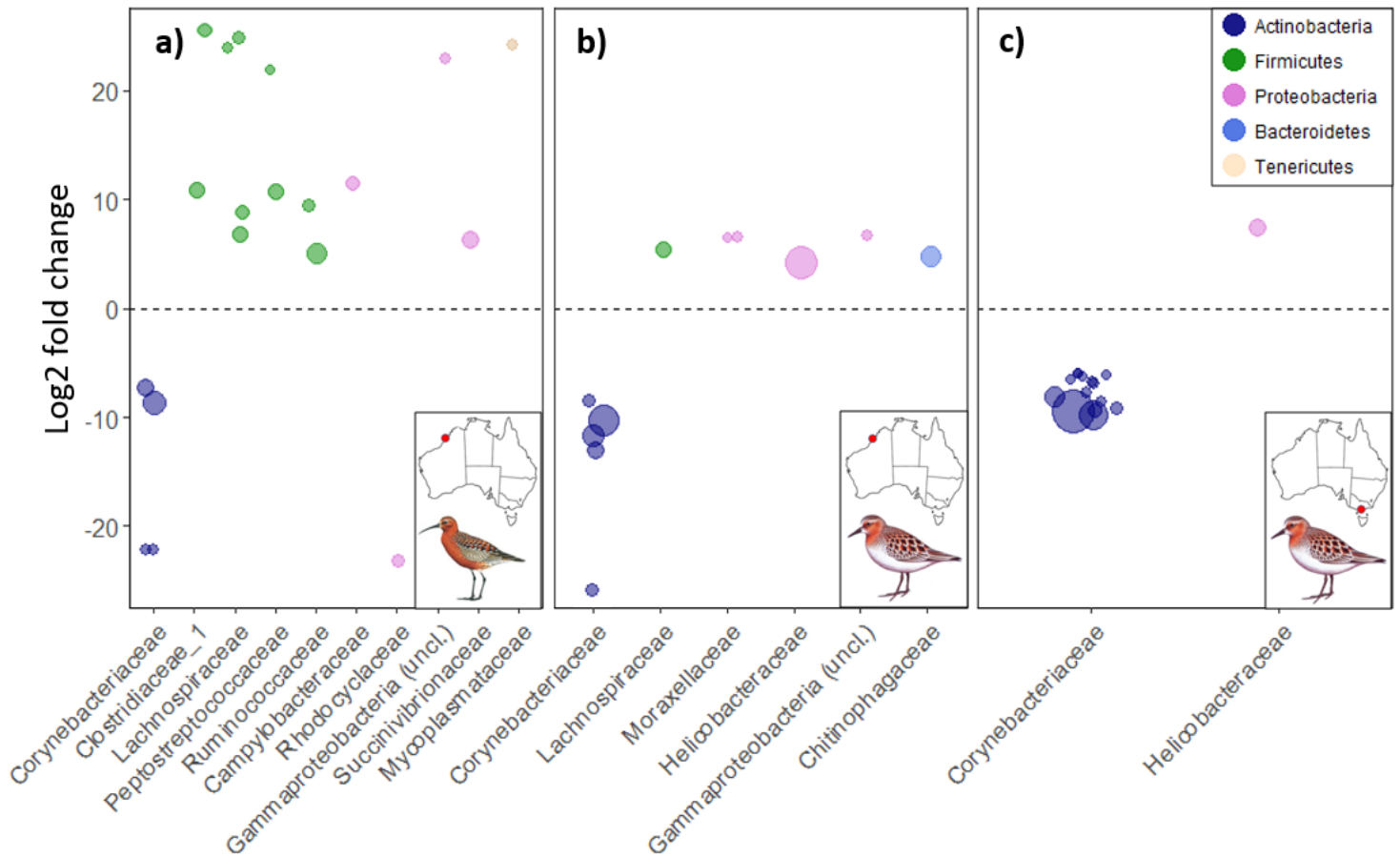
610 Figure 1) Bacterial composition of migrant and resident Curlew sandpiper in Broome (top
 611 panel), Red-necked stint in Broome (middle panel) and Red-necked stint in Victoria (bottom
 612 panel). Bacterial taxonomy is grouped by a) phylum and b) family. For clarity, only bacterial
 613 families that made up more than 5% of total abundance (35 out of 285) are assigned colours.



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627 Figure 2) Fold changes for OTUs (circles) that significantly differed between migrants and
 628 residents for a) Curlew sandpiper in Broome, b) Red-necked stint in Broome, and c) Red-
 629 necked stint in Victoria. OTUs below the dashed line are more abundant in migrants, whilst
 630 those above are more abundant in residents. OTUs are grouped by family, coloured by phyla,
 631 and sized by mean relative abundance across samples.

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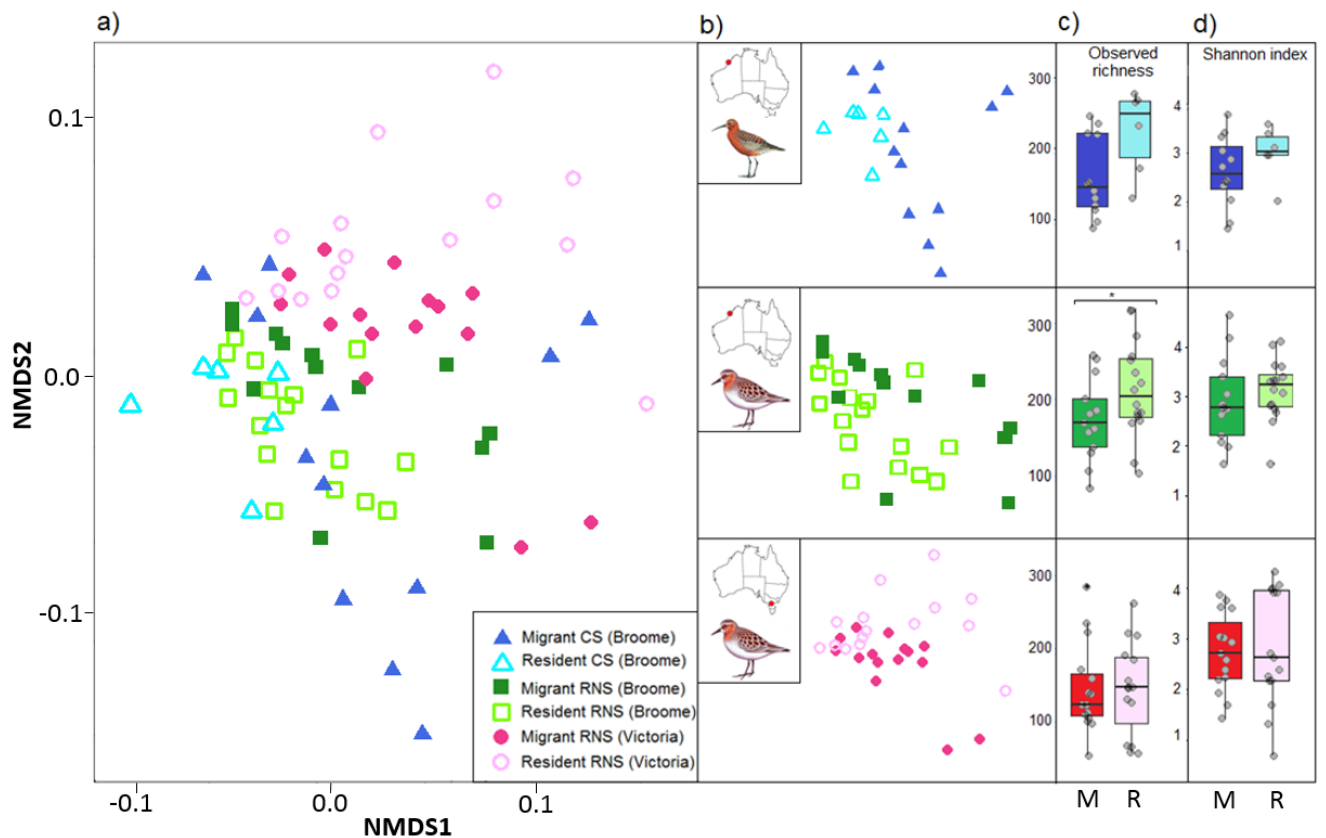
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643 Figure 3a) Non-multidimensional scaling (NMDS) plot based on Bray Curtis distances,
 644 calculated for shorebird gut microbiota communities across all individuals, coded by
 645 migratory status, site and species (CS = Curlew sandpiper, RNS = Red-necked stint); b)
 646 Subsetted NMDS plots for Curlew sandpiper in Broome (top), Red-necked stint in Broome
 647 (middle), and Red-necked stint in Victoria (bottom); c) observed richness and d) Shannon
 648 index calculated for migrant and resident individuals for each group (M = migrants, R =
 649 residents).

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