2018

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

Alice Risely
*Deakin University*

David Waite
*University of Queensland*

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

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

Marcel Klaassen
*Deakin University*

## Publication Details

Risely, A., Waite, D. W., Ujvari, B., Hoye, B. J. & Klaassen, M. (2018). Active migration is associated with specific and consistent changes to gut microbiota in Calidris shorebirds. Journal of Animal Ecology, 87 (2), 428-437.

# 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.

**Disciplines**

Medicine and Health Sciences | Social and Behavioral Sciences

**Publication Details**

Risely, A., Waite, D. W., Ujvari, B., Hoye, B. J. & Klaassen, M. (2018). Active migration is associated with specific and consistent changes to gut microbiota in Calidris shorebirds. Journal of Animal Ecology, 87 (2), 428-437.

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

Alice Risely[1], David Waite[2], Beata Ujvari[1], Bethany Hoye[1,3], & Marcel Klaassen[1]

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

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

[3] School of Biological Sciences, University of Wollongong, Wollongong NSW, 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: Gut microbiota dynamics in migrants

Key words: gut microbiota, host-microbe interactions, microbiome, migration, physiology

ABSTRACT

1. 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).
2. 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.
3. 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 3000 km away in southern Australia.
4. 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*.
5. 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.

## INTRODUCTION

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

However, species that are adapted to predictable physiological challenges may provide valuable study systems in which to investigate adaptive host-microbe interactions. For example, hibernating bears and ground squirrels undergo highly specific and consistent changes in gut microbiota composition between summer, when they must deposit body 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

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

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

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

## METHODS

### Sample collection

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

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*.

In Broome, cloacal samples 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 -20°C. After one week, they were transported from the field facility to a laboratory where they were stored at -80°C. Cloacal samples collected in Victoria were treated in the same manner but stored at -80°C directly after 3-5 hours refrigeration. Differences in bacterial composition resulting from storage conditions have been shown to not eclipse differences between samples, even when left at ambient temperatures for two weeks (Lauber *et al.* 2010; Dominianni *et al.* 2014; Song *et al.* 2016). Therefore we assumed that differences in time spent at -20°C had minimal effect on bacterial composition of our samples. Moreover, our analyses focused on comparisons made between samples treated identically and treatment of samples is therefore not expected to impact our conclusions.

DNA isolation, amplification and sequencing

DNA was isolated using a phenol-chloroform method and washed in ethanol (Green *et al.* 2012). 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). Two technical replicates within each plate, as well as two technical replicates between plates, were included as an additional data quality check.

Sequence processing

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

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

Count data processing

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

Data analysis

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

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

Differences in phylogeny and abundance

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

Differences in species diversity

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

understanding of such interactions are largely based on human or mouse models (Tremaroli & Bäckhed 2012; Sommer & Bäckhed 2013b). However, a powerful study on the relationship between gut microbiota and hibernation experimentally demonstrated functional links between these microbial changes and seasonal host fat deposition (Sommer *et al.* 2016). Correspondingly, *Corynebacterium* may conceivably be involved in functional host-microbe interactions that enable migrating shorebirds to maximise fat deposition and/or energy harvest during migration. Such mechanisms are proposed to be triggered by bacterial endotoxins (produced by gram-negative bacteria) or exotoxins (produced by some gram-positive bacteria), which lead to host inflammatory responses that increase host energy harvest and fat deposition (Tremaroli & Bäckhed 2012; Zhao 2013; Boulangé *et al.* 2016). These mechanisms have been experimentally demonstrated by increased fat deposition in mice inoculated with pathogenic gram-negative bacteria or their associated endotoxins (Cani *et al.* 2007; Schertzer *et al.* 2011; Fei & Zhao 2013). Such associations may potentially explain the unusually high abundances of pathogen-associated bacteria in migrating birds, such as *Corynebacterium* (this study), *Campylobacter* in American shorebird species (Grond *et al.* 2014), and *Escherichia* and *Paracoccus* in passerines (Lewis, Moore & Wang 2016).

In addition to functional interactions between migrants and their gut microbes, gut microbial composition is also influenced by short-term changes to host diet, physiology, and environment (Candela *et al.* 2012; David *et al.* 2014; Carmody *et al.* 2015). Differences in composition between migrant and resident conspecifics may therefore also stem from the presumably distinct range of diets and habitats experienced by migrants in the days or weeks prior to sampling, as well as to physiological effects of exercise and gut atrophy experienced during migration. Although the specificity and repeatability of increased abundances of *Corynebacterium* in migrants suggest a shared physiological response to migration, other weak differences in bacterial abundance and phylogeny still remained when this genus was

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

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

## Conclusions

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

## ACKNOWLEDGEMENTS

We would like to thank the Victorian Wader Study Group and the Australasian Wader Studies Group, in particular Clive Minton, Penny Johns, Chris Hassell and Grace Maglio for facilitating field work. We thank Michelle Wille for helpful comments that improved 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), Western Australia's Department of Parks and Wildlife (DPaW), 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.

## DATA ACCESSIBILITY

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

## AUTHOR CONTRIBUTIONS

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

## REFERENCES


Allen, H.K., Bayles, D.O., Looft, T., Trachsel, J., Bass, B.E., Alt, D.P., Bearson, S.M., Nicholson, T. & Casey, T.A. (2016) Pipeline for amplifying and analyzing amplicons of the V1–V3 region of the 16S rRNA gene. *BMC research notes,* **9,** 380.

Allen, J.M., Miller, M.E.B., Pence, B.D., Whitlock, K., Nehra, V., Gaskins, H.R., White, B.A., Fryer, J.D. & Woods, J.A. (2015) Voluntary and forced exercise differentially alters the gut microbiome in C57BL/6J mice. *Journal of applied physiology,* **118,** 1059-1066.

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

Bäckhed, F., Ding, H., Wang, T., Hooper, L.V., Koh, G.Y., Nagy, A., Semenkovich, C.F. & Gordon, J.I. (2004) The gut microbiota as an environmental factor that regulates fat storage. *Proceedings of the National Academy of Sciences of the United States of America,* **101,** 15718-15723.

Baker, G., Smith, J.J. & Cowan, D.A. (2003) Review and re-analysis of domain-specific 16S primers. *Journal of microbiological methods,* **55,** 541-555.

Barbosa, A., Balagué, V., Valera, F., Martínez, A., Benzal, J., Motas, M., Diaz, J.I., Mira, A. & Pedrós-Alió, C. (2016) Age-Related Differences in the Gastrointestinal Microbiota of Chinstrap Penguins (Pygoscelis antarctica). *PloS one,* **11,** e0153215.

Battley, P.F., Piersma, T., Dietz, M.W., Tang, S., Dekinga, A. & Hulsman, K. (2000) Empirical evidence for differential organ reductions during trans–oceanic bird flight. *Proceedings of the Royal Society of London B: Biological Sciences,* **267,** 191-195.

Boulangé, C.L., Neves, A.L., Chilloux, J., Nicholson, J.K. & Dumas, M.-E. (2016) Impact of the gut microbiota on inflammation, obesity, and metabolic disease. *Genome medicine,* **8,** 42.

Buehler, D.M. & Piersma, T. (2008) Travelling on a budget: predictions and ecological evidence for bottlenecks in the annual cycle of long-distance migrants. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* **363,** 247-266.

Burkovski, A. (2013) Cell envelope of corynebacteria: structure and influence on pathogenicity. *ISRN microbiology,* **2013**.

Caesar, R., Reigstad, C.S., Bäckhed, H.K., Reinhardt, C., Ketonen, M., Lundén, G.Ö., Cani, P.D. & Bäckhed, F. (2012) Gut-derived lipopolysaccharide augments adipose macrophage accumulation but is not essential for impaired glucose or insulin tolerance in mice. *Gut,* gutjnl-2011-301689.

Candela, M., Biagi, E., Maccaferri, S., Turroni, S. & Brigidi, P. (2012) Intestinal microbiota is a plastic factor responding to environmental changes. *Trends in microbiology,* **20,** 385-391.

Cani, P.D., Amar, J., Iglesias, M.A., Poggi, M., Knauf, C., Bastelica, D., Neyrinck, A.M., Fava, F., Tuohy, K.M. & Chabo, C. (2007) Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes,* **56,** 1761-1772.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L. & Bauer, M. (2012) Ultra-high-throughput microbial

community analysis on the Illumina HiSeq and MiSeq platforms. *The ISME journal,* **6,** 1621-1624.
Carey, H.V., Andrews, M.T. & Martin, S.L. (2003) Mammalian hibernation: cellular and molecular responses to depressed metabolism and low temperature. *Physiological reviews,* **83,** 1153-1181.
Carey, H.V., Walters, W.A. & Knight, R. (2013) Seasonal restructuring of the ground squirrel gut microbiota over the annual hibernation cycle. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology,* **304,** R33-R42.
Carmody, R.N., Gerber, G.K., Luevano, J.M., Gatti, D.M., Somes, L., Svenson, K.L. & Turnbaugh, P.J. (2015) Diet dominates host genotype in shaping the murine gut microbiota. *Cell host & microbe,* **17,** 72-84.
David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E., Ling, A.V., Devlin, A.S., Varma, Y. & Fischbach, M.A. (2014) Diet rapidly and reproducibly alters the human gut microbiome. *Nature,* **505,** 559-563.
de Vos, W.M. & de Vos, E.A. (2012) Role of the intestinal microbiome in health and disease: from correlation to causation. *Nutrition reviews,* **70,** S45-S56.
Dill-McFarland, K.A., Neil, K.L., Zeng, A., Sprenger, R.J., Kurtz, C.C., Suen, G. & Carey, H.V. (2014) Hibernation alters the diversity and composition of mucosa-associated bacteria while enhancing antimicrobial defence in the gut of 13-lined ground squirrels. *Molecular ecology,* **23,** 4658-4669.
Dominianni, C., Wu, J., Hayes, R.B. & Ahn, J. (2014) Comparison of methods for fecal microbiome biospecimen collection. *BMC microbiology,* **14,** 1.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C. & Knight, R. (2011) UCHIME improves sensitivity and speed of chimera detection. *Bioinformatics,* **27,** 2194-2200.
Fei, N. & Zhao, L. (2013) An opportunistic pathogen isolated from the gut of an obese human causes obesity in germfree mice. *The ISME journal,* **7,** 880-884.
Gill, R.E., Tibbitts, T.L., Douglas, D.C., Handel, C.M., Mulcahy, D.M., Gottschalck, J.C., Warnock, N., McCaffery, B.J., Battley, P.F. & Piersma, T. (2009) Extreme endurance flights by landbirds crossing the Pacific Ocean: ecological corridor rather than barrier? *Proceedings of the Royal Society of London B: Biological Sciences,* **276,** 447-457.
Green, M.R., Hughes, H., Sambrook, J. & MacCallum, P. (2012) Molecular Cloning: a Laboratory Manual. *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.
Grond, K., Ryu, H., Baker, A.J., Santo Domingo, J.W. & Buehler, D.M. (2014) Gastro-intestinal microbiota of two migratory shorebird species during spring migration staging in Delaware Bay, USA. *Journal of Ornithology,* **155,** 969-977.
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.
Hedenström, A. (2008) Adaptations to migration in birds: behavioural strategies, morphology and scaling effects. *Philosophical Transactions of the Royal Society of London B: Biological Sciences,* **363,** 287-299.
Kamada, N., Chen, G.Y., Inohara, N. & Núñez, G. (2013) Control of pathogens and pathobionts by the gut microbiota. *Nature immunology,* **14,** 685-690.
Khosravi, A. & Mazmanian, S.K. (2013) Disruption of the gut microbiome as a risk factor for microbial infections. *Current Opinion in Microbiology,* **16,** 221-227.

Kohl, K.D., Amaya, J., Passement, C.A., Dearing, M.D. & McCue, M.D. (2014) Unique and shared responses of the gut microbiota to prolonged fasting: a comparative study across five classes of vertebrate hosts. *FEMS microbiology ecology,* **90,** 883-894.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K. & Schloss, P.D. (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., Čížková, D., Kropáčková, L. & Albrecht, T. (2015) Cloacal microbiome structure in a long-distance migratory bird assessed using deep 16sRNA pyrosequencing. *PloS one,* **10,** e0137401.

Kreisinger, J., Kropáčková, L., Petrželková, A., Adámková, M., Tomášek, O., Martin, J.-F., Michálková, R. & Albrecht, T. (2017) Temporal stability and the effect of transgenerational transfer on faecal microbiota structure in a long distance migratory bird. *Frontiers in Microbiology,* **8,** 50.

Lambert, J.E., Myslicki, J.P., Bomhof, M.R., Belke, D.D., Shearer, J. & Reimer, R.A. (2015) Exercise training modifies gut microbiota in normal and diabetic mice. *Applied Physiology, Nutrition, and Metabolism,* **40,** 749-752.

Langille, M.G., Zaneveld, J., Caporaso, J.G., McDonald, D., Knights, D., Reyes, J.A., Clemente, J.C., Burkepile, D.E., Thurber, R.L.V. & Knight, R. (2013) Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature biotechnology,* **31,** 814-821.

Lauber, C.L., Zhou, N., Gordon, J.I., 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.

Lewis, W.B., Moore, F.R. & Wang, S. (2016) Characterization of the gut microbiota of migratory passerines during stopover along the northern coast of the Gulf of Mexico. *Journal of Avian Biology*.

Love, M.I., Huber, W. & Anders, S. (2014) Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome biology,* **15,** 550.

Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S. & Jobb, G. (2004) ARB: a software environment for sequence data. *Nucleic acids research,* **32,** 1363-1371.

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

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

McWilliams, S.R. & Karasov, W.H. (2001) Phenotypic flexibility in digestive system structure and function in migratory birds and its ecological significance. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology,* **128,** 577-591.

Oakley, B.B., Buhr, R.J., Ritz, C.W., Kiepper, B.H., Berrang, M.E., Seal, B.S. & Cox, N.A. (2014) Successional changes in the chicken cecal microbiome during 42 days of growth are independent of organic acid feed additives. *BMC veterinary research,* **10,** 282.

Oksanen, J., Kindt, R., Legendre, P., O'Hara, B., Stevens, M.H.H., Oksanen, M.J. & Suggests, M. (2007) The vegan package. *Community ecology package,* **10**.

Piersma, T. (1998) Phenotypic flexibility during migration: optimization of organ size contingent on the risks and rewards of fueling and flight? *Journal of Avian Biology,* 511-520.

526 Piersma, T. & Gill Jr, R.E. (1998) Guts don't fly: small digestive organs in obese bar-tailed
527 godwits. *The Auk*, 196-203.
528 Piersma, T., Gudmundsson, G.A. & Lilliendahl, K. (1999) Rapid changes in the size of
529 different functional organ and muscle groups during refueling in a long-distance
530 migrating shorebird. *Physiological and Biochemical Zoology*, **72,** 405-415.
531 Piersma, T., PÉREZ-TRIS, J., Mouritsen, H., Bauchinger, U. & Bairlein, F. (2005) Is there a
532 "migratory syndrome" common to all migrant birds? *Annals of the New York*
533 *Academy of Sciences*, **1046,** 282-293.
534 Price, M.N., Dehal, P.S. & Arkin, A.P. (2009) FastTree: computing large minimum evolution
535 trees with profiles instead of a distance matrix. *Molecular biology and evolution*, **26,**
536 1641-1650.
537 Pruesse, E., Peplies, J. & Glöckner, F.O. (2012) SINA: accurate high-throughput multiple
538 sequence alignment of ribosomal RNA genes. *Bioinformatics*, **28,** 1823-1829.
539 Pruesse, E., Quast, C., Knittel, K., Fuchs, B.M., Ludwig, W., Peplies, J. & Glöckner, F.O.
540 (2007) SILVA: a comprehensive online resource for quality checked and aligned
541 ribosomal RNA sequence data compatible with ARB. *Nucleic acids research*, **35,**
542 7188-7196.
543 Ranjitkar, S., Lawley, B., Tannock, G. & Engberg, R.M. (2016) Bacterial Succession in the
544 Broiler Gastrointestinal Tract. *Applied and environmental microbiology*, **82,** 2399-
545 2410.
546 Risely, A. (2017) Riselya/Migration-shorebird-physiology: Data and code for Risely et al.
547 2017 J. ANIM. ECOL. *http://doi.org/10.5281/zenodo.1036852*.
548 Risely, A., Waite, D., Ujvari, B., Klaassen, M. & Hoye, B. (2017) Gut microbiota of a long-
549 distance migrant demonstrates resistance against environmental microbe incursions.
550 *Molecular ecology*.
551 Round, J.L. & Mazmanian, S.K. (2009) The gut microbiota shapes intestinal immune
552 responses during health and disease. *Nature Reviews Immunology*, **9,** 313-323.
553 Schertzer, J.D., Tamrakar, A.K., Magalhães, J.G., Pereira, S., Bilan, P.J., Fullerton, M.D.,
554 Liu, Z., Steinberg, G.R., Giacca, A. & Philpott, D.J. (2011) NOD1 activators link
555 innate immunity to insulin resistance. *Diabetes*, **60,** 2206-2215.
556 Sommer, F., Anderson, J.M., Bharti, R., Raes, J. & Rosenstiel, P. (2017) The resilience of the
557 intestinal microbiota influences health and disease. *Nature Reviews Microbiology*.
558 Sommer, F. & Bäckhed, F. (2013a) The gut microbiota--masters of host development and
559 physiology. *Nature reviews. Microbiology*, **11,** 227.
560 Sommer, F. & Bäckhed, F. (2013b) The gut microbiota—masters of host development and
561 physiology. *Nature Reviews Microbiology*, **11,** 227-238.
562 Sommer, F., Ståhlman, M., Ilkayeva, O., Arnemo, J.M., Kindberg, J., Josefsson, J., Newgard,
563 C.B., Fröbert, O. & Bäckhed, F. (2016) The gut microbiota modulates energy
564 metabolism in the hibernating brown bear Ursus arctos. *Cell reports*, **14,** 1655-1661.
565 Song, S.J., Amir, A., Metcalf, J.L., Amato, K.R., Xu, Z.Z., Humphrey, G. & Knight, R.
566 (2016) Preservation methods differ in fecal microbiome stability, affecting suitability
567 for field studies. *mSystems*, **1,** e00021-00016.
568 Tremaroli, V. & Bäckhed, F. (2012) Functional interactions between the gut microbiota and
569 host metabolism. *Nature*, **489,** 242-249.
570 van Dongen, W.F., White, J., Brandl, H.B., Moodley, Y., Merkling, T., Leclaire, S.,
571 Blanchard, P., Danchin, É., Hatch, S.A. & Wagner, R.H. (2013) Age-related
572 differences in the cloacal microbiota of a wild bird species. *BMC ecology*, **13,** 1.
573 Weber, J.-M. (2009) The physiology of long-distance migration: extending the limits of
574 endurance metabolism. *Journal of Experimental Biology*, **212,** 593-597.

Werner, J.J., Koren, O., Hugenholtz, P., DeSantis, T.Z., Walters, W.A., Caporaso, J.G., Angenent, L.T., Knight, R. & Ley, R.E. (2012) Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. *The ISME journal,* **6,** 94-103.

Wikelski, M., Tarlow, E.M., Raim, A., Diehl, R.H., Larkin, R.P. & Visser, G.H. (2003) Avian metabolism: costs of migration in free-flying songbirds. *Nature,* **423,** 704-704.

Zaneveld, J.R., McMinds, R. & Vega, T.R. (2017) Stress and stability: applying the Anna Karenina principle to animal microbiomes. *Nature microbiology,* **2,** 17121.

Zhao, L. (2013) The gut microbiota and obesity: from correlation to causality. *Nature Reviews Microbiology,* **11,** 639-647.

FIGURES

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

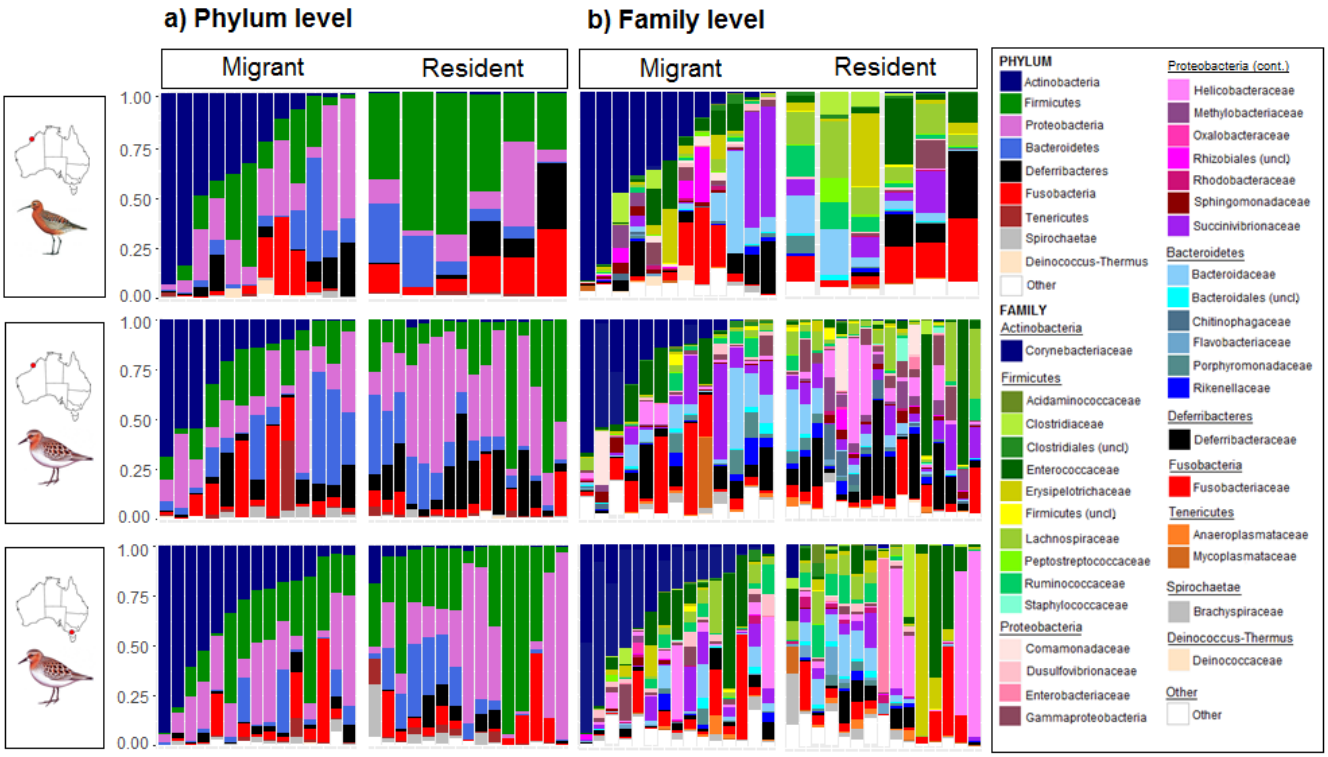
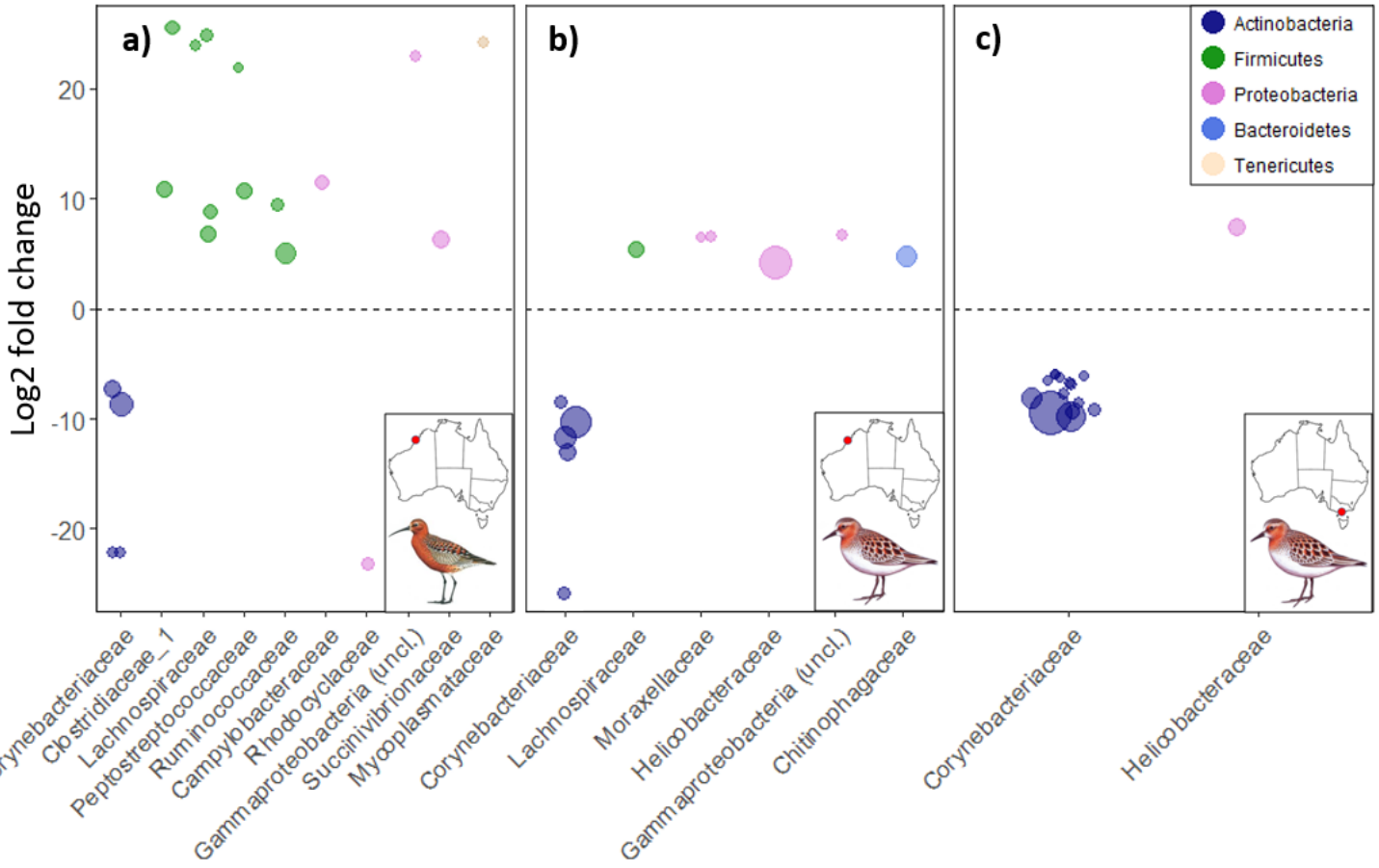
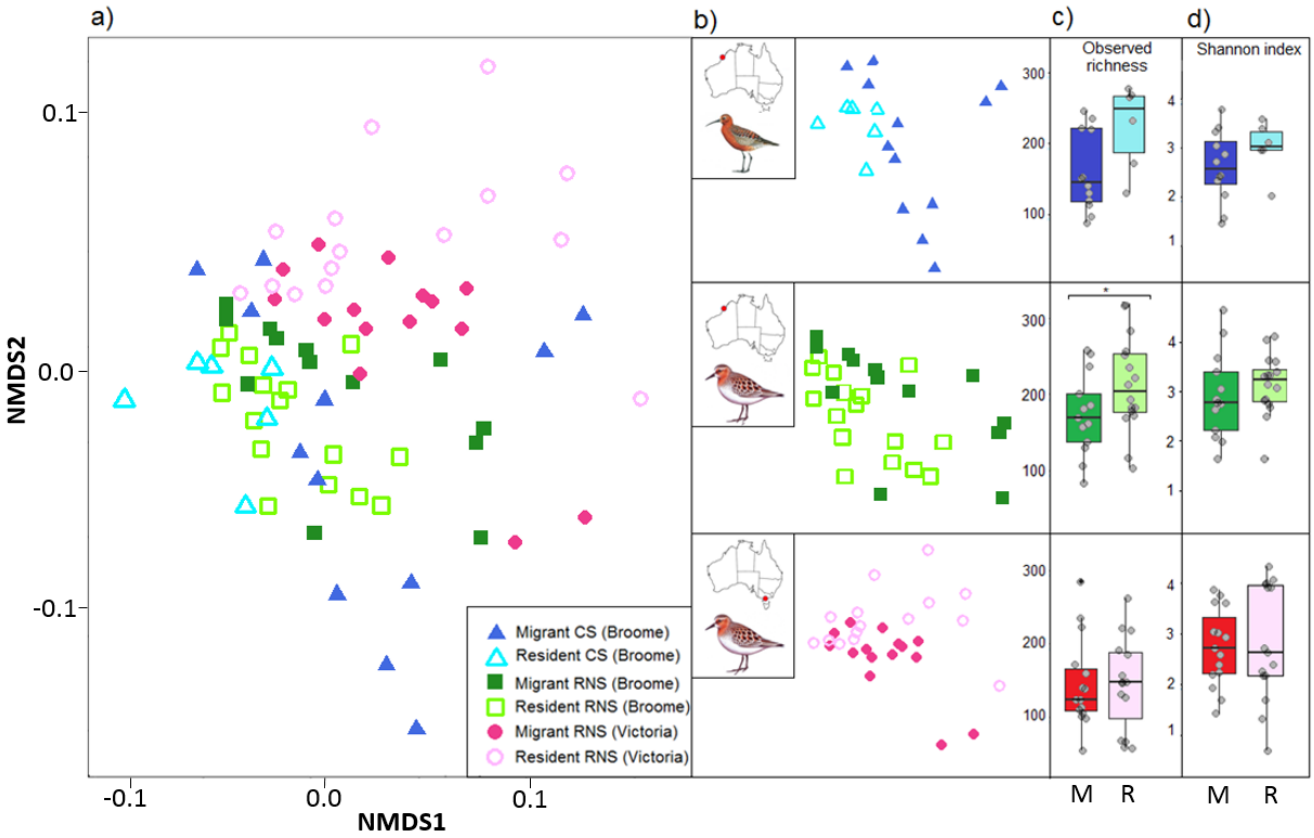


Figure 2) Fold changes for OTUs (circles) that significantly differed between migrants and residents for a) Curlew sandpiper in Broome, b) Red-necked stint in Broome, and c) Red-necked stint in Victoria. OTUs below the dashed line are more abundant in migrants, whilst those above are more abundant in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



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).

650




651

652

653

654

655